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Overexpression of Arl6ip5 in osteoblast regulates RANKL subcellualr localization

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The osteoblastic expression of RANKL, which is essential for the communication between osteoblastic cells and osteoclastogenic cells, is stimulated by locally acting or circulating osteotropic cytokines and hormones such as PTH and 1,25-$(OH)_2$-D_3 during the bone remodeling process. However, mechanisms those control subcellular trafficking events, membrane expression and extracellular secretion of the newly synthesized RANKL are still not well understood. In our previous study, we have found that the deficiency of osteoblastic Arl6ip5 (ADP-ribosylation-like factor 6 interacting protein 5), an endoplasmic reticulum (ER)-localized protein belonging to the prenylated rab-acceptor-family, enhanced osteoclastogenesis by increasing RANKL transcription in an ER stress dependent signaling. Here we found that over-expression of hemagglutinin (HA)-tagged Arl6ip5 in UAMS32 stromal/osteoblastic cells inhibited osteoclastogenesis, decreased the amount of soluble RANKL in culture supernatant and increased RANKL retention in ER. Moreover, Arl6ip5 bound with RANKL and disturbed the RANKL-OPG complex in UAMS-32 cells. Finally, 1 to 36 amino acid deletion on the NH_2 lumen terminus of Arl6ip5 impaired the interaction between Arl6ip5 and RANKL, restored the level of soluble RANKL and the osteoclastogenic ability. These findings indicated that Arl6ip5 was an anti-catabolic factor by binding with RANKL and disturbing its subcellular trafficking in osteoblast.

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1. Introduction

Bone remodeling process is tightly regulated by the signaling coupling between osteoblast lineage cells and osteoclast in “Basic Multicellular Unit (BMU)” [1,2]. This coordinated remodeling process is largely mediated by a wide range of paracrine and endocrine factors that secreted by bone cells. Among them, receptor activator of NF-κB ligand (RANKL), which is mainly expressed and secreted by osteoblast lineages, is the essential mediator for osteoclastogenesis [3–7]. RANKL acts on its receptor RANK on the surface of haematopoietic precursor cells and leads to the development of mature osteoclasts.

In stromal/osteoblast cells, RANKL expression can be induced at the transcriptional level by factors known to stimulate bone resorption such as PTH and 1,25 dihydroxyvitamin D3 (1,25-$(OH)_2$-D_3) [4,8–10]. PTH regulates RANKL transcription via protein kinase A (PKA)-cAMP-cAMP response element binding protein (CREB) pathway [4,8]. RANKL is also regulated at its subcellular trafficking level [11–13]. Osteoprotegerin (OPG), which is a very effective inhibitor of osteoclast formation via its block on RANKL-RANK signaling, mediates lysosome trafficking of osteoblastic RANKL. In osteoblast, OPG interacts with RANKL and helps RANKL form stable complexes with its lysosome trafficking partner Vps33a [11,12]. The lysosome localized RANKL is further transported to the cell surface under the actions of Rab27a/b and their effectors slp4a, slp5 and Munc13-4 [13]. However, the detailed mechanisms about RANKL regulation under different physiological states are still unclear.

ADP-ribosylation-like factor 6 interacting protein 5 (Arl6ip5) is

Abbreviations: Arl6ip5, ADP-ribosylation-like factor 6 interacting protein 5; ER, endoplasmic reticulum; HA, hemagglutin; OPG, Osteoprotegerin; POBs, Primary osteoblasts; RANKL, receptor activator of nuclear factor-κB ligand; sRANKL, soluble RANKL; TRAP, tartrate resistant acid phosphatase.

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an endoplasmic reticulum (ER) localized and a ubiquitously expressed protein that belongs to prenylated rab-acceptor (PRA) protein family [14–16]. Studies from its rat homolog GTRAP3-18 indicated that it can form complex with glutamate transporter excitatory amino acid carrier 1 (EAAC-1), delay the ER exit of EAAC-1 and inhibit EAAC-1 transportation into the transport complexes in a Rab-1 dependent manner [14,16,17]. In osteoblast, Arl6ip5 mainly localizes in ER and its expression is regulated by osteotropic factors [18]. Moreover, osteoblastic Arl6ip5 deficiency leads to ER stress dependent RANKL transcription and thereby induce osteoclastogenesis [18]. It would be interesting to find whether over-expression of Arl6ip5 in osteoblast has anti-catabolic effect by regulating the homeostasis of RANKL. Here, with Arl6ip5 over-expressed osteoblastic cells, we found that Arl6ip5 over-expression in osteoblast inhibited osteoclastogenesis, moreover, Arl6ip5 was a binding partner of RANKL and its over-expression let to the ER retention of RANKL.

2. Materials and methods

2.1. Reagents and antibodies

Fetal bovine serum (FBS), L-glutamine, antibiotics, alpha-modified essential medium (α-MEM) and trypsin/EDTA were obtained from Gibco (Life Technologies, Grand Island, NY, USA). PTH and anti-FLAG antibody, anti-HA antibody and anti-EGFP antibody were from Sigma (Sigma–Aldrich, St. Louis, MO, USA). RANKL, LAMP-1, Calnexin, OPG and β-actin antibodies were from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Arl6ip5 antibody was used as implied before [19].

2.2. Cells and plasmids

UAMS-32 stromal/osteoblastic was a kind gift from Prof. Charles A O'Brien (University of Arkansas for Medical Sciences, Little Rock, AR, USA) and maintained in α-MEM contained 10% FBS, 2 mM L-glutamine and antibiotics [20]. Primary calvarial osteoblasts (POBs) were prepared as indicated before [18]. The POBs used in present study was 2–4 passages. Bone marrow macrophages (BMMs) were harvested from mice femurs and tibia as indicated elsewhere [18]. Raw264.7 cell was purchased from ATCC and maintained in α-MEM with 10% FBS and antibiotics. UAMS-32 cells with stable plasmid expression were selected under 100 μg/ml Geneticin (Sigma–Aldrich, St. Louis, MO, USA).

The cDNA extracted from UAMS-32 cells was used as template for plasmid construction. FLAG-RANKL and HA-Arl6ip5 were constructed into pcDNA-3.1(+) with FLAG tag in COOH terminus of mouse RANKL and HA sequence in the COOH terminus of mouse Arl6ip5. EGFP-RANKL was constructed into EGFP-C1 with primers (5′ to 3′): Sense-CTACTCGAGCTATGCGCCGGGGCCAGCCG; Antisense-GCCAAGCTTGGTCTATGTCCTGAACTT. The truncates of FLAG-RANKL were constructed on the backbone of full length FLAG-RANKL, the primers were showed below (5′ to 3′): FL-FLAG-RANKL-sense: GCCAAGCTTGCCACCATGCGCCGGGCCAGCCG; Δ1-44 sense: GCCAAGCTTATGGCCTCCCGCTCCATGTTC; Δ1-100 sense: GCCAAGCTTATGCAGGACTCGACTCTGGAGAG; FLAG-RANKL-antisense: CGCCTCGAGTCACTTGTCATCGTCGTCCTTGTAGTCGTCTATGTCCTGAACTTTG. EGFP-RANKL with types of Cysteine mutations were constructed with QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA) followed the protocol provided. The NH_2 and COOH terminus truncates of HA-Arl6ip5 were constructed on the backbone of full length HA-Arl6ip5, the primers were showed below (5′ to 3′): FL-HA-Arl6ip5-sense: CGCGGATCCGCCACCATGGACGTGAACCTCGCC; ΔN-HA-Arl6ip5-sense: CGCGGATCCGCCACCATGGTAGTGAGCAATCTGCTCTAT; ΔC-HA-Arl6ip5-antisense: CGGTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTAGCGAAGTCTCAGGGATGC; FL-HA-Arl6ip5-antisense: CGGTCTAGAAATCCGCATCA-GCCCGTGCAGCATTCCTAT CTCCCTCGCTTTGCTGATGTAG. All plasmids were verified by sequencing.

2.3. Quantitative polymerase chain reaction (q-PCR) analysis

q-PCR was done as indicated before [18] and the primers for RANKL, Csf-1, Sema3a, Sema3b, Sema7a, Wnt5a and Actb were retrieved from Primer bank [21]. All detections were in triplicate for each sample and data were normalized to Actb level (ΔΔcT).

2.4. Western blotting and immunoprecipitation analysis

Protein was extracted from whole cell pellets with RIPA lysis buffer (Beyotime Institute of Biotechnology, Nantong, China) containing protease and phosphatase inhibitors (Halt Protease Inhibitor Cocktail, Thermo Fisher Scientific, Rockford, IL, USA). The same amount of protein was used for immunoblotting as indicated before [18]. Quantification of band intensities was performed using Image J software (National Institute of Health, Bethesda, MD, USA).

For Immunoprecipitation analysis, cells with plasmids co-transfected were solubilized in IPB buffer (10 mM Tris pH7.6, 5 mM EDTA, 5 mM EGTA, 1 mM sodium orthovanadate,0.1 mM PMSF) with 0.5% NP40 at 4 °C for 30 min with gentle rotation. Following centrifugation of cell debris (12,000 rpm, 15 min), the supernatants were quantified and incubated with Protein G Agarose (Upstate, Lake Placid, NY, USA) then primary antibodies were added to supernatant and incubated overnight at 4 °C with gentle rotation. The immunobead-bound protein complexes were washed and dissociated by boiling in 2 X Laemmli sample buffer and the eluted protein were then analyzed by immunoblotting.

2.5. Co-culture assay and TRAP staining

POBs were seeded into 48 wells culture plate with density of 1000 per well for co-cultured with RAW264.7 cells and 5000 per well with BMMs, 2 days later, 5000 RAW264.7 or 20,000 BMMs were added. At the same time, 100 nM PTH was added and the medium were half-changed every 2 days with consistent PTH treatment for 4–6 days for RAW264.7 cells and 7–9 days for BMMS, the cells were evaluated for the osteoclast formation with TRAP staining using TRACP & ALP double-stain Kit (TaKaRa Bio Inc., Otsu, Japan).

2.6. ELISA and immunofluorescence

Soluble RANKL in culture medium was detected with mouse RANKL ELISA kit from R&D (R&D Systems, Inc., Minneapolis, MN, USA) followed the protocol provided. For the localization of endogenous RANKL to LAMP-1, Calnexin and HA in HA-Arl6ip5 over-expressed UAMS-32 cells, cells were fixed and blocked in PBST contained normal goat serum at RT for 1 h then incubated with diluted primary antibody at 4 °C overnight. Then cells were treated with corresponded secondary antibodies with Alexa-488 or Alexa-555 tagged and incubated at 37 °C for 2–3 h. Images were acquired with Olympus Laser Scanning Confocal Microscope (Olympus Corporation, Tokyo, Japan) and merged with Image J software.

2.7. Statics

Results were expressed as the mean ± SEM. Statistical significance was identified by *Student's t test* or *one-way ANOVA* where appropriate, with probability $P < 0.05$ being considered significant.

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