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ORIGINAL ARTICLE

miR-142-5p promotes the osteoclast differentiation of bone marrow-derived macrophages via PTEN/PI3K/AKT/FoxO1 pathway

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Abstract

It is increasingly recognized that microRNAs (miRNAs) are a kind of important regulators, which are involved in the pathogenesis and development of various human diseases. However, the underlying effects and mechanism of miR-142-5p on the osteoclast differentiation of bone marrow-derived macrophages (BMMs) have not been elucidated. The aim of the present study is to explore the molecular mechanisms that regulate the osteoclastogenesis of BMMs for providing more efficient methods for treating bone-related diseases. In the present study, BMMs were isolated from rats and cultured. Moreover, receptor activators of NF-kB ligands were used to induce the osteoclast differentiation of BMMs. Furthermore, we analyzed the effects of miR-142-5p mimics/inhibitor on the osteoclastogenesis of BMMs. The results indicated that the downregulation of miR-142-5p inhibited the osteoclastogenesis of BMMs, whereas the overexpression enhanced this process. PTEN was testified to be a direct target of miR-142-5p, and its effects on the osteoclastogenesis were also described. Most importantly, treatment of LY29004 (an inhibitor of the PI3k/Akt pathway) can attenuate miR-142-5p osteoclastogenesis effects, while the inhibition effects of LY29004 on the osteoclastogenesis were abolished by knockdown of FoxO1. Taken together, our findings demonstrated that miR-142-5p promotes the osteoclastogenesis of BMMs through PI3k/Akt/FoxO1 pathway via targeting PTEN.

Keywords miR-142-5p · PTEN · FoxO1 · Osteoclast · Bone marrow-derived macrophages

Introduction

Osteoporosis (OP) is a systemic and metabolic bone disease, which is characterized by decreased bone mass, destructed bone microstructure and increased bone fragility and fracture [1]. OP can occur in different genders and ages, but commonly occurs in postmenopausal women and older men. Since the deficiency of estrogen is the main cause of OP, postmenopausal women are particularly susceptible to this disease. Though estrogen is significant in bone turnover and skeletal homeostasis, there are several other factors implicated in the pathogenesis of OP, including Wnt, Notch and NF-kB [2]. The balance between bone formation of osteoblasts and bone resorption of osteoclasts contributes to the development and remodeling of bone [3, 4]. The osteoclasts are multi-nucleated cells derived from hematopoietic stem cells, the formation and function of which are tightly regulated by the macrophage colony-stimulating factor (M-CSF) and receptor activator ligand of NF-kB (RANKL) [5]. M-CSF facilitates osteoclast differentiation by stimulating the expression of receptor activator of NF-kB (RANK) on osteoclast precursors. RANKL binds to RANK on osteoclast precursors and mature osteoclasts, leading to the recruitment of adaptor molecules including TNF receptor-associated factor 6 (TRAF6). TRAF6 can activate several downstream signaling pathways, such as NF-kB, MAPKs (ERK, JNK and p38), PI3K/

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AKT, AP-1 transcription factor family and NFATc1, initiating the formation and activity of osteoclast [6].

Recent studies have demonstrated that miRNAs are get trapped in the differentiation of osteoblast and osteoclast [7]. For example, miR-34 suppressed the osteoblast proliferation and differentiation in mice though targeting *SATB2* [8]. It was reported that the expression of miR-138 was decreased during osteogenic differentiation, which negatively regulated the osteogenesis of human bone marrow mesenchymal stem cell (hBMSCs) [9]. Other scholars also found that several miRNAs negatively regulated the differentiation of BMSCs, like miR-141, miR-31 and miR-200a [10, 11]. Furthermore, Teng et al. [12] found that miR-142-5p improved the OP, which is responsible for targeting adhesion molecule VCAM-1 and inhibiting cell migration. However, the regulatory role of miR-142-5p in osteoclast differentiation has not been found with sound evidence.

Phosphatase and tensin homolog (PTEN) is one of the most common tumor suppressor genes in human cancers and plays an important role in the regulation of cell growth and apoptosis [13]. Studies have been revealed that PTEN is targeted with many miRNAs. Meanwhile, the PI3K/AKT pathway is involved in the signal translation which is related to cell growth, proliferation, differentiation, motility, survival and metabolism [14]. Interestingly, PTEN negatively regulates the PI3K/AKT pathway. FoxO family is a type of transcription factors, predominant members of which include FoxO1, FoxO3a and FoxO4. Among them, FoxO1 is a major regulator of redox balance, which can stimulate the proliferation and differentiation and inhibit the apoptosis of osteogenic lineage cells [15]. As the downstream signal molecule of PI3k/AKT pathway, the activity of FoxO1 can also be eliminated by phosphorylation. Previous studies have shown that PTEN can be act as a target gene for multiple miRNAs, thereby affecting the process of osteoclast differentiation. For instance, miR-214 can promote osteoclastogenesis by regulating PI3K/AKT pathway via targeting PTEN [16]. Moreover, overexpression miRNA-196a enhanced cell proliferation and inhibited cell apoptosis through PTEN/PI3K/Akt/FoxO1 pathway [17]. However, the modulating regulation between miR-142-5p and PTEN, as well as the role of FoxO1 in RANKL-induced osteoclast differentiation of BMMs, still needs further investigation.

In our study, we examined the dynamic expression of miR-142-5p and PTEN before and after osteoclast differentiation. Our findings indicated that miR-142-5p was involved in the efficient regulation of osteoclast differentiation through targeting PTEN. And we also found that miR-142-5p regulates osteoclast differentiation through PTEN/PI3K/AKT/FoxO1 pathway. This evidence prompted us to conclude that miR-142-5p may serve as a novel therapeutic target for BMMs osteoclastogenesis-related disorders.

Materials and methods

Cells cultivation and differentiation induction

For isolation of BMMs, SD rats were killed and soaked in 75% ethanol for 10 min, and then, femurs and tibias were dissected under sterile condition and washed three times with PBS. After removing epiphyses, bones were flushed with α-MEM supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) and 100 U/ml penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO). The isolated bone marrow samples were made into single cell suspension and centrifuged at 1500 rpm/min for 10 min, and next 5 ml of erythrocyte lysate was used to lyse red blood cells for 5 min. Then, the lysis was stopped by 10 ml α-MEM, and the samples were centrifuged at 1500 rpm/min for 10 min. After the supernatant was discarded, the cells were resuspended in a concentration of 1×10^9/L. To obtain BMMs, BMCs were cultured in α-MEM supplemented with 10% FBS and M-CSF (10 ng/ml) for 1 day. Non-adherent cells were further cultured in the presence of M-CSF (30 ng/ml) for 3 days. Subsequently, the adherent cells were used as BMMs. To induce osteoclast differentiation, BMMs were incubated into six-well plates (1×10^5 cells/well, 2 ml medium per well) and cultured normally. After 2 days of cultivation, the media were replaced with osteoclast-specific induction medium, mainly composed of DMEM, 10% FBS, 30 ng/mL M-CSF and 50 ng/mL RANKL, which were added to the cells. The medium was changed every 3 days. All experiments performed using SD rats were approved by the Animal Experimental Ethical Inspection of the First Affiliated Hospital of Kunming Medical University.

Luciferase assay and transfection

A pmirGLO Dual-Luciferase miRNA target expression vector was used for 3′-untranslated region (UTR) luciferase assays (Promega, Madison, WI, USA). Mimics, inhibitor and negative control oligonucleotides for miR-142-5p were obtained from RiboBio Co. Ltd. (GenePharma, Shanghai, China). 293T cells were plated (5×10^4 cells per well) in 24-well plates, and cells in each well were co-transfected with miR-142-5p and wild-type or mutant target sequence using Lipofectamine 2000 (Invitrogen). Cells were then harvested 48 h after transfection, and the activities of firefly and Renilla luciferases were measured by using the Dual-Luciferase Reporter Assay System with the miR-control set at 1.0. Moreover, cells were seeded in six-well plates (1×10^6 cells/well, 2 ml medium per well) and cultured normally for 24 h. After one-day cultivation

(to 90–95% confluence), the media were replaced. The fresh medium without serum and penicillin–streptomycin was added to the cells. siRNA AS1842856 was used to construct FoxO1 knockdown. The synthesis of miR-142-5p mimics, miR-142-5p inhibitor and negative control oligonucleotides was purchased from Invitrogen (Carlsbad, CA, USA). The synthesis of FoxO1 siRNA (AS1842856) was conducted by Shanghai Sangon Company. The synthetics were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). qRT-PCR was used to detect the results of transfection.

Cell proliferation CCK-8 assay

Cell proliferation assay was performed using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). According to the manufacturer's protocol, the cells were seeded in a 96-well plate and the total volume of each well was 100 µl (1×10^5 cells in each well); three duplicated wells were set for each group. After cells were treated with different doses of RANKL for 24 h, 10 µl of CCK-8 solution was added to each well and then the plate was kept in a humidified incubator at 37 °C with 5% CO_2. The OD value was recorded after 0, 1, 3 and 5 days of culture. The absorbance was measured at 450 nm wavelength on a microplate reader (Molecular, USA). The proliferation rate of the cells was calculated by the following formula: cell viability = (the OD values of treated groups/the OD values of control group) × 100%.

TRAP and phalloidin staining

For TRAP staining, BMMs were transfected with miR-142-5p mimic, inhibitor, NC or other treatments and cultured for 3 days in a 96-well plate (0.5×10^4 cells/well) in the presence and absence of 50 ng/mL RANKL. TRAP was stained using an acid phosphatase leukocyte kit (Sigma). In our study, TRAP-positive cells were defined as those that contained three or more nuclei. Meanwhile, to further verify the formation of multi-nucleated cells challenged with miR-142-5p mimic and RANKL, phalloidin staining assay was performed. Briefly, cells were fixed in 3.7% formaldehyde/PBS solution for 3 min and washed three times with PBS. 0.1% Triton X/PBS was used to penetrate osteoclasts; after adding DAPI for 10 min and being washed with PBS, 400 µl of phalloidin-conjugated AF633 was added to each well. Osteoclasts were determined by laser scanning confocal microscope.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Trizol reagent (LifeTech, USA) was used to extract total RNA from cells according to the manufacturer's instruction. After that, cDNA was then synthesized using 1 µg total RNA as a template and RevertAid^{TM} First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Quantitative real-time PCR (qRT-PCR) analyses were performed with SYBR® Premix Ex Taq™ (Takara, Japan) using a StepOnePlus Real-Time PCR System (Applied Biosystems). The PCR amplification included an initial denaturation at 95 °C for 1 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 2 min and extension for 30 s at 72 °C. Results of the log-linear phase of the growth curve were analyzed and relative quantification was performed using the $2^{-\Delta\Delta CT}$ method with β-actin as a housekeeping gene. U6 snRNA was used as internal controls to normalize the expression levels of miRNAs. The primers were synthesized by Shanghai Sangon Company. The primer sequences were as follows: U6 forward primer 5′-GCTTCGGCAGCACATATACTAAAAT-3′ and reverse primer 5′-CGCTTCACGAATTTGCGTGTCAT-3′; miR-142-5p forward primer 5′-CCGGTCA TAAAGTAGAAAGC-3′ and reverse primer 5′-GTGCAGGGTCCGAGGT-3′; miR-142-3p forward primer 5′-GCCCTGTAGTGTTTCCTACTT-3′ and reverse primer 5′-GTGCAGGGTCCGAGGT-3′; miR-214 forward primer 5′-GCCCTGTAGTGTTTCC TACTT-3′ and reverse primer 5′-GTGCAGGGTCCGAGGT-3′; miR-455-3P forward primer 5′-GCAGTCCACGGGCATATACAC-3′ and reverse primer 5′-GTGCAGGGTC CGAG-3′; miR-5112 forward primer 5′-TAGCTCAGCGGGAGAGCA-3′ and reverse primer 5′-GTGCAGGGTCCGAG-3′; miR-92a-3p forward primer 5′-TATTGCACTTG TCCCGGCCT-3′ and reverse primer 5′-GTGCAGGGTCCGAG-3′; Acp5 forward primer 5′-GCGACCATTGTTAGCCACATACG-3′ and reverse primer 5′-CGTTGATG TCGCACAGAGGGAT-3′; MMP-9 forward primer 5′-GATCCCCAGAGCGTTACTC G-3′ and reverse primer 5′-GTTGTGGAAACTCACACGCC-3′; Ctsk forward primer 5′-TCCTCAACAGTGCAAGCGAA-3′ and reverse primer 5′-CCAGCGTCTATCAGC ACAGA-3′; Clcn7 forward primer 5′-CTGTTCTTGTGGAGTTTGGTCC-3′ and reverse primer 5′-ATCCAGCTCCACGTTGTTCA-3′; PTEN forward primer 5′-AGGCCCTGGATTTTTATGGGG-3′ and reverse primer 5′-AGCGCCTCTGACTGG GAATA-3′; Nfatc1 forward primer 5′-GTGCAAGCCAAATTCCCTGG-3′ and reverse primer 5′-TCAGAGCTGGCTCAAAGTCG-3′; ATP6v0d2 forward primer 5′-GTTAGCCCAAGCCGAAGACT-3′ and reverse primer 5′-CTACGGCGTCAAACA AAGGC-3′; β-actin forward primer 5′-GCAGGAGTACGATGAGTCCG-3′ and reverse primer 5′-ACGCAGCTCAGTAACAGTCC-3′.

Protein extraction and western blot analysis

Total protein was extracted, and protein concentration was quantified using a BCA protein assay kit (Beyotime, Shanghai, China). A total of 20 µg of protein from each sample

was used for western blot. The samples were separated by SDS–PAGE (10%) at 200 V, 300 mA for 50 min. After transferring the proteins onto polyvinylidene fluoride membranes, the blotting was performed at 200 V, 300 mA for 45 min. After blocking with 5% (w/v) dry milk in TBS for 1 h at room temperature, membranes were incubated with the primary antibodies. The primary antibodies used in this study included monoclonal anti-PTEN (1:1000), FoxO1 (1:500), AKT (1:500), Trap (1:1000) and p-AKT (1:500, Cell Signaling Technology, Beverly, MA), Nfatc1(1:1000), MMP-9 (1:500), cathepsin K (1:500) and anti-Acp-5 (1:500, Proteintech, Chicago, IL, USA) and anti-β-actin polyclonal antibody (1:2000, Santa Cruz, CA, USA) at 4 °C overnight. Then, the membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse antibody (Cell Signaling Technology, Beverly, MA) for 2 h at room temperature. Finally, the blots were developed with an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA) and the bands were quantified densitometrically using a Bio-Rad imaging system (Hercules, CA). β-Actin was used as the loading control. The relative band intensity of each sample was normalized to β-actin signal in the same lane.

Statistical analysis

Statistical difference between two groups was analyzed using Student's *t* test. Differences among three or above groups were analyzed using one-way analysis of variance and Tukey's post hoc test. Data analysis was performed at GraphPad Prism 5(GraphPad Software, La Jolla, CA, USA) and presented as mean ± SD. Difference was considered as significant when $p < 0.05$.

Results

miR-142-5p is upregulated in M-CSF and RANKL-induced osteoclastogenesis

To investigate the role of miR-142-5p in osteoclastogenesis, qRT-PCR was performed. BMMs osteoclast precursor cells were induced by RANKL. The results showed that six miRNAs were significantly increased during the differentiation of BMMs cells into osteoclasts (Fig. 1a), including miR-92a-3p, miR-142-3p, miR-5112, miR-142-5p, miR-455-3p and miR-214. Among them, miR-142-5p was the most obviously upregulated miRNA. BMMs were cultured with M-CSF (10 ng/ml) alone for 1 day, followed by M-CSF (30 ng/ml) and RANKL (50 ng/ml) for 0, 3 and 5 days, respectively. The expressions of miR-142-5p were progressively increased during the process of osteoclastogenesis with a time-dependent manner (Fig. 1b). The expressions of miR-142-5p were significantly increased on day 3 ($p < 0.05$) and day 5 ($p < 0.01$). Moreover, the expression of miR-142-5p on day 5 nearly up to sixfold of that before induction. Additionally, with the increase in induction time, the expressions of PTEN were gradually decreased (Fig. 1c). As shown in Fig. 1d-f, the expressions of Acp5 and Trap were both significantly increased on day 3 ($p < 0.01$) and day 5 ($p < 0.01$). The results suggested that miR-142-5p may play an important role in osteoclastogenesis.

Overexpression miR-142-5p promotes osteoclastogenesis

To evaluate the effects of miR-142-5p on osteoclast differentiation, BMMs osteoclast precursor cells were treated with miR-142-5p mimics or inhibitor in the course of osteoclastogenesis after M-CSF and RANKL induction for 5 days. The results showed that miR-142-5p expression was substantially upregulated by mimics treatment ($p < 0.01$), but markedly decreased by its inhibitor ($p < 0.05$, Fig. 2a). Acp5, MMP-9, Clcn7 and Ctsk are important markers for the differentiation and activity of osteoclast [18]. After 5 days of induction, the expressions of Acp5, Clcn7, Mmp9 and Ctsk were significantly increased in mimics treatment group, but reversed by inhibitor compared with treatment negative control, respectively ($p < 0.05$, Fig. 2b). Functionally, the number of TRAP-positive cells, multi-nucleated osteoclasts per well, was substantially increased in the miR-142-5p overexpression group, but markedly suppressed by the inhibitor (Fig. 2c, d). These observations were supported by phalloidin staining assay showing miR-142-5p mimic expressing more actin sealing rings than the one in control and while remarkably inhibited by its inhibitor (Fig. 2c, e). These results showed that overexpression miR-142-5p can promote osteoclastogenesis.

PTEN is a target of miR-142-5p

Using the miRNA target prediction program PicTar (http://pictar.mdc-berlin.de/) and miRNA. org (http://www.microrna.org/microrna/home.do), we obtained PTEN which is a target gene of miR-142-5p, playing a critical role in RANKL-induced osteoclast differentiation [17]. Therefore, we validated the interaction between PTEN and miR-142-5p. We found that 3′-UTR of rat PTEN (WT) contains a putative region that was complementary to the seed sequence of rno-miR-142-5p (Fig. 2f). Moreover, to explore whether miR-142-5p targets PTEN, we examined the effects of miR-142-5p inhibitor, miR-142-5p mimics and NC on luciferase activity in BMMs cells which were transfected with luciferase reporters containing the wild-type (WT) PTEN or mutant (Mut) PTEN, respectively. The results showed that the luciferase reporter activity of WT was significantly decreased by miR-142-5p mimic,

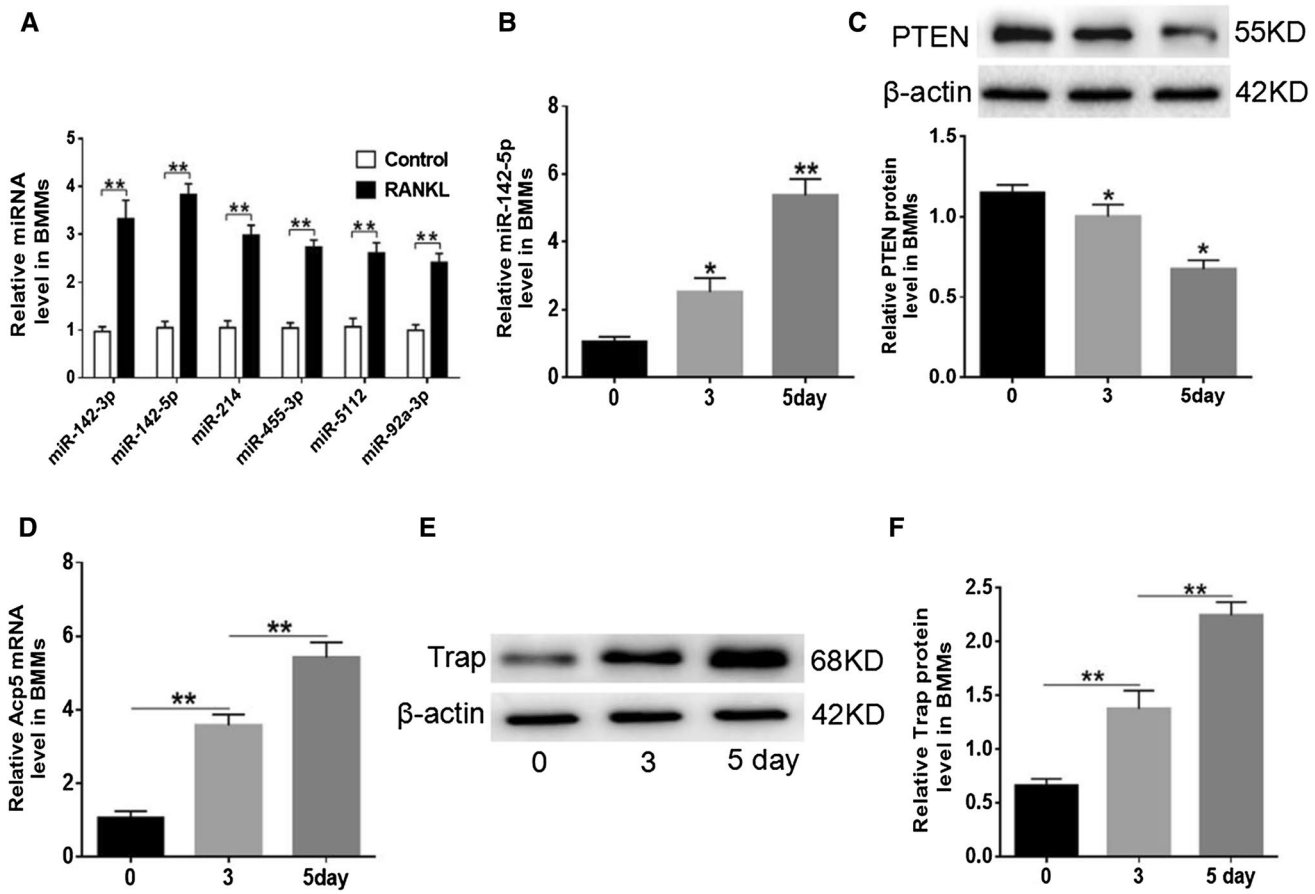

Fig. 1 miR-142-5p was upregulated during osteoclastogenesis. **a** Relative miRNA levels in RANKL-induced BMMs were analyzed by qRT-PCR; miRNA levels were normalized to U6. **b** Relative miR-142-5p levels in M-CSF + RANKL-induced BMMs were analyzed by qRT-PCR. **c** Relative PTEN protein levels in M-CSF + RANKL-induced BMMs were analyzed by western blot. **d** Relative Acp5 mRNA expression in M-CSF + RANKL-induced BMMs was analyzed by qRT-PCR. **e**, **f** Relative trap levels in M-CSF + RANKL-induced BMMs were analyzed by western blot. $^{*}p < 0.05$; $^{**}p < 0.01$

while the effect was abolished by miR-142-5p inhibitor (Fig. 2g). Meanwhile, the miR-142-5p mimics had no significant effect on the luciferase activity in the Mut one. The results demonstrated that PTEN was physically associated with miR-142-5p via their binding site. Moreover, the luciferase reporter activity was increased after reducing the endogenous levels of miR-142-5p by treating with miR-142-5p inhibitor. We then examined the effect of miR-142-5p mimics and miR-142-5p inhibitor on the PTEN mRNA and protein expression in RANKL-induced BMMs osteoclast precursors. The results demonstrated that miR-142-5p mimics leads to a decrease in PTEN protein level in RANKL-induced BMMs, and miR-142-5p inhibitor leads to an increase in PTEN protein level compared with the cells transfected with miR-142-5p-negative control (Fig. 2h, i). However, there was no detectable change in PTEN mRNA level (Fig. 2j). These results showed that PTEN is the target of miR-142-5p in osteoclast differentiation.

miR-142-5p promotes osteoclast differentiation through PTEN-involved pathway

It has been demonstrated that PTEN regulates RANKL-induced osteoclast differentiation from RAW 264.7 osteoclast precursors through PI3K/AKT pathway [17]. To test the effects of miR-142-5p on the PTEN/AKT pathway during RANKL-induced osteoclast differentiation, BMMs cells were treated with RANKL, RANKL with miR-142-5p inhibitor, miR-142-5p mimics and miR-142-5p mimics with PI3K inhibitor (LY29004), respectively. Intracellular miR-142-5p levels were upregulated by RANKL induction and decreased by miR-142-5p inhibitor treatment. LY29004 has no effect on the expression of miR-142-5p (Fig. 3a). We also verified that LY29004 can significantly inhibit the expressions of Nfatc1 and p-AKT in BMMs ($p < 0.05$, Fig. 3b–d) and can remarkably increase the FoxO1 protein expression ($p < 0.05$, Fig. 3e, f).

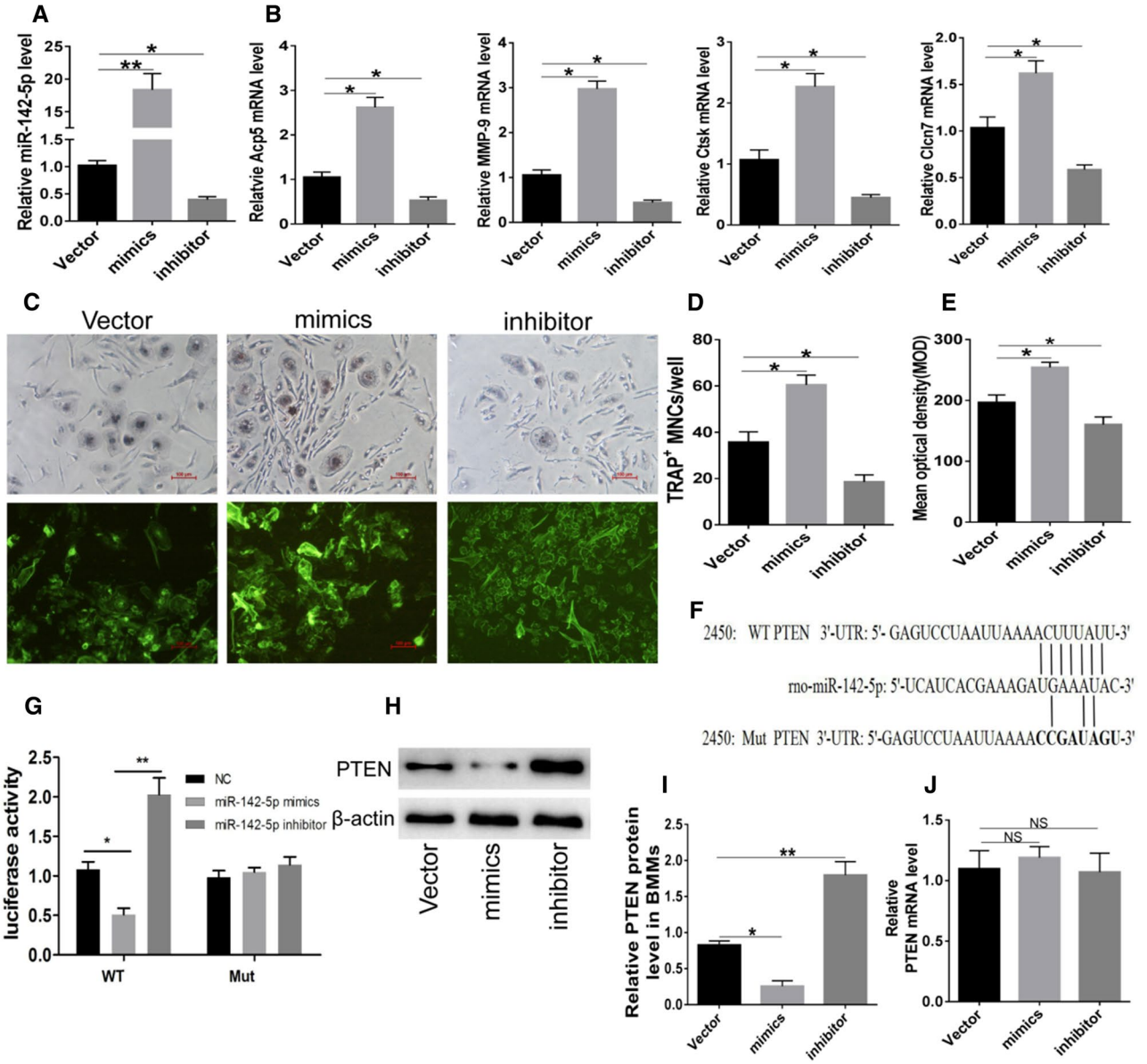

Fig. 2 miR-142-5p directly targets PTEN to regulate the osteoclastogenesis of BMMs. **a**–**b** Expressions of miR-142-5p, Acp5, Mmp9, Ctsk and Clcn7 after cells treated with miR-142-5p mimics, inhibitor and negative control (vector). **c** Osteoclast formation was measured by TRAP and phalloidin staining, respectively. **d** The number of TRAP-positive multi-nucleated cells (MNCs) is shown. **e** The ring structure of F-actin (Green) was evaluated by optical density. **f** Sequence alignment of rat miR-142-5p with 3′-UTR of PTEN. **g** The effects of miR-142-5p mimics, inhibitor and NC on luciferase activity in M-CSF + RANKL-induced BMMs transfected with the wild-type (WT) PTEN or mutant (MUT) PTEN 3′-UTR receptors, respectively. (**h**–**j**) Effects of miR-142-5p mimics, inhibitor and NC on PTEN expressions by western blot analysis and qRT-PCR. (*$p < 0.05$; **$p < 0.01$; NS, no significance)

To explore the effects of FoxO1 on the PI3K/AKT pathway during RANKL-induced osteoclast differentiation, BMMs cells were treated with RANKL, RANKL with miR-142-5p inhibitor, miR-142-5p mimics, miR-142-5p mimics with PI3K inhibitor (LY29004), miR-142-5p mimics with PI3K inhibitor (LY29004) plus FoxO1 inhibitor (AS1842856), respectively. As shown in Fig. 3e, f, knockdown FoxO1 by AS1842856 significantly decreased the protein expression of FoxO1. In addition, the effect of AS1842856 on FoxO1 expression was inhibited by LY29004. Meanwhile, we further detected the expressions of PTEN, cathepsin K, Nfatc1, Acp5 and MMP-9 in BMMs. The results demonstrated that cathepsin K, Nfatc1, Acp5 and MMP-9 expressions were upregulated after RANKL induction. Furthermore, miR-142-5p mimics increased the expression of cathepsin K, Nfatc1, Acp5 and MMP-9 by decreasing the PTEN protein level in the process, which can be inhibited by LY29004. Moreover, these effects of LY29004

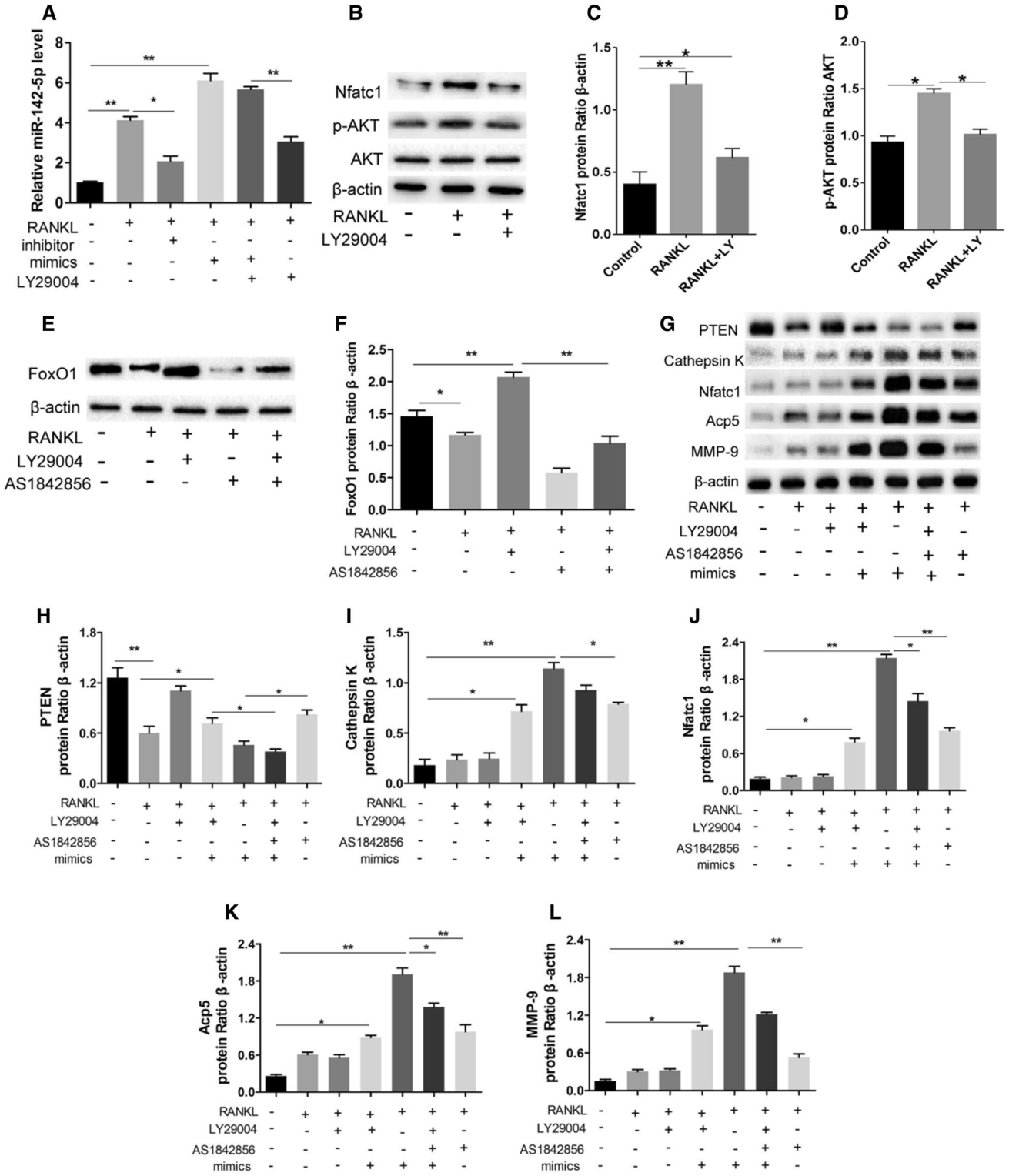

Fig. 3 miR-142-5p promotes osteoclast differentiation through PTEN-involved pathway. **a** miR-142-5p expressions were determined by qRT-PCR in BMMs after treating with RANKL, miR-142-5p mimics, miR-142-5p inhibitor and LY29004 (PI3K inhibitor). **b**, **d** Effects of RANKL and LY29004 on the expressions of Nfatc 1 and p-AKT in BMMs. **e**, **f** Effects of RANKL, LY29004 and AS1842856 (FoxO1-siRNA) on the expressions of FoxO1 in BMMs. **g**–**l** Effects of miR-142-5p mimics, LY29004 and AS1842856 on the expressions of PTEN, cathepsin K, Nfatc1, Acp5 and MMP-9 in BMMs, which were analyzed by western blot. (*$p<0.05$; **$p<0.01$)

can be markedly reversed by AS1842856 (Fig. 3g–l). These data suggested that miR-142-5p regulates RANKL-activated PI3K/AKT/FoxO1 signaling pathway for osteoclast differentiation by targeting PTEN.

miR-142-5p promotes the osteoclast activity

To investigate the role of miR-142-5p in osteoclasts activity, we detected the proliferation of BMMs with a CCK-8 assay. The results showed that the treatment of RANKL can inhibit BMMs proliferation, which can be significantly inhibited by miR-142-5p mimics and enhanced by miR-142-5p inhibitor (Fig. 4a). Moreover, the activity of TRAP as the mark of osteoclast differentiation is also detected in our study. The results indicated that RANKL can markedly increase the activity of TRAP, which can be enhanced by miR-142-5p mimics. Nevertheless, the effect of miR-142-5p mimics can be attenuated by LY29004 but promoted by AS1842856 (Fig. 4b). On the other hand, the results of TRAP staining and bone resorption assay showed that LY29004 can markedly attenuate the effect of miR-142-5p mimics in promoting osteoclast differentiation of BMMs and improved by AS1842856 (Fig. 4c, d). These data suggested that miR-142-5p regulates RANKL-activated osteoclast differentiation through PI3K/AKT/FoxO1 signaling pathway.

Discussion

Emerging reports have demonstrated that miRNAs take part in the progression of osteoclast and osteoblast differentiation via regulation of expression of multiple target genes related to the development and progression [16]. Therefore, identification of specific miRNAs and their targets related

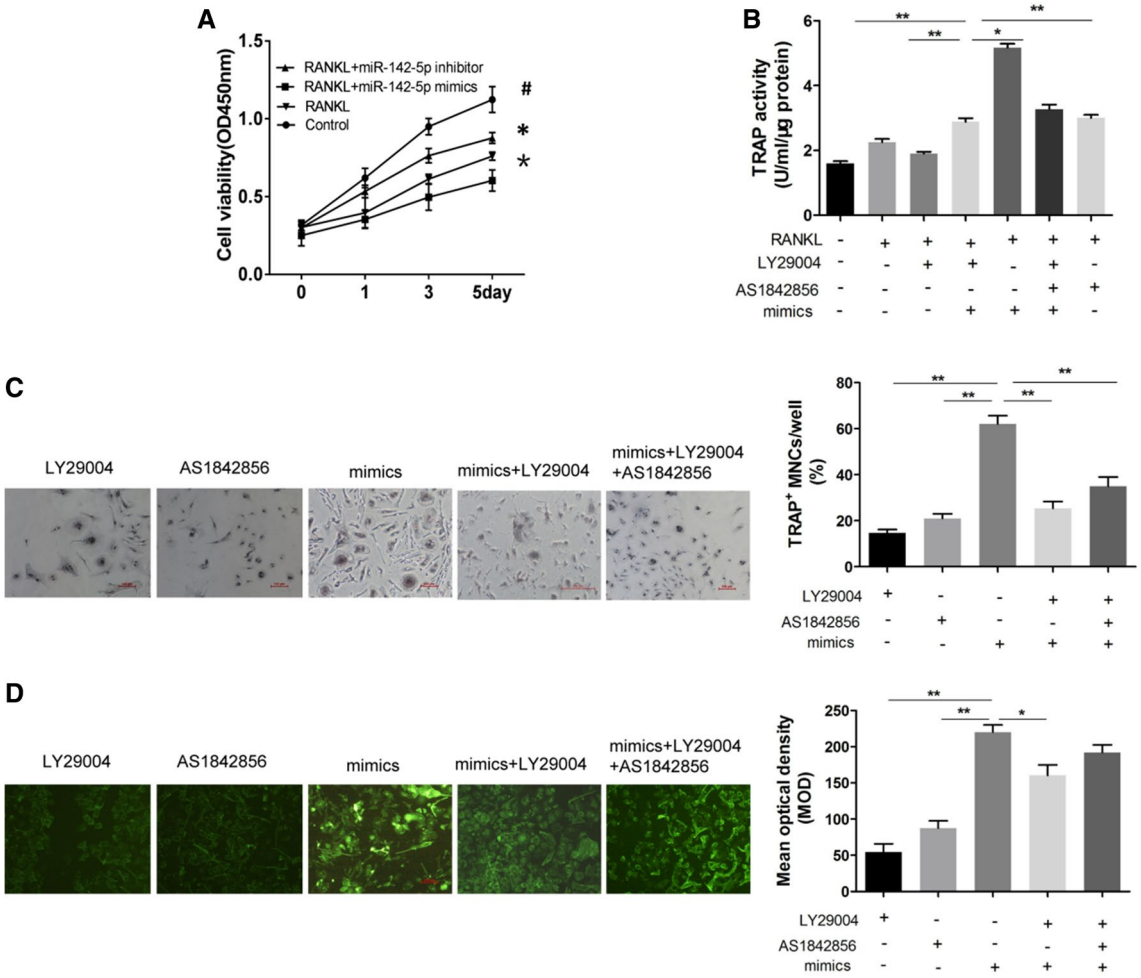

Fig. 4 miR-142-5p promotes osteoclast activity. **a** Viability of osteoblasts treated with RANKL or transfected with miR-142-5p mimics/inhibitor was determined by CCK-8 assay. **b** Effects of miR-142-5p mimics, LY29004 and AS1842856 on the activities of TRAP were detected by ELISA assay. **c**, **d** Effects of miR-142-5p mimics, LY29004 and AS1842856 on the osteoclast formation (the number of TRAP-positive multi-nucleated cells was analyzed) and the ring structure of F-actin was evaluated and image analysis was performed. (*$p < 0.05$; **$p < 0.01$)

to osteoclast differentiation would supply valuable insight for the diagnosis and treatment of patients with human OP. Additionally, increasing miRNAs are found as negative factors involved in the regulation of osteoclast differentiation [7, 8, 19]. Only a few miRNAs have been found to promote osteoclast differentiation [20]. However, in the present study, we identified miR-142-5p can act as an important enhancer of osteoclast differentiation of BMMs. Moreover, we also found that miR-142-5p is upregulated during RANKL-induced osteoclast differentiation from BMMs. In vitro, miR-142-5p can promote osteoclast differentiation by targeting PTEN.

PTEN acts as a major tumor suppressor gene, and its deletion, mutation or gene silencing has been reported in many cancers [21]. PTEN mediated growth and survival factor signaling by dephosphorylating phosphatidylinositol 3, 4, 5-triphosphate (PIP3) and decreasing the activity of class I phosphatidylinositol 3-kinases (PI3K), through PI3K effectors such as AKT and mTOR [22]. It has been found that microRNA-196a overexpression promotes cell proliferation and inhibits cell apoptosis through PTEN/AKT/FoxO1 pathway [17]. Moreover, studies indicated that miR-145 regulates osteogenic differentiation of human adipose-derived mesenchymal stem cells through targeting FoxO1 [15] and resveratrol prevents OP by upregulating FoxO1 transcriptional activity [23], which all emphasized the pivotal effects of FoxO1 on the osteoclastogenesis and differentiation. Hence, based on the previous studies, we believe that FoxO1 plays a key role during osteoclast differentiation, yet its regulation by miR-142-5p in rat BMMs has not been elucidated until the present study. Therefore, we focused on the investigation of the biological roles of miR-142-5p via targeting PTEN and PI3k/Akt/FoxO1 pathway on the progression of osteoclastogenesis.

Acp-5, MMP9, integrin av and cathepsin K are osteoclast-specific differentiation markers, and these expressions can indirectly reflect the degree of osteoclasts differentiation. Cathepsin K is a key protease that matures osteoclasts to degrade collagen, plays a role in osteoclastogenesis, and determines the bone resorption activity of osteoclasts. Additionally, TRAP staining, F-actin staining and the activity of TRAP can also reflect the osteoclasts differentiation. In the present study, we found that miR-142-5p can target PTEN, increase the expression of AKT, p-AKT, Acp-5, MMP9, integrin and cathepsin K and increase the positive results of TRAP and F-actin staining. Moreover, the miR-142-5p inhibitor can produce contrary results and the effects of miR-142-5p mimics can be attenuated by LY29004 and promoted by AS1842856. Together, our study indicated that miR-142-5p regulates osteoclasts differentiation of BMMs through PTEN/PI3K/AKT/FoxO1 pathway.

In summary, our study provided evidence that miR-142-5p is involved in the process of osteoclasts differentiation of BMMs via PTEN/PI3K/AKT/FoxO1 signal pathway. Moreover, our findings suggested that miR-142-5p and its target gene PTEN may be potential targets for the therapy of human osteoclast-related disorders. However, we have only studied the effect of miR-142-5p on regulating osteoclast differentiation via PTEN target in vitro. Therefore, in vivo studies and osteoclast-related studies still need to be performed.

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Conflict of interest Authors declare that they have no conflict of interest.

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