**IncRNA-Gm5532 regulates osteoclast differentiation through miR-125a-3p/TRAF6 axis**

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Short Communication

**IncRNA-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, IncRNA microarray analysis was used to identify differentially expressed lncRNAs in differentiated osteoclasts. IncRNA-Gm5532 is highly expressed during osteoclast differentiation. IncRNA-Gm5532 knockdown impairs osteoclast formation and bone resorption. Mechanistic experiments shows that IncRNA-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 suppresses osteoclast differentiation and TAK1/NF-κB/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)

### 1 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 remodelling 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 remodelling 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 a miRNA-125a-3p sponge. miRNA-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.

2 Materials and Methods

2.1 Cell culture and osteoclast differentiation

The murine monocyte/macrophage line RAW264.7 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/cm^2 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 with 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 by ImageJ software (National Institutes of Health, Bethesda, USA). TRAP activity was determined with 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 10% sodium hypochlorite and observed with a light microscope (Olympus). The resorption pits were analysed by 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).
2.2 LncRNA microarray analysis

Undifferentiated RAW264.7 cells and RAW264.7-derived osteoclasts on day 4 were lysed by TRIzol Reagent, 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. The labelled cRNAs were hybridized onto the microarray. After washing, the arrays were scanned by an Agilent Scanner G2505C (Agilent Technologies; Santa Clara, USA). Feature Extraction software (version 10.7.1.1, Agilent Technologies) was used to analyse 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.

2.3 siRNA and miRNA mimic transfection

Short interfering RNAs (siRNA) targeting LncRNA-Gm5532 and miRNA-125a-3p mimics were synthesized by GenePharma (Shanghai, China). The sequences of siGm5532 and miRNA-125a-3p mimics were as follows: siGm5532, sense 5'-GCUACUAGAGACCAUGAUATT-3', and antisense 5'-UAUCAUGGUCUCUAGUAGCTT-3'; miRNA-125a-3p mimics, sense 5'-ACAGGUGAGGUUCUUGGAGCC-3', and antisense 5'-CUCCAAGAACCUCACCUGUUU-3'; negative control (NC) siRNA, sense 5'-UUCUCCGAACGUGACGU-3', and antisense 5'-ACGUGACGUUCGAATT-3'; miR-125a-3p inhibitor, 5'-GGCUCCCAAGAACCUCACCUGU-3'; and inhibitor NC, 5'-CAGUACUUUUGUGUAGUACAAA-3'. After RAW264.7 cells were seeded overnight, siGM5532 and miRNA-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.

2.4 Quantitative PCR

Total RNA was extracted by an RNA isolation kit (Beyotime) and reverse transcribed with 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 following primer pairs 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 analysed via the $2^{-\Delta\Delta Ct}$ method.

2.5 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](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/](http://www.targetscan.org/vert_72/)) web tool. 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 with the dual-luciferase reporter assay system (Promega) and normalized to Renilla luciferase activity.

2.6 Western blot analysis

Total cellular protein was extracted with RIPA buffer (Beyotime). Cell lysates (20 μg per sample) were loaded and run on a 10% SDS-PAGE gel, transferred to a PVDF membrane (Merck Millipore, MA, 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. The bands were detected with a Bio-Rad Imaging System (Bio-Rad) 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),
IκBα (Beyotime), p-IκBα (Beyotime), p65 (Beyotime), and p-p65 (Beyotime). GAPDH was used as an internal control.

2.7 Statistical analysis

All experiments were performed in triplicate. Data were expressed as the mean ± 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.

3 Results

3.1 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.

3.2 lncRNA-Gm5532 regulates osteoclast differentiation

To explore whether lncRNA-Gm5532 modulated 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 significantly decreased pit formation (Figure 2C) and the expression 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.

3.3 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 LncRNA2Target 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.

3.4 miR-125a-3p suppresses osteoclast differentiation

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

### 3.5 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 levels 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 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.

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

Through bioinformatic analysis, 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
We further investigated the influence of miR-125a-3p on the TAK1/NF-κB/MAPKs pathway, the downstream signaling pathway of TRAF6. Compared with NC, miR-125a-3p inhibited the phosphorylation of TAK1, IKK, IκBα 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. miR-125a-3p regulates TRAF6 expression.

4 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 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-Bmnncr 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 (ERα) 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, lncRNA-Gm5532 was identified as a ceRNA by targeting miR-125a-3p. miR-125a-3p is 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 expression 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 bioinformatic predictions via TargetScan (http://www.targetscan.org/vert_72/) and dual-luciferase reporter assay, TRAF6 was identified 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-κB, MAPK, NFATc1, and Src/PI3K/Akt pathways [29]. TRAF6 is essential for osteoclast differentiation. TRAF6 deficiency leads to osteopetrosis due to defects in NF-κB signaling [30]. During osteoclast differentiation, TRAF6 was upregulated. miR-125a-3p mimic treatment reduced TRAF6 expression and suppressed the TAK1/NF-κB/MAPK pathway. TRAF6 is the target of miR-125a-3p.
In summary, we found a novel lncRNA, lncRNA-Gm5532, which regulated osteoclast differentiation by functioning as a competing endogenous RNA. lncRNA-Gm5532 knockdown decreased osteoclast formation and bone resorption. Mechanistically, lncRNA-Gm5532 competitively bound to miR-125a-3p and blocked 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 anti-resorptive drug.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

**Reference**


[18] Chen RS, Zhang XB, Zhu XT, Wang CS. LncRNA Bmncr alleviates the progression of


**Figure legends**

**Figure 1. lncRNA-Gm5532 was 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 were presented as the mean ± SD. ***P < 0.001 vs Un-Diff.

**Figure 2. lncRNA-Gm5532 knockdown suppressed 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 analysed by the area of TRAP-positive multinucleated cells. Scale bar: 200 μm. (C) Bone resorption was analysed by the area of resorption pits. Scale bar: 200 μm. (D) TRAP activity was expressed as nanomoles of pNPP hydrolysed 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 were presented as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs NC.

**Figure 3. miR-125a-3p was 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. 293 T cells cotransfected with miRNA-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 were presented as the mean ± SD. *P<0.05 vs NC mimics or NC.

Figure 4. miR-125a-3p suppressed osteoclast differentiation (A) RAW264.7 cells were transfected with miR125a-3p mimics and then incubated with 50 ng/mL RANKL. Osteoclast formation was analysed by the area of TRAP-positive multinucleated cells. Scale bar: 200 μm. (B) Bone resorption was analysed by the area of resorption pits. Scale bar: 200 μm. (C) miR-125a-3p expression during osteoclast differentiation. (D) TRAP activity was expressed as nanomoles of pNPP hydrolysed 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 were presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 vs NC or Un-Diff.

Figure 5. LncRNA-Gm5532 regulated osteoclast differentiation by targeting miR-125a-3p (A) RAW264.7 cells were cotransfected with siGm5532 and miR125a-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 analysed by the area of TRAP-positive multinucleated cells. Scale bar: 200 μm. (D,E) Bone resorption was analysed by the area of resorption pits. Scale bar: 200 μm. (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 were presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 6. TRAF6 was 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. 293 T cells cotransfected with miRNA-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, IκBα, p65, ERK, JNK, and p38 antibodies. *P<0.05, **P<0.01, ***P<0.001 vs NC mimic, Un-Diff, or NC.
A

RAW264.7
+RANKL
4 days

RAW264.7 derived osteoclasts

TRAP positive multinucleated cells

F. actin
Nucleus

Actin ring

B

CTSK
MMP9
NFATc1
GAPDH

Un-Diff
diff

C

Volcano Plot

D

140x78mm (300 x 300 DPI)
161x83mm (300 x 300 DPI)
A

miR-125a-3p 3' CCGAGGGUUUCUUGGAGUGGACA 5'

IncRNA-Gm5532 WT 5' UAGCAAGUUCAUGUUCACCUGU 3'
IncRNA-Gm5532 MT 5' UAGCAAGUUCAUGUAGUGGACA 3'

B

![Bar chart showing relative luciferase activity for Gm5532 WT and MT with NC mimics and miR-125a-3p mimics.](image)

C

![Bar chart showing miR-125a-3p (U6) levels for NC and siGm5532.](image)
172x89mm (300 x 300 DPI)
Table 1. Primers sequences used for quantitative real-time PCR

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<td>CCTTTGCCGTTGGGATTAC</td>
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<tr>
<td>GAPDH</td>
<td>TGCACCACCAACTGCTAG</td>
<td>GGATGCAGGGATGATGTC</td>
</tr>
<tr>
<td>Integrin β3</td>
<td>CCACCTCCAACCAATATCAC</td>
<td>CCAATCCCCACCATAC</td>
</tr>
<tr>
<td>MMP9</td>
<td>GCCCTGGAACCTCACCACGCA</td>
<td>TGGGAACATCCACGAGCCAGAA</td>
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<tr>
<td>V-ATPase</td>
<td>CCACTGGAAGCCCATCAGTAACAGA</td>
<td>GAACGATGAGGCAGGTGAC</td>
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High activation of osteoclast differentiation is regarded as one of the major reasons for osteoporosis occurrence. Here, we show that lncRNA-Gm5532 is involved in osteoclast differentiation through miR-125a-3p/TRAF6 axis. (1) lncRNA-Gm5532 is found to be highly expressed during osteoclast differentiation. lncRNA-Gm5532 knockdown impairs osteoclast formation and bone resorption. (2) miR-125a-3p acts as a sponge for LncRNA-Gm5532, and suppresses osteoclast differentiation. (3) TRAF6 is the target of miR-125a-3p. miR-125a-3p mimics inhibits TAK1/NF-κB/MAPK signaling, the downstream signaling of TRAF6.
RANKL
RANK
TRAF6

IncRNA-Gm5532

miR-125a-3p

Osteoclast differentiation

53x45mm (300 x 300 DPI)