

Original Article

lncRNA-Gm5532 regulates osteoclast differentiation through the miR-125a-3p/TRAF6 axis

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Abstract

Long noncoding RNAs (lncRNAs) are important regulators of bone metabolism. In this study, lncRNA microarray analysis was used to identify differentially expressed lncRNAs in differentiated osteoclasts. lncRNA-Gm5532 is highly expressed during osteoclast differentiation. lncRNA-Gm5532 knockdown impairs osteoclast formation and bone resorption. Mechanistic experiments show that lncRNA-Gm5532 functions as a competing endogenous RNA (ceRNA) and acts as a sponge for miR-125a-3p, which promotes TNF receptor-associated factor 6 (TRAF6) expression. miR-125a-3p mimics suppress osteoclast differentiation and TAK1/NF- κ B/MAPK signaling. The miR-125a-3p inhibitor reverses the negative effects of siGm5532 on osteoclast differentiation. In summary, our study reveals that lncRNA-Gm5532 functions as an activator in osteoclast differentiation by targeting the miR-125a-3p/TRAF6 axis, making it a novel biomarker and potential therapeutic target for osteoporosis.

Key words lncRNA-Gm5532, osteoclast differentiation, miR-125a-3p, TNF receptor-associated factor 6 (TRAF6)

Introduction

Osteoporosis is a progressive metabolic bone disease characterized by low bone mineral density (BMD) and deterioration of bone microarchitecture, resulting in bone fragility and an increased risk of fracture. Osteoporosis is common in postmenopausal women, older adults, and patients with prolonged bed rest or treated with corticosteroids. Osteoporosis-related fractures seriously affect quality of life and cause an enormous economic burden to families and society [1]. Bone is a dynamic tissue. The balance of bone remodeling is orchestrated by osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Osteoclasts derived from hematopoietic stem cells are multinucleated giant cells responsible for clearing away bone that is aged or damaged [2]. Highly increased osteoclast activity can lead to the dysregulated balance of bone remodeling and loss of bone, which is one of the main reasons for osteolytic diseases [3].

Long noncoding RNAs (lncRNAs) are a type of noncoding RNA with lengths exceeding 200 nucleotides and are involved in a variety of cellular activities, including differentiation, proliferation, apoptosis, invasion, and metastasis. A number of lncRNAs have been identified to be involved in bone homeostasis [4]. During osteoclast differentiation, lncRNA expression profiles are altered, indicating that lncRNAs may play pivotal roles in osteoclastogenesis [5]. The

identification of novel lncRNAs involved in osteoclast differentiation is of great importance for understanding the mechanism of bone homeostasis. The present study focused on lncRNA-Gm5532, which has been found to be altered in developing retinas, spermatogenesis, and *Candida albicans* infection [6–8]. However, whether lncRNA-Gm5532 regulates osteoclastogenesis and its regulatory mechanism remain elusive.

In the present study, we identified that lncRNA-Gm5532 was significantly upregulated during osteoclast differentiation. Knockdown of lncRNA-Gm5532 inhibited osteoclast differentiation. lncRNA-Gm5532 acted as an miR-125a-3p sponge. miR-125a-3p suppressed osteoclast differentiation by targeting TNF receptor-associated factor 6 (TRAF6). lncRNA-Gm5532 drives osteoclast differentiation by acting as a ceRNA to regulate the miR-125a-3p/TRAF6 axis.

Materials and Methods

Cell culture and osteoclast differentiation

The murine monocyte/macrophage line RAW264.7 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) was cultured in α -MEM (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 2 mM L-glutamine (Beyotime, Shanghai, China). For osteoclast differentiation, the cells were seeded at a density of 1×10^4 cells/cm² and cultured overnight. After

that, the cells were incubated with 50 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL; PeproTech, Rocky Hill, USA) for 4 days. The formed multinucleated osteoclasts were determined by tartrate-resistant acid phosphatase (TRAP) staining using a Leukocyte Acid Phosphatase kit (Sigma-Aldrich, St Louis, USA). The osteoclasts were observed and photographed with a light microscope (Olympus, Tokyo, Japan). TRAP-positive cells with more than three nuclei were defined as osteoclasts. The area of osteoclasts was quantified using ImageJ software (National Institutes of Health, Bethesda, USA). TRAP activity was determined using a TRAP assay kit (Beyotime) on days 2 and 4. For the bone resorption assay, RAW264.7 cells were seeded on a Corning Osteo Assay plate (Corning, Tewksbury, USA) and incubated with 50 ng/mL RANKL for 7 days. After that, the plate was bleached with 0.5% sodium hypochlorite and observed with a light microscope (Olympus). The resorption pits were analyzed using ImageJ software. Actin ring formation was determined by F-actin staining on day 4. The cells were fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton X-100. Then, the cells were stained with rhodamine-labelled phalloidin (1:20; Invitrogen, Carlsbad, USA) and counterstained with 100 ng/mL DAPI (Invitrogen). The formation of actin rings was observed with a fluorescence microscope (Olympus).

LncRNA microarray analysis

Undifferentiated RAW264.7 cells and RAW264.7-derived osteoclasts on day 4 were lysed using TRIzol Reagent (Invitrogen), frozen in dry ice, and sent to Oebiotech (Shanghai, China) for lncRNA microarray analysis. Briefly, total RNA was transcribed to double-stranded cDNA, synthesized into cRNA and labelled with cyanine-3-CTP (Agilent Technologies, Santa Clara, USA). The labelled cRNAs were hybridized onto the Agilent mouse lncRNA microarray (4*180K; Agilent Technologies). After washing, the arrays were scanned with an Agilent Scanner G2505C (Agilent Technologies). Feature Extraction software (version 10.7.1.1; Agilent Technologies) was used to analyze array images to obtain raw data. Genespring (version 13.1; Agilent Technologies) was employed to complete the basic analysis with the raw data. The threshold set for up- and downregulated genes was a fold change ≥ 2.0 and a *P* value ≤ 0.05.

siRNA and miRNA mimic transfection

Short interfering RNAs (siRNAs) targeting lncRNA-Gm5532 and miR-125a-3p mimics were synthesized by GenePharma (Shanghai, China). The sequences of siGm5532 and miR-125a-3p mimics were

as follows: siGm5532, sense 5'-GCUACUAGAGACCAUGAUATT-3', and antisense 5'-UAUCAUGGUCUCUAGUAGCTT-3'; miR-125a-3p mimics, sense 5'-ACAGGUGAGGUUCUUGGGAGCC-3', and antisense 5'-CUCCCAAGAACCUCACCUGUUU-3'; negative control (NC) siRNA, sense 5'-UUCUCCGAACGUGUCACGUTT-3', and antisense 5'-ACGUGACGUUCGGAGAATT-3'; miR-125a-3p inhibitor, 5'-GGCUCCCAAGAACCUCACCUGU-3'; and inhibitor NC, 5'-CAGUACUUUUGUGUAGUACAA-3'. After RAW264.7 cells were seeded and cultured overnight, siGm5532 and miR-125a-3p mimics were transfected using siRNA mate (GenePharma) according to the manufacturer's instructions. siRNA or miRNA mimic transfection complexes were prepared with siRNA mate in α-MEM and were added into the culture medium to achieve a final concentration of 30 nM. Twenty-four hours posttransfection, the cells were induced to differentiate into osteoclasts in the presence of 50 ng/mL RANKL.

Quantitative PCR

Total RNA was extracted using an RNA isolation kit (Beyotime) and reverse transcribed using an RT reagent kit (Beyotime) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using SYBR Green PCR master mix (Beyotime). The amplification program was as follows: pre-denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s. The primer pairs of the following genes were used: *lncRNA-Gm5532*, *miR-125a-3p*, *U6*, *carbonic anhydrase II (Car2)*, *cathepsin K (CTSK)*, *GAPDH*, *integrin β3*, *MMP9*, and *vacuolar-type H⁺-ATPase (V-ATPase)*. The sequences of the primers are listed in Table 1. *GAPDH* was used as an internal control for gene expression assays, and *U6* was used as an internal control for miRNA assays. The expression levels of mRNA and miR-125a-3p were analyzed using the 2^{-ΔΔCt} method.

Dual-luciferase reporter assay

DNA sequences including or lacking the predicted lncRNA-Gm5532 or TRAF6 binding sequence were inserted into the psiCHECK-2 plasmid (Promega, Madison, USA). The potential binding sites between lncRNA-Gm5532 and miR-125a-3p were predicted by using the LncRNA2Target (v2.0; (<http://123.59.132.21/lncrna2target/index.jsp>) web tool. The potential binding sites between miR-125a-3p and TRAF6 were predicted using the TargetScan (http://www.targetscan.org/vert_72/) web tool. Then, 293T cells were seeded in a 96-well plate and cotransfected with these plasmids, pRL-TK *Renilla* luciferase plasmid, and miR-125a-3p mimics using GP-transfect-Mate reagent (GenePharma). Luciferase activity was measured using the dual-luciferase reporter assay system (Promega)

Table 1. Sequences of primers used for quantitative real-time PCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
<i>lncRNA-Gm5532</i>	GCCACCACTCACACTGCTCT	CCTCTATGCTGGCTGACACG
<i>miR-125a-3p</i>	ACACTCCAGCTGGGACAGGTGAGGTTCTTG	CTCAACTGGTGTCTGAGTTCGGCAATTCAGTTGAGGGCTCCCA
<i>U6</i>	AGAGCCTGTGGTGTCCG	CATCTTCAAAGCACTTCCCT
<i>Car2</i>	CATTACTGTCAGCAGCGAGCA	GACGCCAGTTGTCCACCATC
<i>CTSK</i>	CAGCAGAACGGAGGCATTGA	CCTTTGCCGTGGCGTTATAC
<i>GAPDH</i>	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC
<i>integrin β3</i>	CCACCTTCACCAATATCAC	CCAAATCCCACCCATACAC
<i>MMP9</i>	GCCCTGGAATCACACGACA	TTGGAAATCACACGCCAGAA
<i>V-ATPase</i>	CCACTGGAAGCCCAGTAAACAGA	GAACGTATGAGGCCAGTGAGCA

and normalized to *Renilla* luciferase activity.

Western blot analysis

Total cellular protein was extracted with RIPA buffer (Beyotime). Cell lysates (20 μ g per sample) were subjected to 10% SDS-PAGE, transferred to a PVDF membrane (Merck Millipore, Billerica, USA), blocked with 5% fat-free milk, and incubated with primary antibody (1:1000) at 4°C overnight. After that, the membrane was incubated with HRP-conjugated secondary antibodies (Beyotime). The bands were detected with a Bio-Rad Imaging System (Bio-Rad, Hercules, USA) after incubation with ECL substrate. The following primary antibodies were used: CTSK (Beyotime), MMP-9 (Beyotime), nuclear factor of activated T cells 1 (NFATc1; Abcam, Cambridge, UK), GAPDH (Cell Signaling Technology, Boston, USA), p38 (Cell Signaling Technology), p-p38 (Cell Signaling Technology), ERK (Cell Signaling Technology), p-ERK (Cell Signaling Technology), JNK (Cell Signaling Technology), p-JNK (Cell Signaling Technology), TAK1 (Beyotime), p-TAK1 (Beyotime), IKK (Beyotime), p-IKK (Beyotime), I κ B α (Beyotime), p-I κ B α (Beyotime), p65 (Beyotime), and p-p65 (Beyotime). GAPDH was used as an internal control.

Statistical analysis

All experiments were performed in triplicate. Data are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was performed using GraphPad Prism software (GraphPad, La Jolla, USA) with Student's *t*-test for two groups or by one-way ANOVA using Tukey's *post hoc* test for multiple groups. Differences with $P < 0.05$ were considered statistically significant.

Results

lncRNA-Gm5532 is highly expressed during osteoclast differentiation

The lncRNA microarray analysis provided an overview of the

lncRNAs that were differentially expressed during osteoclast differentiation. In the present study, RAW264.7 cells were incubated with 50 ng/mL RANKL for 4 days. During differentiation, the RAW264.7 monocytes formed TRAP-positive multinucleated cells (>3 nuclei) and ring-like actin structures (Figure 1A). Compared with nondifferentiated cells, osteoclast markers such as CTSK, MMP9 and NFATc1 were highly expressed during osteoclast differentiation on day 2 (Figure 1B). These results indicate that RAW264.7-derived TRAP-positive multinucleated cells possess osteoclast characteristics and can be used for osteoclast studies. Next, the differentiated cells were used for lncRNA microarray analysis. In total, 1404 lncRNAs were upregulated in RAW264.7-derived osteoclasts, and 1065 lncRNAs were downregulated (Figure 1C). Among the 20 most upregulated lncRNAs, lncRNA-Gm5532 was chosen for further study (Figure 1D). The expression changes of lncRNA-Gm5532 were verified by qPCR. The results showed that the expression level of lncRNA-Gm5532 significantly increased to about 50 folds in RAW264.7-derived osteoclasts (Figure 1E). These findings suggest that lncRNA-Gm5532 may play a regulatory role during osteoclast differentiation.

lncRNA-Gm5532 regulates osteoclast differentiation

To explore whether lncRNA-Gm5532 modulates osteoclast differentiation *in vitro*, the siRNA method was used to downregulate lncRNA-Gm5532 expression. Compared with NC, siGm5532 treatment led to a nearly 50% reduction in lncRNA-Gm5532 expression (Figure 2A). RAW264.7 cells treated with siGm5532 formed fewer TRAP-positive osteoclasts on day 4 (Figure 2B). TRAP activity and NFATc1, a master transcription regulator of osteoclast formation, were also decreased in siGm5532 cells (Figure 2D,E). Since lncRNA-Gm5532 downregulation had a negative impact on osteoclast formation, we next examined the effects of siGm5532 on bone resorption. The results showed that siGm5532 treatment signifi-

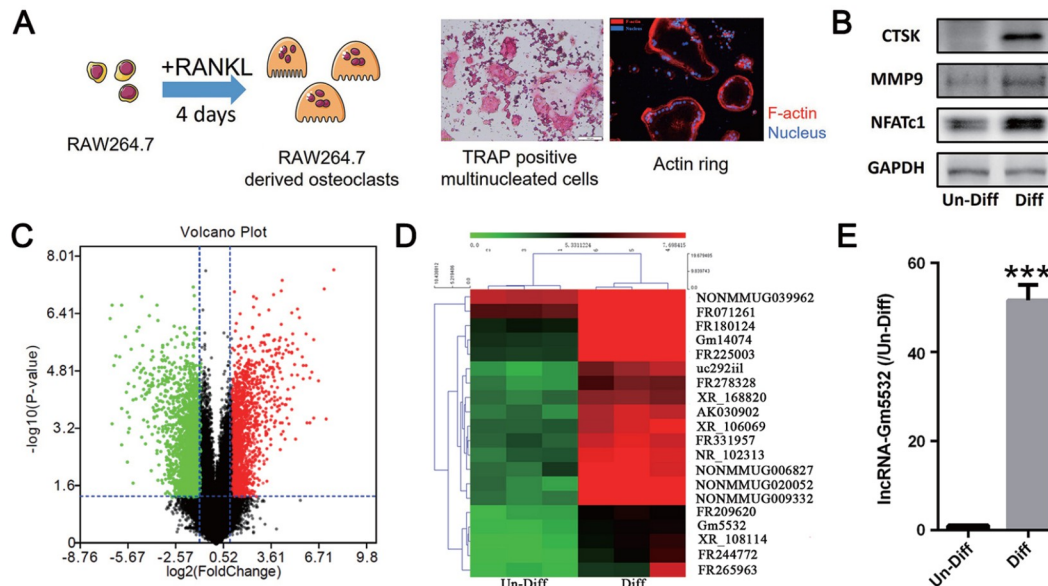


Figure 1. lncRNA-Gm5532 is highly expressed during osteoclast differentiation (A) RAW264.7 cells were differentiated into osteoclasts upon treatment with 50 ng/mL RANKL for 4 days. TRAP staining and F-actin staining were used to detect TRAP-positive multinucleated cells and ring-like actin structures. (B) Western blot analysis of the protein levels of CTSK, MMP9 and NFATc1 in RAW264.7-derived osteoclasts. (C) Volcano plot of the lncRNA expression signature in undifferentiated RAW264.7 cells and RAW264.7-derived osteoclasts. (D) The hierarchical clustering heatmap of lncRNAs that are highly expressed in RAW264.7-derived osteoclasts. (E) lncRNA-Gm5532 expression in undifferentiated RAW264.7 cells and RAW264.7-derived osteoclasts. Data are presented as the mean $\pm$ SD. *** $P < 0.001$ vs Un-Diff.

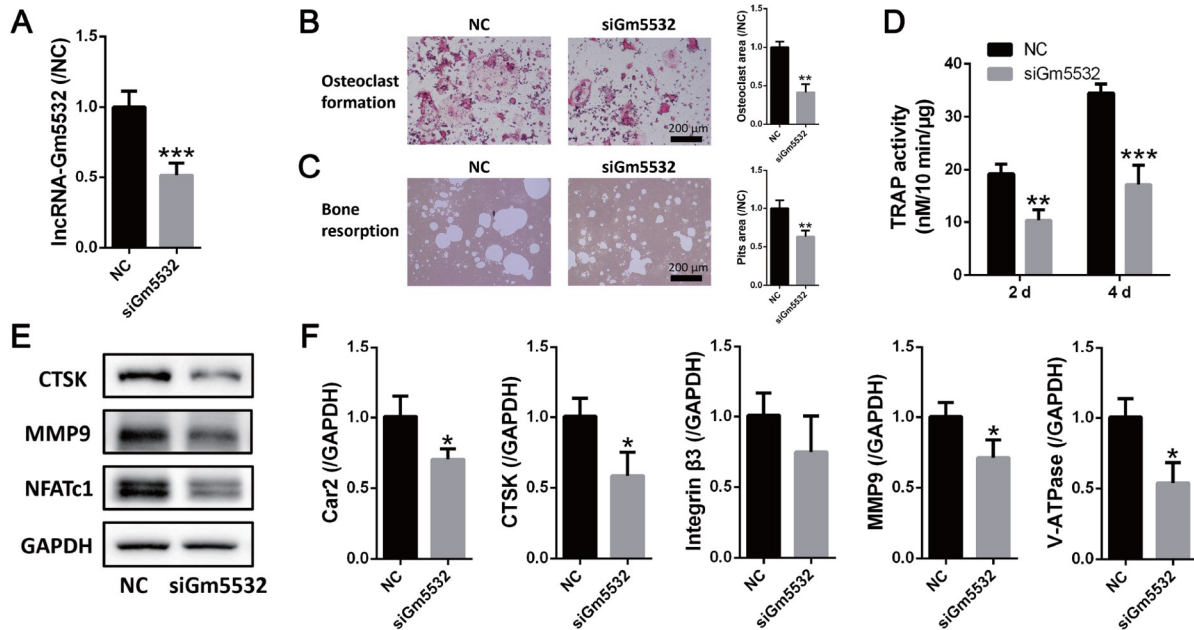


Figure 2. lncRNA-Gm5532 knockdown suppresses osteoclast formation and bone resorption (A) The siRNA method was used to downregulate lncRNA-Gm5532 expression. (B) RAW264.7 cells were transfected with siGm5532 and then incubated with 50 ng/mL RANKL. Osteoclast formation was analyzed by the area of TRAP-positive multinucleated cells. Scale bar: 200 μ m. (C) Bone resorption was analyzed by the area of resorption pits. Scale bar: 200 μ m. (D) TRAP activity was expressed as nanomoles of pNPP hydrolyzed per 10 min per microgram of protein. (E) Western blot analysis of the protein levels of CTSK, MMP9 and NFATc1. (F) mRNA expression of bone resorption-associated genes was measured on day 2. Data are presented as the mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 vs NC.

cantly decreased pit formation (Figure 2C) and the expressions of genes associated with bone resorption, including Car2, MMP9, V-ATPase, and CTSK (Figure 2E,F). These results suggest that lncRNA-Gm5532 plays a pivotal role in osteoclast formation and bone resorption. lncRNA-Gm5532 downregulation alleviates osteoclast differentiation.

miR-125a-3p acts as a sponge for lncRNA-Gm5532

lncRNAs can function as ceRNAs by binding to target miRNAs and indirectly regulating gene transcription. Here, we used lncRNA2-Target to identify the potential miRNA targets, and found that lncRNA-gm5532 could bind to miR-125a-3p to form a sponge structure (Figure 3A). To confirm the specific interaction between lncRNA-Gm5532 and miR-125a-3p, dual-luciferase reporters comprising the 5' end of lncRNA-Gm5532, which included either the wild-type (WT) or the mutated miR-125a-3p binding sites, were constructed. When miR-125a-3p was cotransfected with the pRL-TK plasmid, the luciferase activity was decreased, but the luciferase activities of NC and Gm5532 MT were not affected (Figure 3B). During osteoclast differentiation, lncRNA-Gm5532 downregulation by siGm5532 significantly increased miR-125a-3p level (Figure 3C). These findings suggest that lncRNA-Gm5532 functions as a ceRNA by binding to miR-125a-3p. miR-125a-3p is the target of lncRNA-Gm5532.

miR-125a-3p suppresses osteoclast differentiation

During osteoclast differentiation, miR-125a-3p was downregulated on day 2. To determine whether miR-125a-3p is involved in osteoclast differentiation, we transfected RAW264.7 cells with miR-125a-3p mimics and incubated them with 50 ng/mL RANKL. Our results showed that miR-125a-3p mimics had a negative impact on

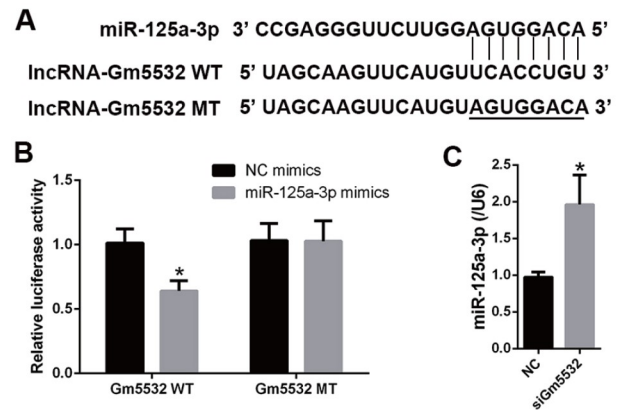


Figure 3. miR-125a-3p is the target of lncRNA-Gm5532 (A) Complementary sequences of lncRNA-Gm5532 and miR-125a-3p. (B) The putative binding sites of lncRNA-Gm5532 were mutated and inserted into the psiCHECK-2 plasmid. 293T cells cotransfected with miR-125a-3p mimics and Gm5532-WT or Gm5532-MT plasmid were detected for luciferase activity. (C) miR-125a-3p expression after siGm5532 treatment during osteoclast differentiation. Data are presented as the mean $\pm$ SD. * P < 0.05 vs NC mimics or NC.

osteoclast formation (Figure 4A), and bone resorption (Figure 4B). miR-125a-3p was downregulated on day 2 during osteoclast differentiation (Figure 4C). TRAP activity was downregulated on day 4 upon miR-125a-3p mimics treatment (Figure 4D). Accordingly, the expressions of marker genes associated with bone resorption, including CTSK, MMP9, NFATc1, Car2, and V-ATPase, were downregulated after miR-125a-3p mimic treatment (Figure 4E,F). These results suggest that miR-125a-3p is a negative regulator of osteoclast differentiation.

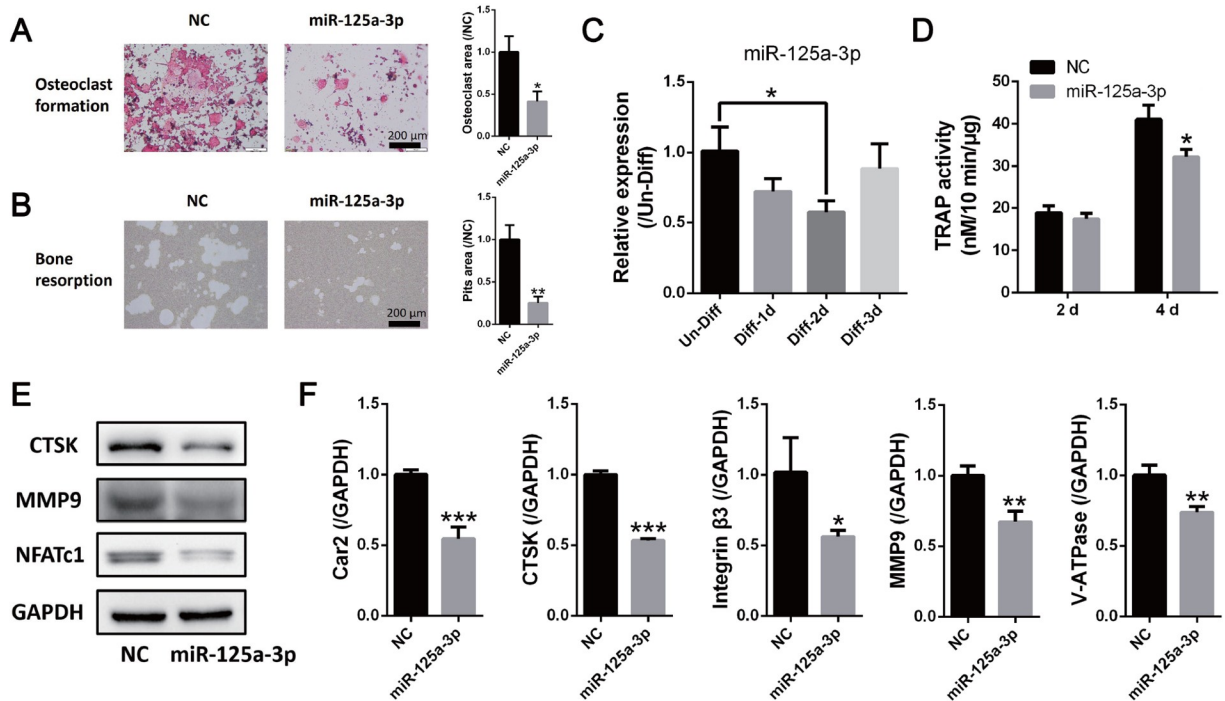


Figure 4. miR-125a-3p suppresses osteoclast differentiation (A) RAW264.7 cells were transfected with miR-125a-3p mimics and then incubated with 50 ng/mL RANKL. Osteoclast formation was analyzed by the area of TRAP-positive multinucleated cells. Scale bar: 200 μ m. (B) Bone resorption was analyzed by the area of resorption pits. Scale bar: 200 μ m. (C) miR-125a-3p expression during osteoclast differentiation. (D) TRAP activity was expressed as nanomoles of pNPP hydrolyzed per 10 min per microgram of protein. (E) Western blot analysis of the protein levels of CTSK, MMP9 and NFATc1. (F) mRNA expression of bone resorption-associated genes was measured on day 2. Data are presented as the mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 vs NC or Un-Diff.

lncRNA-Gm5532 regulates osteoclast differentiation by targeting miR-125a-3p

RAW264.7 cells were transfected with siGm5532 alone or siGm5532 combined with miR-125a-3p inhibitor to determine the regulatory role of lncRNA-Gm5532/miR-125a-3p in osteoclast differentiation. siGm5532 combined with miR-125a-3p inhibitor NC treatment increased the level of miR-125a-3p, but the increased expression was abrogated by miR-125a-3p inhibitor treatment (Figure 5A). The miR-125a-3p inhibitor upregulated osteoclast differentiation, including osteoclast formation, bone resorption and TRAP activity (Figure 5B–F). lncRNA-Gm5532 silencing inhibited osteoclast formation, bone resorption and TRAP activity. The negative effects were completely inhibited in the cells cotransfected with siGm5532 and miR-125a-3p inhibitor (Figure 5B–F). Western blot analysis results further demonstrated that siGm5532 decreased the levels of protein markers associated with osteoclast differentiation, including CTSK, MMP9 and NFATc1, while these effects could be reversed by miR-125a-3p inhibitor treatment (Figure 5G). These results suggest that lncRNA-Gm5532 regulates osteoclast differentiation by targeting miR-125a-3p.

TRAF6 is the target of miR-125a-3p

Bioinformatics analysis showed that the 3'UTR of the TRAF6 mRNA transcript had binding sites for miR-125a-3p (Figure 6A). We generated luciferase reporter constructs containing the TRAF6 3'UTR with or without a miR-125a-3p binding site-null mutant sequence and then tested their luciferase activity in 293T cells by cotransfection with miR-194-3p mimics and the pRL-TK plasmid. The results showed that miR-125a-3p had an obvious inhibitory

effect on TRAF6 3'UTR reporter activity (Figure 6B). Since miR-125a-3p was downregulated during osteoclast differentiation and TRAF6 was the potential target of miR-125a-3p, we speculate that TRAF6 expression should be increased during osteoclast differentiation. Our results demonstrated that TRAF6 expression was increased in RAW264.7-derived osteoclasts on day 2 (Figure 6C,D). miR-125a-3p mimics inhibited the mRNA expression of TRAF6 (Figure 6E). We further investigated the influence of miR-125a-3p on the TAK1/NF- κ B/MAPKs pathway, the downstream signaling pathway of TRAF6. Compared with NC, miR-125a-3p inhibited the phosphorylation of TAK1, IKK, I κ B α and p65 (Figure 6F). The phosphorylation of ERK, JNK and p38 was also downregulated after miR-125a-3p mimics treatment (Figure 6G). Collectively, TRAF6 is the target of miR-125a-3p, and miR-125a-3p regulates TRAF6 expression.

Discussion

Excessive bone resorption is an important cause of osteoporosis. The identification of novel molecules involved in osteoclast differentiation is of great importance for understanding the pathogenesis of osteoporosis and developing novel antiresorptive drugs with fewer side effects [9].

lncRNAs play pivotal roles in numerous biological processes and disease progression by modulating transcription, protein/RNA stability, translation, posttranslational modifications, and interaction with signaling molecules [10]. Some lncRNAs have been reported to be associated with osteoporosis risk by modulating bone resorption [5]. Actually, lncRNA profiles are altered during osteoclast differentiation. The altered potential of differentiation

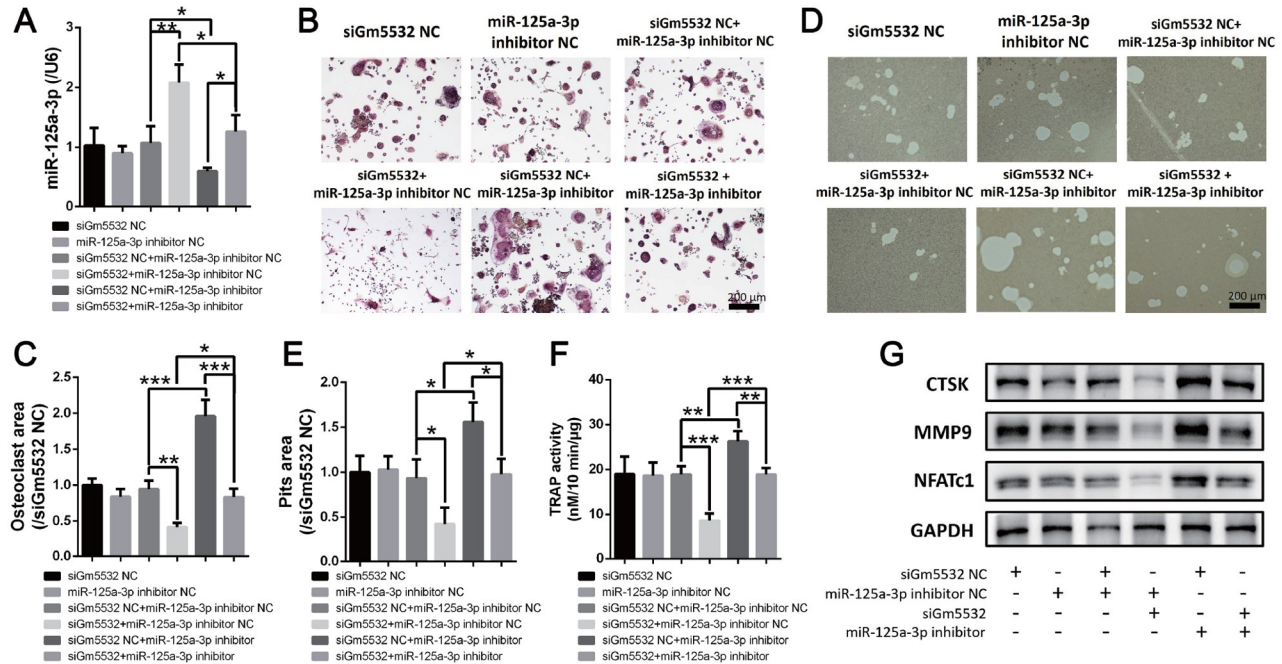


Figure 5. lncRNA-Gm5532 regulates osteoclast differentiation by targeting miR-125a-3p (A) RAW264.7 cells were cotransfected with siGm5532 and miR-125a-3p inhibitor. After that, the cells were incubated with 50 ng/mL RANKL. miR-125a-3p expression was detected on day 2. (B,C) Osteoclast formation was analyzed by the area of TRAP-positive multinucleated cells. Scale bar: 200 μ m. (D,E) Bone resorption was analyzed by the area of resorption pits. Scale bar: 200 μ m. (F) TRAP activity was expressed as nanomoles of pNPP hydrolysed per 10 min per microgram of protein. (G) Western blot analysis of the protein levels of CTSK, MMP9 and NFATc1. Data are presented as the mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001.

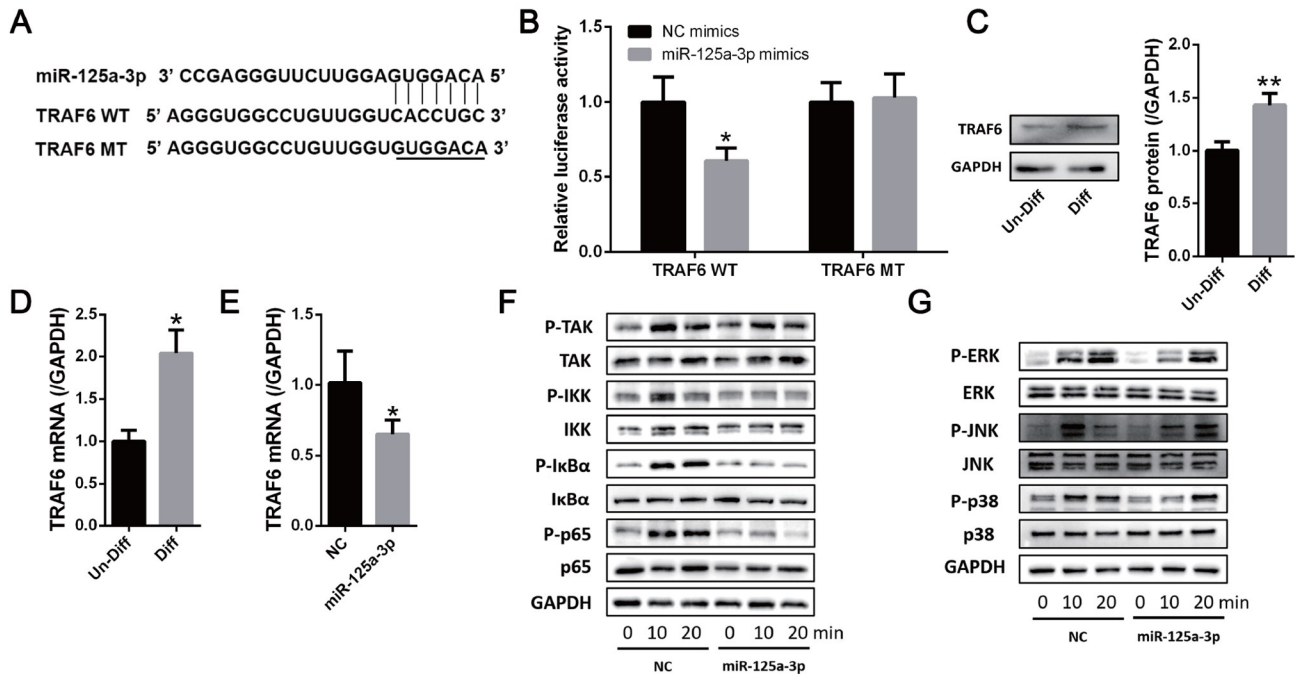


Figure 6. TRAF6 is the target of miR-125a-3p (A) Complementary sequences of TRAF6 and miR-125a-3p. (B) The putative binding sites of TRAF6 were mutated and inserted into the psiCHECK-2 plasmid. 293T cells cotransfected with miR-125a-3p mimics and TRAF6-WT or TRAF6-MT plasmid were detected for luciferase activity. (C) TRAF6 protein expression was detected on day 2 during osteoclast differentiation. (D) TRAF6 mRNA expression was detected on day 2 during osteoclast differentiation. (E) miR-125a-3p suppressed the mRNA expression of TRAF6 on day 2 during osteoclast differentiation. (F,G) After RAW264.7 cells were transfected with miR-125a-3p mimics, the cells were incubated with 50 ng/mL RANKL. Cell lysates were collected at 0 min, 10 min, and 20 min and immunoblotted with anti-phosphorylated and total TAK1, IKK, IkB α , p65, ERK, JNK, and p38 antibodies. * P < 0.05, ** P < 0.01, *** P < 0.001 vs NC mimic, Un-Diff, or NC.

also influences lncRNA profiles [11]. lncRNAs are involved in modulating osteoclast differentiation. For instance, lncRNA-XIST promotes osteoclast differentiation by interacting with fused in sarcoma (FUS) [12]. lncRNA-SOX2OT regulates osteoclast differentiation by functioning as a ceRNA via the miR-194-5p/Rac family small GTPase 1 (RAC1) axis [13]. lncRNA-SNHG15, as a ceRNA, modulates osteoclast differentiation, proliferation, and metastasis by sponging the miR-381-3p/NIMA-related kinase 2 (NEK2) axis [14]. lncRNA-Jak3 stimulates osteoclast differentiation via the Jak3/NFATc1/CTSK axis [15]. lncRNA-TUG1 positively regulates osteoclast differentiation by targeting v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MafB) [16]. lncRNA-Xist serves as a ceRNA of miR-590-3p to promote TGF- β -induced factor homeobox 2 (Tgif2) level and then promotes osteoclast differentiation [17]. lncRNA-Bmncr is expressed at low levels in the marrow and spleen of osteoporosis mice and inhibits RANKL-induced osteoclast differentiation [18]. lncRNA-Nron inhibits osteoclast differentiation by interacting with the E3 ubiquitin ligase cullin 4B (CUL4B) to regulate estrogen receptor α (ER α) stability [19]. lncRNA-HOTAIR promotes osteoclast differentiation by targeting the TGF- β /PTHrP/RANKL pathway [20]. lncRNA-Neat1 stimulates osteoclastogenesis by sponging miR-7 [21]. In the present study, we found that lncRNA-Gm5532 was highly expressed upon RANKL-induced osteoclast differentiation. Knockdown of lncRNA-Gm5532 attenuated osteoclast formation and bone resorption. lncRNA-Gm5532 is a novel molecule regulating osteoclast differentiation.

Accumulating evidence indicates that the competing endogenous RNA (ceRNA) network between lncRNAs and miRNAs affects cell differentiation. Through lncRNA2Target prediction and dual-luciferase reporter assay, we identified lncRNA-Gm5532 as a ceRNA by targeting miR-125a-3p. miR-125a-3p was reported to be involved in the progression of multiple diseases, including atherosclerosis [22], cataracts [23], rheumatoid arthritis [24], papillary thyroid carcinoma [25], breast cancer [26], and colorectal cancer [27]. In addition, miR-125a-3p is a potential regulator of bone homeostasis. miR-125a-3p inhibits osteoblast proliferation and differentiation by targeting G protein-coupled receptor kinase interacting protein 1 (GIT1) [28]. To date, there have been no studies regarding its role in osteoclasts. During osteoclast differentiation, miR-125a-3p was downregulated. miR-125a-3p mimics significantly inhibited osteoclast formation, bone resorption, TRAP activity, and the expressions of critical genes associated with osteoclast differentiation. miR-125a-3p is a negative regulator of osteoclast differentiation. Furthermore, miR-125a-3p stimulated osteoclast differentiation and reversed the inhibitory effects of lncRNA-Gm5532 downregulation on osteoclast differentiation.

Through bioinformatics predictions via TargetScan (http://www.targetscan.org/vert_72/) and dual-luciferase reporter assay, we identified TRAF6 as a potential target of miR-125a-3p. TRAF6 is a critical RANK downstream signaling molecule that regulates osteoclast differentiation, bone-resorbing function and the survival of mature osteoclasts. The binding of RANKL to its receptor RANK recruits TRAF6 to the conserved TRAF domains within the C-terminal cytoplasmic tail of RANK. Subsequently, TRAF6 activates the TAK1, NF- κ B, MAPK, NFATc1, and Src/PI3K/Akt pathways [29]. TRAF6 is essential for osteoclast differentiation. TRAF6 deficiency leads to osteopetrosis due to defects in NF- κ B signaling [30]. During osteoclast differentiation, TRAF6 was upregulated. miR-125a-3p mimic treatment reduced TRAF6 expression and

suppressed the TAK1/NF- κ B/MAPK pathway. TRAF6 is the target of miR-125a-3p.

In summary, in this study we identified a novel lncRNA, lncRNA-Gm5532, which regulates osteoclast differentiation by functioning as a competing endogenous RNA. lncRNA-Gm5532 knockdown decreased osteoclast formation and bone resorption. Mechanistically, lncRNA-Gm5532 competitively binds to miR-125a-3p and blocks its function by regulating TRAF6. Our results provide new insight into the mechanisms of osteoclast differentiation and suggest that lncRNA-Gm5532 may be a potential target for osteoporosis treatment as an antiresorptive drug.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

- Reid IR. A broader strategy for osteoporosis interventions. *Nat Rev Endocrinol* 2020, 16: 333–339
- Katsimbri P. The biology of normal bone remodelling. *Eur J Cancer Care* 2017, 26: e12740
- Park-Min KH. Metabolic reprogramming in osteoclasts. *Semin Immunopathol* 2019, 41: 565–572
- Aurilia C, Donati S, Palmieri G, Miglietta F, Iantomasi T, Brandi ML. The involvement of long non-coding RNAs in bone. *Int J Mol Sci* 2021, 22: 3909
- Liu W, Li Z, Cai Z, Xie Z, Li J, Li M, Cen S, *et al.* lncRNA-mRNA expression profiles and functional networks in osteoclast differentiation. *J Cell Mol Med* 2020, 24: 9786–9797
- Wan Y, Liu X, Zheng D, Wang Y, Chen H, Zhao X, Liang G, *et al.* Systematic identification of intergenic long-noncoding RNAs in mouse retinas using full-length isoform sequencing. *BMC Genomics* 2019, 20: 559
- Joshi M, Rajender S. Long non-coding RNAs (lncRNAs) in spermatogenesis and male infertility. *Reprod Biol Endocrinol* 2020, 18: 103
- Gonçalves GF, de Faria Poloni J, Dorn M. Transcriptomic analysis of long non-coding RNA during *Candida albicans* infection. *Genes* 2023, 14: 251
- Awasthi H, Mani D, Singh D, Gupta A. The underlying pathophysiology and therapeutic approaches for osteoporosis. *Med Res Rev* 2018, 38: 2024–2057
- Bridges MC, Daulagala AC, Kourtidis A. LNCcation: lncRNA localization and function. *J Cell Biol* 2021, 220: e202009045
- Tian Y, Shao Q, Gu J, Tang Y, Bie M, Zhou Y, Cheng C, *et al.* lncRNA-mRNA expression profiles of osteoclast after conditional knockout HIF-1 α . *Front Genet* 2022, 13: 909095
- Zhang DW, Wang HG, Zhang KB, Guo YQ, Yang LJ, Lv H. lncRNA XIST facilitates S1P-mediated osteoclast differentiation via interacting with FUS. *J Bone Miner Metab* 2022, 40: 240–250
- Ni J, Zhang X, Li J, Zheng Z, Zhang J, Zhao W, Liu L. Tumour-derived exosomal lncRNA-SOX2OT promotes bone metastasis of non-small cell lung cancer by targeting the miRNA-194-5p/RAC1 signalling axis in osteoclasts. *Cell Death Dis* 2021, 12: 662
- Wang Y, Zhu G, Pei F, Zhao Z. lncRNA SNHG15 as a ceRNA modulates

- osteoclast differentiation, proliferation, and metastasis by sponging miR-381-3p/NEK2 axis. *J Immunol Res* 2022, 2022: 8634820
15. Lee CP, Huang YN, Nithiyanantham S, Huang CM, Ko YC. LncRNA-Jak3: Jak3 coexpressed pattern regulates monosodium urate crystal-induced osteoclast differentiation through Nfatc1/Ctsk expression. *Environ Toxicol* 2019, 34: 179–187
 16. Du Y, Yu Q, Zheng X, Wang S. LncRNA TUG1 positively regulates osteoclast differentiation by targeting v-maf musculoaponeurotic fibrosarcoma oncogene homolog B. *Autoimmunity* 2020, 53: 443–449
 17. Shao Y, Hu X, Wu X. LncRNA X inactive-specific transcript promotes osteoclast differentiation through Tgfb2 by acting as a ceRNA of miR-590-3p in a murine model. *Regen Med* 2021, 16: 643–653
 18. Chen RS, Zhang XB, Zhu XT, Wang CS. LncRNA Bmncr alleviates the progression of osteoporosis by inhibiting RANML-induced osteoclast differentiation. *Eur Rev Med Pharmacol Sci* 2019, 23: 9199–9206
 19. Jin F, Li J, Zhang YB, Liu X, Cai M, Liu M, Li M, *et al.* A functional motif of long noncoding RNA Nron against osteoporosis. *Nat Commun* 2021, 12: 3319
 20. Zhang C, Yang J, Zhu Z, Qin J, Yang L, Zhao X, Su W, *et al.* Exosomal lncRNA HOTAIR promotes osteoclast differentiation by targeting TGF- β /PTHrP/RANKL pathway. *Basic Clin Pharma Tox* 2023, 132: 242–252
 21. Zhang Y, Chen XF, Li J, He F, Li X, Guo Y. LncRNA neat1 stimulates osteoclastogenesis via sponging miR-7. *J Bone Miner Res* 2020, 35: 1772–1781
 22. Xia F, Zeng Q. miR-125a-3p aggravates ox-LDL-induced HUVEC injury through BAMBI. *J Biochem Mol Tox* 2022, 36: e23198
 23. Kang L, Luo J, Li P, Zhang G, Wei M, Ji M, Guan H. miR-125a-3p regulates apoptosis by suppressing TMBIM4 in lens epithelial cells. *Int Ophthalmol* 2023, 43: 1261–1274
 24. Wang Y, Yin Z, Zhang N, Song H, Zhang Q, Hao X, Wang Z. MiR-125a-3p inhibits cell proliferation and inflammation responses in fibroblast-like synovial cells in rheumatoid arthritis by mediating the Wnt/ β -catenin and NF- κ B pathways via targeting MAST3. *J Musculoskelet Neuronal Interact* 2021, 21: 560–567
 25. Song M, Wang N, Li Z, Zhang Y, Zheng Y, Yi P, Chen J. miR-125a-3p suppresses the growth and progression of papillary thyroid carcinoma cell by targeting MMP11. *J Cell Biochem* 2020, 121: 984–995
 26. Zheng L, Meng X, Li X, Zhang Y, Li C, Xiang C, Xing Y, *et al.* miR-125a-3p inhibits ER α transactivation and overrides tamoxifen resistance by targeting CDK3 in estrogen receptor-positive breast cancer. *FASEB J* 2018, 32: 588–600
 27. Liang L, Gao C, Li Y, Sun M, Xu J, Li H, Jia L, *et al.* miR-125a-3p/FUT5-FUT6 axis mediates colorectal cancer cell proliferation, migration, invasion and pathological angiogenesis via PI3K-Akt pathway. *Cell Death Dis* 2017, 8: e2968
 28. Tu XM, Gu YL, Ren GQ. miR-125a-3p targetedly regulates GIT1 expression to inhibit osteoblastic proliferation and differentiation. *Exp Ther Med* 2016, 12: 4099–4106
 29. Zhang J. The osteoprotective effects of artemisinin compounds and the possible mechanisms associated with intracellular iron: a review of *in vivo* and *in vitro* studies. *Environ Toxicol Pharmacol* 2020, 76: 103358
 30. Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, Morony S, *et al.* TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* 1999, 13: 1015–1024