LAPTM5 is transactivated by RUNX2 and involved in RANKL trafficking in osteoblastic cells

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Abstract. The present study aimed to investigate the role of lysosomal-associated transmembrane protein 5 (LAPTM5) in osteoclast differentiation induced by osteoblasts. The results demonstrated that the expression levels of LAPTM5 were downregulated following runt-related transcription factor 2 (RUNX2) silencing and upregulated following RUNX2 overexpression in ST2 cells. Chromatin immunoprecipitation analysis identified the binding of RUNX2 to the LAPTM5 promoter at the -1176 to -1171 position. Dual-luciferase reporter assays confirmed that RUNX2 directly activated the LAPTM5 gene. The concentration of receptor activator of nuclear factor-κB ligand (RANKL) protein in the cytoplasm and in the media was significantly increased following LAPTM5 knockdown. LAPTM5 silencing in ST2 cells enhanced osteoclastic differentiation of co-cultured RAW264.7 cells. The present study indicated that expression of LAPTM5 was regulated by the interaction of RUNX2 with its promoter region and that LAPTM5 was involved in the trafficking of RANKL. These findings suggested a possible coupling mechanism between osteogenesis and osteoclastogenesis in which RUNX2 may be involved in osteoclast differentiation through the regulation of the lysosome-associated genes that modulate RANKL expression.

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Introduction

The coupling of osteogenesis and osteoclastogenesis is mediated through intercellular signaling during bone remodeling (1). Receptor activator of nuclear factor-κB ligand (RANKL) is a central player in osteoblast-induced osteoclast differentiation (2). RANKL is expressed in osteoblasts and osteocytes and binds the receptor activator of nuclear factor-κB on the surface of osteoclast precursors to induce osteoclast differentiation through the NF-κB pathway (2). Osteoprotegerin (OPG) functions as a soluble decoy receptor of RANKL, and thereby inhibits osteoclastogenesis (2).

Runt-related transcription factor 2 (RUNX2) is a master transcription factor in osteogenesis (3). The targeted disruption of RUNX2 results in a complete loss of bone formation owing to the arrest of osteoblast maturation (4). Transgenic mice overexpressing RUNX2 in osteoblasts show increased osteoclast differentiation and dramatically enhanced bone resorption (5). The dual role of RUNX2 in osteogenesis and osteoclastogenesis indicates its involvement in integrating signals between osteoblasts and osteoclasts (6). RUNX2 transactivates many essential genes in osteogenesis (7,8), yet its role in osteoclastogenesis remains unclear. There are putative RUNX2 binding sites in the RANKL promoter sequence (9), however, RUNX2 does not significantly affect RANKL expression in osteoblasts (10). It has also been reported that RUNX2 may regulate osteoclastogenesis through RANKL trafficking (6). This observation suggests that RUNX2 may target genes required for the regulation of the intracellular trafficking of RANKL.

A previous genomic analysis reported that the forced expression of RUNX2 upregulated several genes related to intracellular trafficking (11); one of these genes encoded for lysosomal-associated protein transmembrane 5 (LAPTM5). LAPTM5 is a transmembrane protein that resides in lysosomes and functions as a regulator of protein trafficking (12). The transport of LAPTM5-positive vesicles from the Golgi to the lysosome is modulated by its binding to the E3 ubiquitin ligase NEDD4, a HECT-type E3 ligase that belongs to the Nedd4 family (13). LAPTM5 is involved in the negative regulation of antigen receptor expression as a mediator of lysosomal degradation (14-16). The role of LAPTM5 in lysosomal function and its possible regulation by RUNX2 led to the hypothesis

that LAPTM5 may be a modulator of RANKL trafficking in osteoblasts, the assessment of which could further the understanding of the coupling of osteogenesis and osteoclastogenesis. In the present study, this hypothesis was initially tested by analyzing RUNX2 binding sites in the LAPTM5 promoter to investigate their role in transcription regulation. In addition, the role of LAPTM5 in the regulation of RANKL expression was assessed.

Materials and methods

Cell culture. The bone marrow-derived mesenchymal pluripotent cell line ST2 was a gift from Dr Xin Ye of Shandong University. The murine monocyte/macrophage cell line RAW264.7 was purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Primary osteoblasts (POBs) were isolated from the calvaria of female newborn C57BL/6 mice, as previously described (17). A total of 12 mice were purchased from Guangdong Medical Laboratory Animal Center. Cell isolation was performed upon arrival of the mice. The procedure was approved by The Institutional Animal Care and Use Committee of Guangzhou Medical University. The cells were cultured in α-minimal essential medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The cells were co-cultured using Transwell chambers (Corning, Inc.). For co-culture, RAW264.7 cells were cultured in 24-well plates and ST2 cells were cultured in Transwell chambers and were supplemented with 10 nM 1,25-$(OH)_2D_3$ (Sigma-Aldrich; Merck KGaA) and 100 nM dexamethasone (Sigma-Aldrich; Merck KGaA). To explore the effect of co-culture on osteoclastogenesis, RAW264.7 cells cultured with transwell containing no ST2 cells served as the control.

Reverse transcription-quantitative PCR (RT-qPCR). The cells in culture plates were washed three times with PBS. TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to isolate the RNA. The concentration and purity of the total RNA were measured using a UV spectrophotometer. First-strand complementary DNA synthesis was performed using M-MLV Reverse Transcriptase kit (Promega Corporation) according to the manufacturer's instruction. qPCR was performed using a Real-Time Quantitative PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) with ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme Biotech Co., Ltd.) based on SYBR-Green method. The thermal condition was 40 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 30 sec. The primers used are listed in Table I. β-Actin was used as the control gene. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression level of each mRNA (18).

Western blotting. Cells were washed three times with PBS and lysed in 50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and a protease inhibitor cocktail. Total protein concentrations were determined using the bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Protein samples (20 μg) were separated by 8% SDS-PAGE, transferred to PVDF membranes and soaked in a blocking solution (5% nonfat dry milk and 0.1% Tween-20) for 30 min at room temperature. The following primary antibodies were used: RUNX2 (clone D1H7; 1:1,000; cat. no. 8486; Cell Signaling Technology, Inc.), LAPTM5 (clone H-178; 1:500; cat. no. sc-134676; Santa Cruz Biotechnology, Inc.) and GAPDH (1:5,000; cat no. HC301; Beijing Transgen Biotech Co., Ltd.). The secondary antibodies used were Peroxidase-AffiniPure goat anti-rabbit or anti-mouse immunoglobulin G (1:10,000; cat. nos. 111-035-003 and 115-035-003; Jackson ImmunoResearch Laboratories, Inc.). Protein bands were visualized using an ECL kit (Forevergen). GAPDH was used as an endogenous control.

ELISA. The concentration of RANKL in the medium was determined using the Mouse TNFSF11/RANKL PicoKine™ ELISA kit (cat. no. EK0843; Wuhan Boster Biological Technology, Ltd.) in accordance with the manufacturer's instructions.

Fluorescence microscopy. Immunofluorescence staining was performed as previously described (19). Briefly, cell monolayers were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.01% Triton X-100 in PBS at room temperature for 10 min. After blocking with 3% BSA at room temperature for 1 h, cells were incubated with a primary anti-RANKL mouse monoclonal antibody (1:50; cat. no. ab45039; Abcam) at 4˚C overnight, followed by incubation with an Alexa Fluor 555 donkey anti-mouse secondary antibody (1:500; cat. no. ab150106; Abcam) in the dark at room temperature for 1 h. The coverslips were mounted using DAPI solution (Invitrogen; Thermo Fisher Scientific, Inc.). Slides were viewed using a fluorescent microscope (Imager Z1; Zeiss AG). The fluorescence intensity was analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc.).

Tartrate-resistant acid phosphatase (TRAP) staining. RAW264.7 cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature for 30 min. Cells were then stained with a TRAP kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Cells containing three or more nuclei were counted as positive.

Transfections. The RUNX2 overexpression plasmid was synthesized with LV003 vector by Forevergen. Small interfering (si) RNA targeting RUNX2 (5'-CCACTTACCACAGAGCTAT-3') and control sequences siRNA (5'-UUCUCCGAACGUGUC ACGUTT-3') were purchased from Forevergen. Short hairpin (sh) RNA (5'-GGTAAAGTGTCCTGTAGGTT-3') targeting LAPTM5 was purchased from General Biosystems, Inc. The shRNA sequences were inserted into the pSicoR vector (Forevergen), according to the manufacturer's protocol. Empty pSicoR vector was used as control. Transfections were carried out using lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.; 2 μg overexpression plasmid, 2 μg shRNA, 200 pmol si RNA, per well in 6-well plate), according to the manufacturer's protocol, respectively.

Construction of the LAPTM5 promoter-luciferase reporter plasmids. Promoter fragments of LAPTM5 were obtained from ST2 cells by PCR amplification using KOD-Plus-Neo with 10X PCR Buffer for KOD-Plus-Neo (Toyobo Life

Table I. Primers used in reverse transcription-quantitative PCR.

Target	Forward (5'-3')	Reverse (5'-3')
Oscar	CCTCCAGACTCCACCAGATA	GGAAATAAGGCACAGGAAGG
TRAP	GCCCCAAAGAAATGACCATC	CTGTAAGTAAGCCCCTTGGT
Nfatc1	GTCTCTTTCCCCGACATCAT	TCTCCAAGTAACCGTGTAGC
RUNX2	GACTGTGGTTACCGTCATGGC	ACTTGGTTTTTCATAACAGCGGA
RANKL	AGGCTGGGCCAAGATCTCTA	GTCTGTAGGTACGCTTCCCG
LAPTM5	CGTACCTCAGGATGGCTGAC	CAAGCTTCAAGTACGCTGGC
β-actin	AGACCTCTATGCCAACACAG	ACTCATCGTACTCCTGCTTG

RUNX2, runt related transcription factor 2; LAPTM5, lysosomal-associated transmembrane protein 5; RANKL, receptor activator of nuclear factor-κB ligand; Oscar, osteoclast-associated immunoglobulin-like receptor; TRAP, tartrate-resistant acid phosphatase; Nfatc1, nuclear factor of activated T cells 1.

Science). The following primers were used: -714 to -1 forward, 5'-ATCGAGCTCGTTTTTTCATCTGTGCAATGGGG-3' and reverse, 5'-CTAGCTAGCGGTGCGCAGTCCCCTCTTC-3'; and -1572 to -1 forward, 5'-ATCGAGCTCGATTAAGCTATCCCCCCAGTGC-3' and reverse, 5'-CTAGCTAGCGGTGCGCAGTCCCCTCTTC-3'. The thermal condition was 35 cycles of 94˚C for 20 sec, 58˚C for 30 sec and 68˚C for 2 min and 15 sec. The amplified fragment was isolated and purified following 1% agarose gel electrophoresis using the EasyPure Quick Gel Extraction kit (Beijing TransGen Biotech) and digested with *Sac*I and *Nhe*I. The fragment was ligated into the equivalent sites of the pGL3-BASIC vector (Promega Corporation) to yield pGL3-714, which contains no RUNX2 binding sites, and pGL3-1572, which contains several RUNX2 binding sites. A DNA fragment (-1 to -1572) with a substitution mutation (-1176 to -1171; ACCACA to ACTGTA) was obtained from GeneRay Biotech Co., Ltd. to construct pGL3-1572m.

Dual-luciferase reporter assays. Cells were co-transfected with LAPTM5 promoter constructs and a *Renilla* luciferase plasmid (pRL-TK; Promega Corporation) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were harvested 48 h after transfection, and the activities of firefly and *Renilla* luciferases were assessed using the Stop & Glo kit (Promega Corporation). A vector without the promoter was used as a negative control. pGL3-1572 and pGL3-1572m were co-transfected with the RUNX2 overexpression plasmids, using an empty vector as a control.

Chromatin immunoprecipitation (ChIP). ChIP assays were carried out using an EZChIP kit (cat. no. 17-371; Merck KGaA), according to the manufacturer's protocol. Briefly, 1% formaldehyde was added to the medium to crosslink DNA-bound proteins to chromatin. After incubation of 10 min at room temperature, unreacted formaldehyde was quenched with 0.125 mol/l glycine. Cells were harvested and resuspended in 1 ml of SDS lysis buffer containing a protease inhibitor cocktail and the DNA was sheared by sonication (amplitude: 20%; for 3 min and 5 sec ON, 10 sec OFF) (JY88-IIN Ultrasonic Homogenizer; Ningo Scientz Biotechnology Co., Ltd.). The fragmented DNA was diluted 10-fold with dilution buffer [0.01% SDS, 1% Triton X-100, 1.2 mmol/l EDTA, 167 mmol/l NaCl, 16.7 mmol/l Tris-HCl (pH 8.1)] containing protease inhibitor cocktail (Merck KGaA). After preclearing with protein G agarose slurry (Merck KGaA), 5% of the supernatant was collected as input DNA. To the remaining supernatant, 5 μg RUNX2 antibody (1:500; cat. no. 8486; Cell Signaling Technology, Inc.) or control immunoglobulin G (1:500; cat. no. 2729; Cell Signaling Technology, Inc.). was added and incubated at 4˚C overnight. The immunoprecipitated complex was centrifuged (5,000 x g for 1 min at 4˚C) and washed with low salt, high salt, LiCl and TE buffers in the kit (EZChIP, Merck KGaA), according to the manufacturer's protocols. The complex was eluted from the antibody using a solution of 1% SDS, 0.1 mol/l $NaHCO_3$ and 200 mmol/l NaCl. The DNA-protein crosslinking was reversed by incubation with 5 M NaCl at 65˚C overnight. All samples were treated with RNase for 30 min and proteinase K at 37˚C for 2 h. DNA was purified using spin columns provided with the kit. Samples were subjected to qPCR (as described above). Primers specific for the LAPTM5 promoter region were used (Table II).

Statistical analysis. Statistical analysis was conducted using IBM SPSS version 22.0 for Windows (IBM Corp.). The experiments were repeated three times. All data were presented as the mean ± SD. Statistical differences between two groups were assessed using unpaired Student's t-test. Statistical differences among multiple groups were assessed using one-way ANOVA and the Newman-Keuls test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Altered expression of RUNX2, LAPTM5 and RANKL in ST2 cells co-cultured with RAW264.7 cells. ST2 and RAW264.7 cells were co-cultured using Transwell chambers for 5 days. The expression levels of the osteoclast-specific genes osteoclast-associated immunoglobulin-like receptor (Oscar), TRAP and nuclear factor of activated T cells 1 (Nfatc1) were upregulated significantly in RAW264.7 cells following co-culture with ST2 cells (Fig. 1A-C). The number of TRAP-positive RAW264.7 cells also increased significantly in the co-culture (Fig. 1D-F). These results suggested that the

Table II. Primers used in chromatin immunoprecipitation.

Name	Forward (5'-3')	Reverse (5'-3')	Product
P1	CGGTTCTCAACCTTCCTG	ACAGTTATGAGGTAGCAACA	-1034 to -930 (105 bp)
P2	AACGCACAATCCCAGGTTTC	GGGCTTCTCACACATCTCCA	-1262 to -1071 (192 bp)
P3	CTGGGGGCCGTTTCTAATCTC	CCTGGGATTGTGCGTTCTTC	-1495 to -1247 (249 bp)

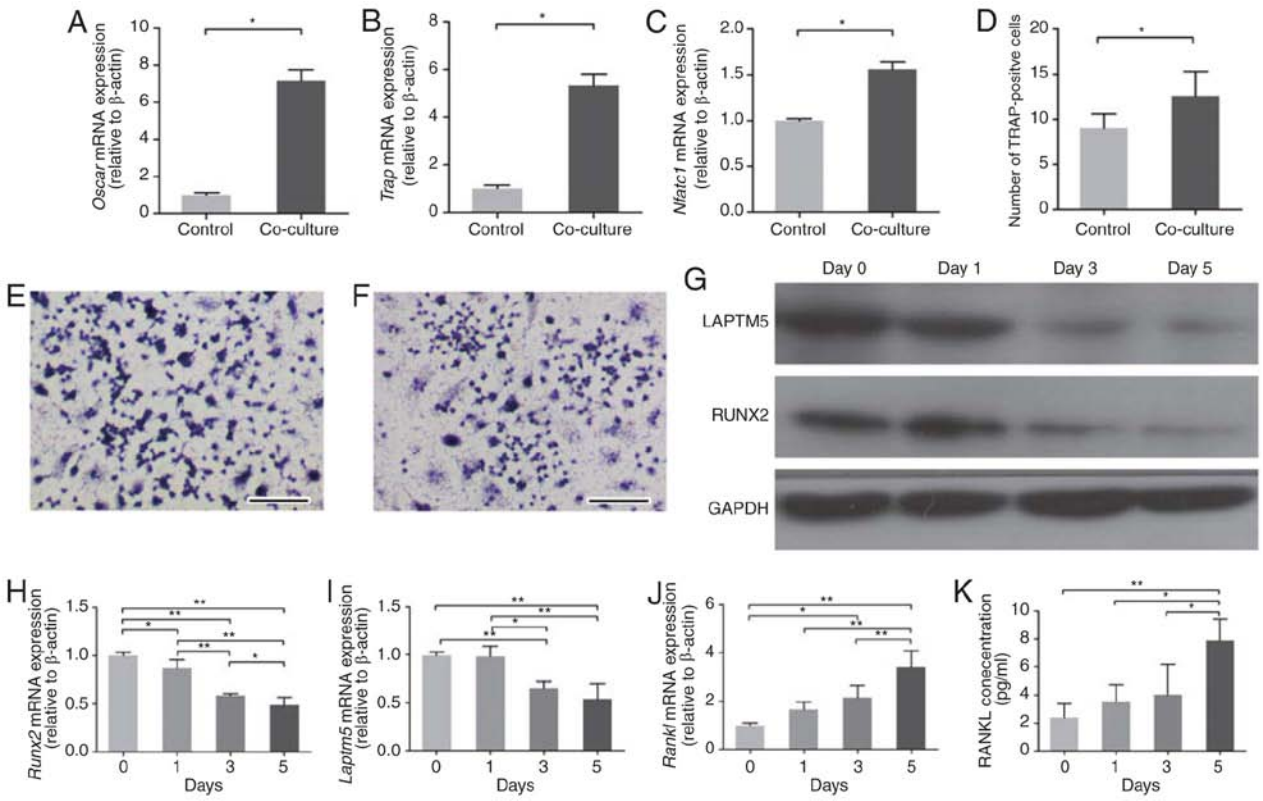

Figure 1. Altered expression of RUNX2, LAPTM5 and RANKL in ST2 cells co-cultured with RAW264.7. (A) In the RAW264.7 cells, the mRNA expression levels of Oscar, (B) TRAP and (C) Nfatc1 were detected following co-culture with ST2 cells. (D) The number of TRAP-positive RAW264.7 cells increased significantly after 5 days of co-culture with ST2 cells. (E) Representative TRAP-stained images of control and (F) co-cultured RAW264.7 cells. Scale bar, 500 μm. (G) Western blotting of RUNX2 and LAPTM5 protein expression levels in ST2 cells following the initiation of co-culture. A representative image is shown from two independent repeats. (H) mRNA expression levels of RUNX2, (I) LAPTM5 and (J) RANKL were detected in ST2 cells following the indicated days of co-culture. (K) Levels of secreted RANKL protein in the media were detected by ELISA. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01. Oscar, osteoclast-associated immunoglobulin-like receptor; TRAP, tartrate-resistant acid phosphatase; Nfatc1, nuclear factor of activated T cells 1; RUNX2, runt related transcription factor 2; LAPTM5, lysosomal-associated transmembrane protein 5; RANKL, receptor activator of nuclear factor-κB ligand.

osteoclastic differentiation of RAW264.7 cells was enhanced following co-culture with ST2 cells.

Next, the effect of the co-culture on the ST2 cell gene expression was examined. The mRNA expression levels of RUNX2, LAPTM5 and RANKL in ST2 cells exhibited time-dependent alterations following co-culture with RAW264.7 cells. Runx2 mRNA was downregulated significantly on day 1 of co-culture, and the downregulation continued over the following days (Fig. 1H). The protein levels of RUNX2 showed a similar trend (Fig. 1G). The expression levels of LAPTM5 mRNA were downregulated significantly on day 3 and 5 of co-culture (Fig. 1I). Accordingly, LAPTM5 protein expression was also markedly decreased on day 5 (Fig. 1G). The mRNA expression levels of RANKL were upregulated throughout the experiment (Fig. 1I). The secreted RANKL protein levels in the culture medium were measured using ELISAs and the results demonstrated that RANKL secreted levels increased significantly on day 5 of o-culture (Fig. 1J).

Expression of LAPTM5 is associated with RUNX2. To explore the underlying association between RUNX2 and LAPTM5 expression, RUNX2 was silenced in ST2 cells using siRNA (Fig. 2A and C). RUNX2 silencing in ST2 cells resulted in reduced expression of LAPTM5 at the mRNA and protein levels (Fig. 2B and C). Overexpression of Runx2 in ST2 cells (Fig. 2D and F) resulted in a significant increase in the expression of LAPTM5 at the mRNA and protein levels (Fig. 2E and F). To further investigate the association between these two genes, RUNX2 was also silenced (Fig. 2G and I) or overexpressed (Fig. 2J and L) in POBs. The expression of LAPTM5 was decreased following RUNX2 silencing (Fig. 2H and I), while the expression of LAPTM5 was increased following Runx2

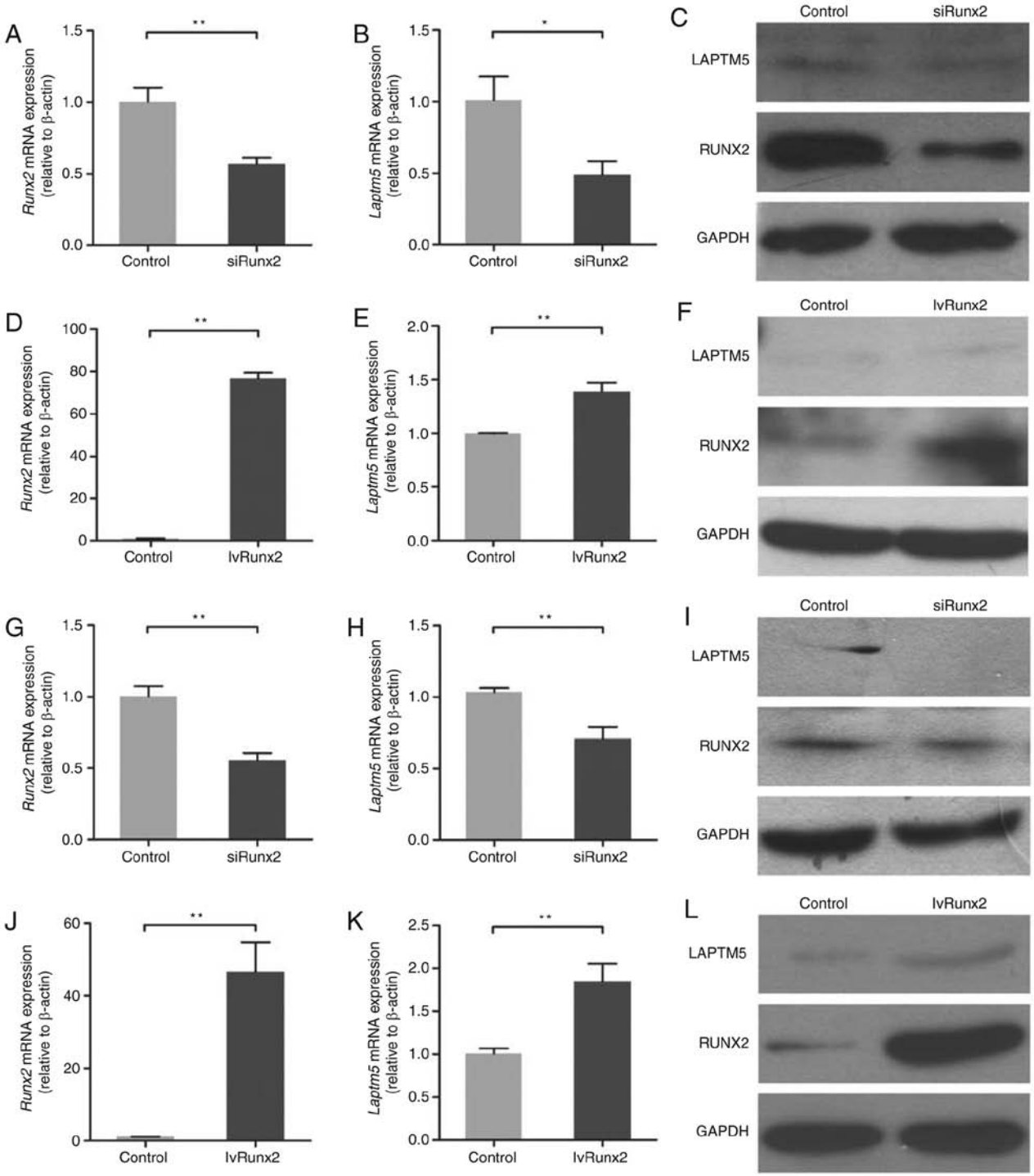

Figure 2. Expression of LAPTM5 is associated with RUNX2. (A) RUNX2 was silenced in ST2 cells using siRNA. (B) LAPTM5 mRNA expression levels were reduced following RUNX2 silencing. (C) Downregulation of RUNX2 and LAPTM5 protein expression was determined by western blotting. (D) Runx2 was overexpressed in ST2 cells. (E) LAPTM5 mRNA expression levels were increased following RUNX2 overexpression. (F) Upregulation of RUNX2 and LAPTM5 protein expression was determined by western blotting. (G) RUNX2 was silenced in POBs using siRNA. (H) LAPTM5 mRNA expression levels were inhibited in POBs following RUNX2 silencing. (I) Downregulation of RUNX2 and LAPTM5 protein expression was determined by western blotting. (J) RUNX2 was overexpressed in POBs. (K) LAPTM5 mRNA expression levels were increased following RUNX2 overexpression. (L) Upregulation of RUNX2 and LAPTM5 protein expression was determined by western blotting. Western blot images are representative from two independent repeats. Data are presented as the mean ± SD of three independent experiments (n=3). Cells transfected with a scramble sequence were used as a control. *P<0.05, **P<0.01. RUNX2, runt related transcription factor 2; LAPTM5, lysosomal-associated transmembrane protein 5; si, small interfering.

overexpression (Fig. 2K and L). These results suggested that the expression of LAPTM5 may be associated with that of RUNX2.

RUNX2 regulates LAPTM5 expression by binding to its promoter region. To test the hypothesis that LAPTM5 may be a target gene regulated by RUNX2, the 5'-flanking region (-1 to -1500) of the LAPTM5 gene was analyzed manually and three putative RUNX2 binding sites (ACCACA) were identified [-1012 to -1007/-980 to -975 (P1), -1176 to -1171 (P2), and -1456 to -1451 (P3); Fig. 3A].

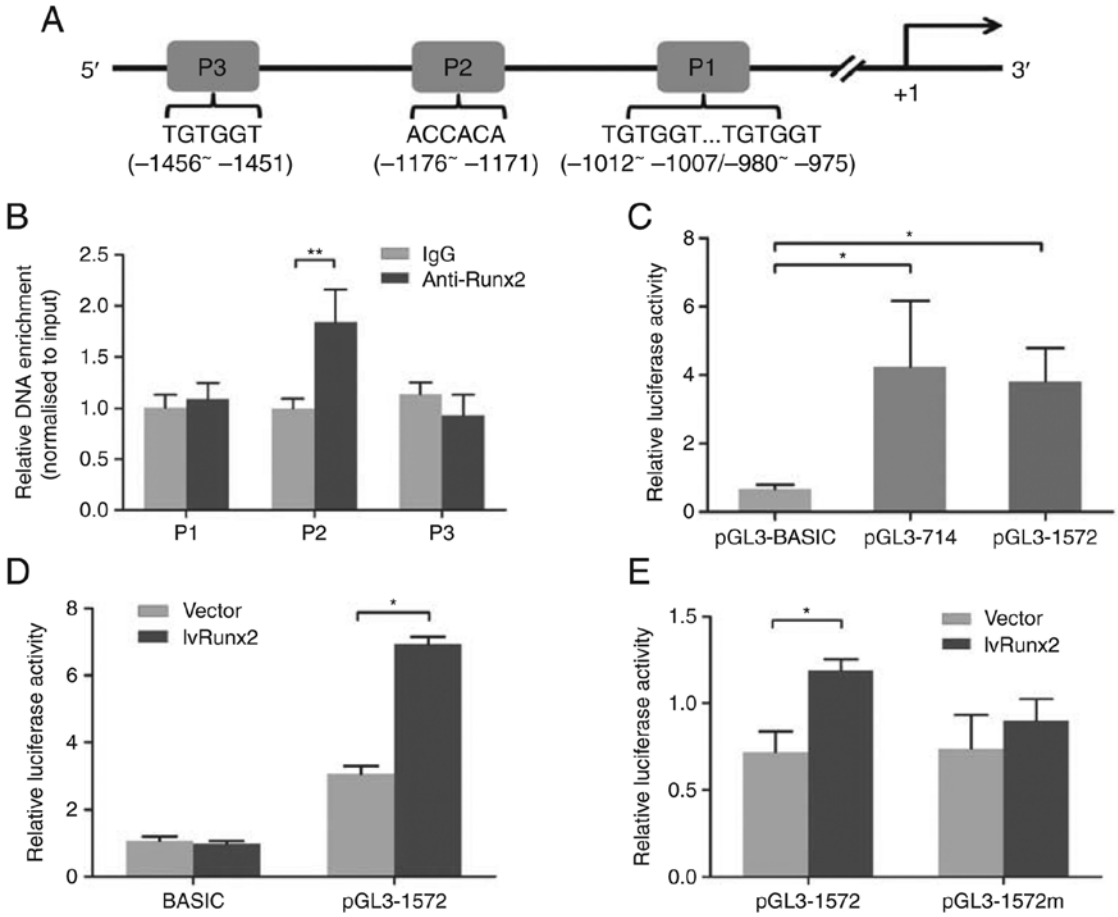

Figure 3. RUNX2 regulates LAPTM5 expression by binding its promoter region. (A) Schematic diagram of the LAPTM5 promoter region, where P1, P2 and P3 indicate putative RUNX2 binding sites. The transcription start site is marked as +1. (B) Chromatin immunoprecipitation assays were used to assess RUNX2 binding to the LAPTM5 promoter in ST2 cells. (C) ST2 cells were transfected with the pGL3-derived reporter constructs pGL3-714, which contains no RUNX2 binding sites, or pGL3-1572, which contains the P1, P2 and P3 binding sites. The empty vector pGL3-BASIC was used as a control. The luciferase activity, normalized to *Renilla* luciferase activity, was analyzed 48 h post-transfection. (D) Cells were co-transfected with the pGL3-1572 vector (using the empty vector pGL3-BASIC as a control) alongside the lvRUNX2 overexpression vector (using the empty LV003 vector as a control). The luciferase activity, normalized to *Renilla* luciferase activity, was analyzed 48 h post-transfection. (E) A substitution mutation in the P2 site was introduced into the pGL3-1572 vector, yielding the pGL3-1572m reporter. Cells co-transfected with the pGL3-1572m and the lvRUNX2 overexpression vector and relative luciferase activity was analyzed 48 h post-transfection. Data are presented as the mean ± SD of two independent experiments. *P<0.05, **P<0.01. RUNX2, runt related transcription factor 2; LAPTM5, lysosomal-associated transmembrane protein 5; IgG, immunoglobulin G.

A ChIP assay was performed to determine whether RUNX2 binds to the LAPTM5 promoter. DNA-protein complexes were immunoprecipitated using a RUNX2 antibody. DNA enrichment in the complexes was analyzed by qPCR. The results revealed that the sequence containing P2 was enriched in DNA-protein immune complexes, while those containing P1 and P3 were not (Fig. 3B), suggesting that RUNX2 was able to bind the LAPTM5 promoter at the -1176 to -1171 position. Next, dual-luciferase reporter assays were used to investigate the effect of RUNX2 on LAPTM5 promoter activation. The relative luciferase activities were significantly increased in cells transfected with pGL3-1572 and pGL3-714 compared with the control group. There was no significant difference between the activities of pGL3-714 and pGL3-1572 (Fig. 3C). Considering the putative RUNX2 binding sites, pGL3-1572 was used for further study. The relative luciferase activity of pGL3-1572 in RUNX2-overexpressing cells displayed a significant increase in activity relative to the control, while pGL3-BASIC displayed no significant alteration (Fig. 3D). Based on the results of the ChIP assay, a substitution mutation in the RUNX2 binding site (-1176 to -1171; ACCACA to ACTGTA) was introduced into the pGL3-1572 reporter (pGL3-1572m). Overexpression of Runx2 increased pGL3-1572 reporter activities significantly compared with the control, however, the mutation diminished this effect (Fig. 3E). Together, these results demonstrated that RUNX2 transactivated the LAPTM5 gene by binding to the LAPTM5 promoter at position -1176 to -1171.

LAPTM5 is involved in the trafficking of RANKL. To investigate the relationship between the expression of LAPTM5 and RANKL, LAPTM5 was silenced using shRNA (Fig. 4A and B). The expression of RANKL mRNA was not influenced by the knockdown of LAPTM5 (Fig. 4C). However, the concentration of secreted RANKL protein in the culture medium was increased significantly following LAPTM5 knockdown (Fig. 4D). RANKL was detected in the cytoplasm using immunofluorescence staining (Fig. 4E). Notably, the fluorescence intensity in the cytoplasm was significantly increased

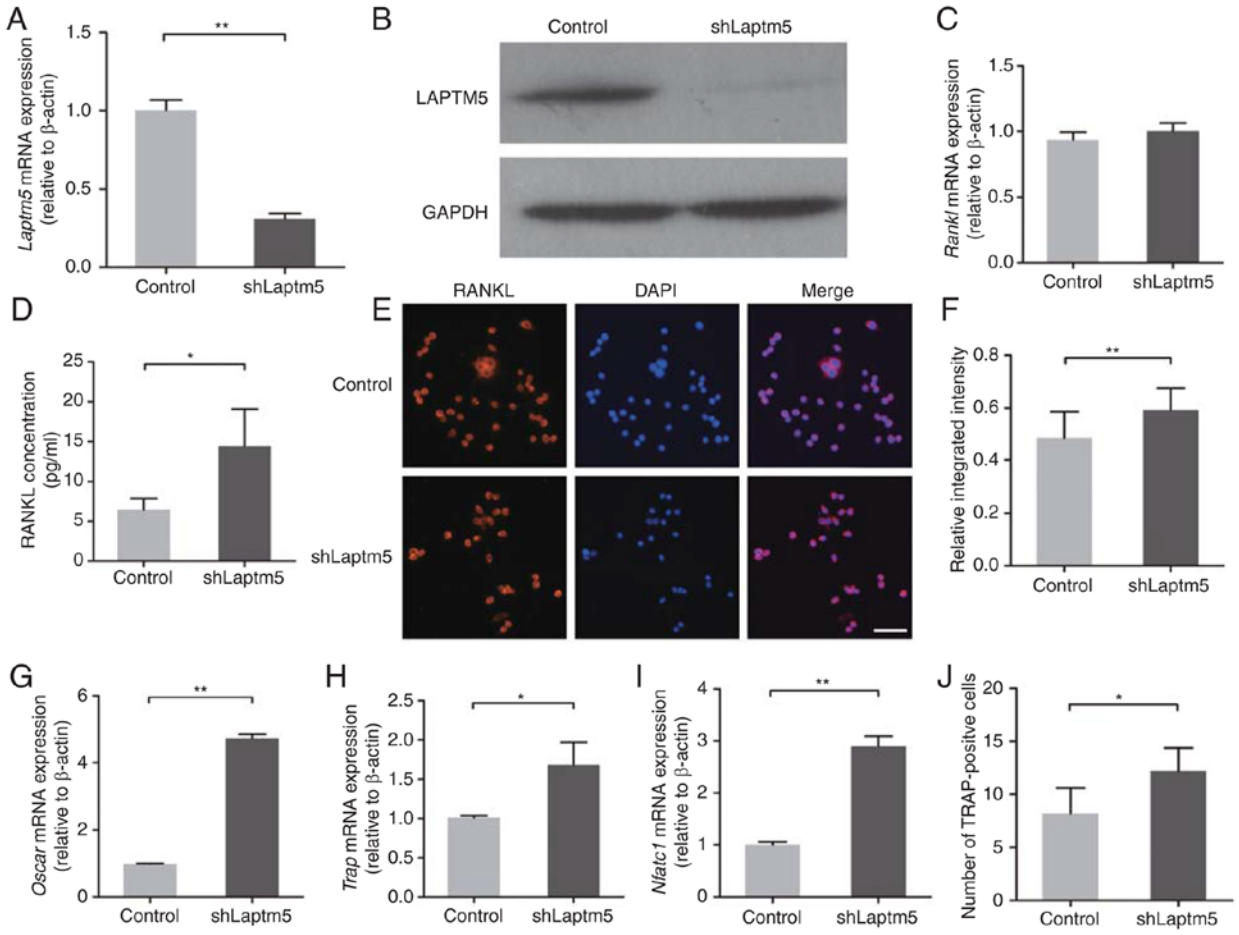

Figure 4. LAPTM5 is involved in RANKL trafficking. (A) LAPTM5 was silenced in ST2 cells using shRNA, and successful knockdown was confirmed at the mRNA and (B) the protein level. (C) RANKL mRNA expression levels exhibited no significant alteration following LAPTM5 knockdown. (D) The concentration of secreted RANKL protein in the culture medium was increased significantly following LAPTM5 knockdown. (E) RANKL expression in ST2 cells was determined using immunofluorescence staining. Nuclei were stained with DAPI. Scale bar, 50 μm. (F) Relative integrated intensity was calculated as the ratio of integrated fluorescence intensity of RANKL in the cytoplasm to that of the total cell. All cells in three random fields were analyzed using Image-Pro Plus 6.0. (G) mRNA expression levels of RUNX2, (H) TRAP and (I) Nftac1 were detected in RAW264.7 cells co-cultured with either control or LAPTM5-silenced ST2 cells. (J) The number of TRAP-positive cells increased significantly in RAW264.7 cells following co-culture with LAPTM5-silenced ST2 cells. Cells transfected with empty vectors were used as the control. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01. RUNX2, runt related transcription factor 2; LAPTM5, lysosomal-associated transmembrane protein 5; RANKL, receptor activator of nuclear factor-κB ligand; sh, short hairpin; Oscar, osteoclast-associated immunoglobulin-like receptor; TRAP, tartrate-resistant acid phosphatase; Nfatc1, nuclear factor of activated T cells 1.

following LAPTM5 knockdown (Fig. 4F), suggesting that LAPTM5 knockdown enhanced RANKL accumulation in the cytoplasm. Furthermore, the expression levels of Oscar, TRAP and Nfatc1 were significantly upregulated (Fig. 4G-I) and the number of TRAP-positive cells was significantly increased (Fig. 4J) in RAW264.7 cells when co-cultured with LPTM5-depleted ST2 cells. Taken together, these results suggested that LAPTM5 is involved in the trafficking of RANKL.

Discussion

The results of the present study indicated that RUNX2 transactivated the expression of the LAPTM5 gene by binding to its promoter region and that LAPTM5 was involved in the expression trafficking of RANKL in ST2 cells.

In the current study, utilizing the co-culture of ST2 and RAW264.7 cells, increased concentrations of RANKL in the medium were observed. Subsequently, the expression levels of Oscar, TRAP and Nfatc1 were demonstrated to be elevated in RAW264.7 cells. These genes are RANKL-inducible and are generally considered to be markers of osteoclast differentiation (20). In addition, an increased number of TRAP-positive cells was detected following co-culture. These results indicated that osteoclastogenic signals were induced by ST2 cells. By using this conventional co-culture model, a previous study has indicated that soluble RANKL mediates osteoclastogenesis (21).

In the presence of 1,25-$(OH)_2D_3$, RUNX2 and LAPTM5 were downregulated in ST2 cells with time. The downregulation of RUNX2 can be attributed to the binding of the vitamin D receptor to the promoter of RUNX2 (22), however, the mechanism of regulation of LAPTM5 expression is not clear. As it has been suggested that the forced expression of RUNX2 upregulates LAPTM5 expression (11), the relationship between RUNX2 and LAPTM5 was explored in the present study by overexpressing and depleting RUNX2. The results demonstrated that the expression of LAPTM5 was regulated by RUNX2. To investigate the underlying mechanism of how RUNX2 regulated LAPTM5 expression, luciferase reporter

and ChIP assays were conducted with a focus on the -1 to -1500 5'-flanking region of the LAPTM5 gene. The ChIP assay results revealed that RUNX2 directly bound to the -1176 to -1171 position within the LAPTM5 promoter. The combination of the luciferase reporter assay and the overexpression of RUNX2 revealed that RUNX2 could directly activate the LAPTM5 promoter and that this activation was prevented by introducing a mutation in the binding site at -1176 to -1171. These findings provided evidence that RUNX2 regulated the expression of LAPTM5 through its role as a transcription factor. When validating this finding using luciferase reporters, it was found that a reporter construct without the RUNX2 putative binding site exhibited an elevated level of activity. This result indicated that the LAPTM5 gene is likely to be regulated by multiple transcription factors.

The lysosomal system is not only involved in osteogenesis but also regulates osteoclastogenesis (23). In differentiating osteoblasts, lysosome levels are increased and are dispersed towards the cell periphery (24). Bone matrix-containing vesicles are partially associated with lysosomes in osteoblasts (24,25). Amorphous calcium/phosphate crystals are secreted through lysosomal exocytosis (26). The osteoclastogenic cytokine RANKL is delivered to the cell surface through secretory lysosomes (27,28) and OPG regulates RANKL-induced osteoclastogenesis by mediating the transport of RANKL to the lysosomal storage compartments (29). The fact that LAPTM5 is transactivated by RUNX2, a transcription factor involved in both osteogenesis and osteoclastogenesis, indicates that LAPTM5 may be an important protein for understanding the role of the lysosomal system in the coupling of osteogenesis and osteoclastogenesis.

In the present study, the upregulation of RANKL was accompanied by the downregulation of LAPTM5 and RUNX2. To the best of our knowledge, RUNX2 has not previously been shown to have a role in RANKL transactivation (30), therefore, the relationship between LAPTM5 and RANKL was examined in the present study. Knockdown of LAPTM5 yielded no significant effect on the mRNA expression of RANKL, however, it significantly increased the levels of RANKL protein in the cytoplasm and its secretion into the culture medium. These results suggested that the upregulation of RANKL could be attributed to suppressed lysosomal proteolytic activity following LAPTM5 silencing. This is consistent with a previous study that reported the increased expression of the surface pre-B cell receptor as a result of LAPTM5 deficiency (31). Future work is required to determine whether LAPTM5 is involved in the lysosomal trafficking of RANKL to the cell surface, or if it regulates the degradation of RANKL by targeting it to lysosomal vesicles; this is specifically relevant in the cellular context, in which RANKL expression on the plasma membrane is lysosome-dependent (23).

The present study indicated that the downregulation of RUNX2 may increase the expression of RANKL in the cytoplasm by suppressing the expression of LAPTM5. These findings add to the existing evidence that RUNX2 serves an important role in osteoclastogenesis (32). However, it appears that both the down and upregulation of RUNX2 can induce osteoclastogenesis through the regulation of RANKL (6). Upregulation of RUNX2 promotes osteoblast-induced osteoclastogenesis by regulating the association of RANKL with the membrane (6). Aside from regulation at the transcriptional level, the function of RUNX2 is also regulated by additional mechanisms, including posttranslational modifications under variable conditions (33). It was reported that the phosphorylation of RUNX2 is required to induce gene expression in response to mechanical stimuli, which is thought to regulate bone remodeling (34); this finding emphasizes the importance of the posttranslational modification of Runx2 in the coupling of osteogenesis and osteoclastogenesis.

In conclusion, the present study demonstrated that RUNX2 transactivated LAPTM5 gene expression and that LAPTM5 was involved in the trafficking of RANKL. These findings suggested that RUNX2 may regulate osteoclast differentiation through lysosome-associated genes that modulate RANKL trafficking in bone cells. The present results suggested a possible coupling mechanism between osteogenesis and osteoclastogenesis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YG performed experiments, performed the statistical analysis and drafted the manuscript. CL, WYL, PL, PY and WLL performed the experiments. PX designed the study and drafted the manuscript. XS designed the study and participated in the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Procedures involving animals were approved by The Institutional Animal Care and Use Committee of Guangzhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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