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Role of anoctamin 5, a gene associated with gnathodiaphyseal dysplasia, in osteoblast and osteoclast differentiation

Jung Ha Kim[a], Kabsun Kim[a], Inyoung Kim[a], Semun Seong[a,b], Sang Wan Kim[c], Nacksung Kim[a,b,*]

[a] Department of Pharmacology, Chonnam National University Medical School, Gwangju 61469, Republic of Korea
[b] Department of Biomedical Sciences, Chonnam National University Medical School, Gwangju 61469, Republic of Korea
[c] Department of Internal Medicine, Seoul National University College of Medicine and Boramae Medical Center, Seoul 07061, Republic of Korea

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ABSTRACT

Anoctamin 5 (Ano5) mutations are responsible for gnathodiaphyseal dysplasia, a rare skeletal syndrome. Despite the close linkage of Ano5 to bone remodeling, the molecular mechanisms underlying the role of Ano5 in bone remodeling remain unknown. In this study, we investigated whether Ano5 regulates osteoblast or osteoclast differentiation to maintain normal bone remodeling. Downregulation of Ano5 expression did not affect osteoblast differentiation and mineralization, while ectopic expression of Ano5 significantly enhanced receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclast differentiation. Furthermore, Ano5-mediated Akt phosphorylation resulted in nuclear factor of activated T-cells c1 (NFATc1) activation, indicating that Ano5 regulates osteoclast differentiation through activation of the Akt-NFATc1 signaling pathway. Thus, our results suggest a possibility that Ano5 is involved in bone remodeling through regulating the function of osteoclasts rather than that of osteoblasts.

1. Introduction

Anoctamin 5 (Ano5), also known as TMEM16E, belongs to the anoctamin family, the members of which share common structural characteristics, including eight transmembrane domains, a re-entrant loop between the fifth and sixth transmembrane domains, and a unique sequence motif called the annotated domain [1,2]. The 10 identified anoctamin family members play critical roles in various physiological processes, such as ion transport, phospholipid scrambling, and regulation of other ion channels [3–9]. Due to the structural similarity between all family members, until recently, it was proposed that all members of this family have the same function of encoding Ca^{2+}-activated Cl^- channels (CaCC). Some anoctamins (Ano1, Ano2, Ano8, and Ano9) possess CaCC activities, while some others (in particular Ano3 to Ano7) lack CaCC activities due to their intracellular localization [10–12]. In this respect, it was suggested that members of the anoctamin family might have evolved to possess different functional properties.

Among the anoctamin family members, Ano5 was the first to be identified as being involved in human diseases. Presence of Ano5 mutations has been associated with the development of gnathodiaphyseal dysplasia (GDD), an autosomal dominant inherited disease. GDD, a rare skeletal syndrome, is characterized by fibro-osseous lesions of the jaw bones, sclerosis and deformations of the tubular bones, and increased bone fragility [2,13,14]. Despite the obvious relevance of Ano5 to skeletal development, the molecular mechanisms by which Ano5 regulates bone remodeling are incompletely understood.

Bone homeostasis is maintained by constant bone remodeling involving the removal of old or damaged bone by bone-resorbing osteoclasts and the replacement of new bone by bone-forming osteoblasts. Normal bone remodeling is controlled by the tight coupling of bone resorption to bone formation. Therefore, an imbalance of activities between osteoclasts and osteoblasts leads to the development of various bone diseases, such as osteoporosis and osteopetrosis [15,16]. Osteoporosis, a common disorder of bone remodeling, is characterized by low bone mass, deformations of the tubular bones, and increased bone fragility [16,17]. Osteoporosis is caused by an imbalance in bone remodeling due to excessive bone resorption compared to bone formation

Abbreviations: GDD, gnathodiaphyseal dysplasia; Ano5, anoctamin 5; BMM, bone marrow-derived macrophage-like cell; NFATc1, nuclear factor of activated T-cells c1; TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of nuclear factor kappa B ligand; M-CSF, macrophage colony stimulating factor; OSCAR, osteoclast-associated receptor; BMP2, bone morphogenetic protein 2; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase

* Corresponding author at: Department of Pharmacology, Chonnam National University Medical School, 160 Baekseo-ro, Dong-Ku, Gwangju 61469, Republic of Korea.
E-mail address: nacksung@jnu.ac.kr (N. Kim).

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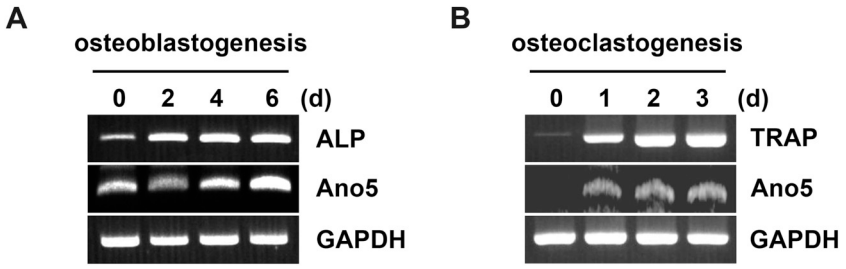

Fig. 1. Expression of Ano5 during osteoblastogenesis and osteoclastogenesis. (A) Primary osteoblast precursor cells were cultured in OGM containing BMP2, ascorbic acid, and β-glycerophosphate for the indicated times. (B) BMMs were cultured for indicated times with M-CSF alone or M-CSF and RANKL. (A and B) Total RNA was extracted at each indicated time point and reverse transcription (RT)-PCR was performed to assess the expression of the indicated genes.

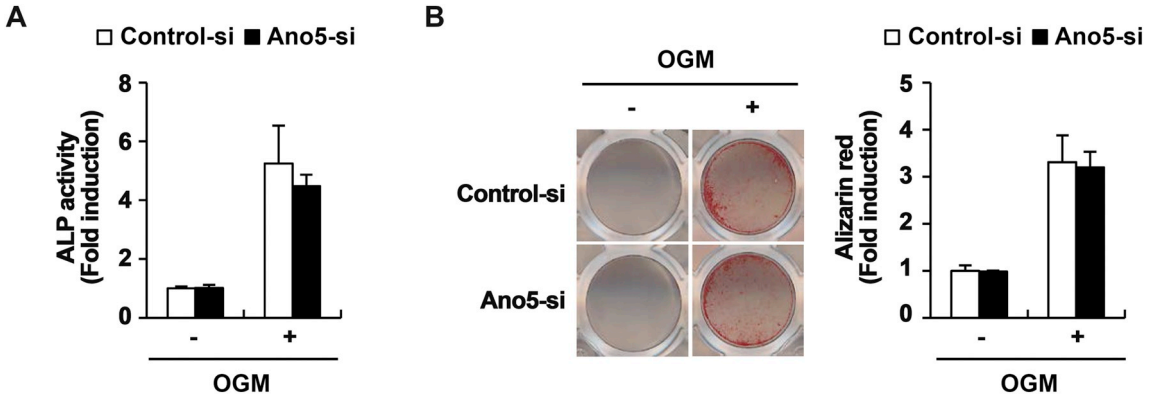

Fig. 2. Effects of Ano5 on osteoblast differentiation and nodule formation. (A and B) Osteoblasts were transfected with control or Ano5 siRNA and cultured in OGM. (A) Cells were cultured for 3 days and subjected to ALP activity assay. (B) Cells were cultured for 6 days and fixed and stained with alizarin red (left panel). Staining intensities were quantified at 562 nm via densitometry (right panel).

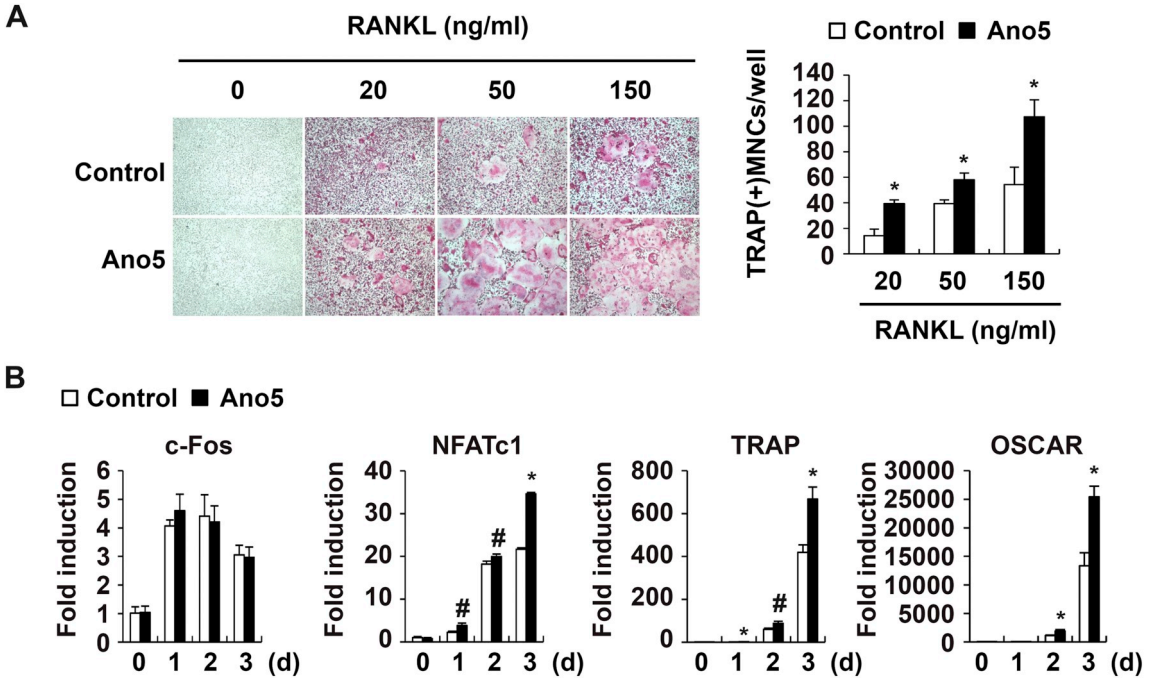

Fig. 3. Effects of Ano5 on osteoclast differentiation. (A and B) BMMs were transduced with pMSCV-IRES-EGFP (control) or Ano5 retrovirus and cultured for 3 days with M-CSF alone or M-CSF and RANKL. (A) Cultured cells were fixed and stained for TRAP (left panel). Numbers of TRAP$^+$ MNCs were counted (right panel). (B) Total RNA was collected at each indicated time point, and real-time PCR was performed to evaluate the expression of the target genes. Data are expressed as the mean ± SD of triplicate samples. $^{\#}p < 0.05$, $^{*}p < 0.01$ versus control.

[16]. Osteopetrosis, a rare heritable disorder of bone remodeling, is characterized by increased bone density and increased susceptibility to fractures, and is caused due to an absence of osteoclasts or inability to resorb bone [16].

Osteoclast differentiation from hematopoietic stem cells is principally stimulated by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL). The binding of RANKL to its sole receptor, RANK, induces the activation of NF-κB, activator protein 1 (AP-1), and nuclear factor of activated T-cells c1 (NFATc1) as well as the mitogen-activated protein kinase and Akt signaling pathways via TNF receptor associated factor 6 (TRAF6) recruitment. RANKL further stimulates NFATc1 activation via

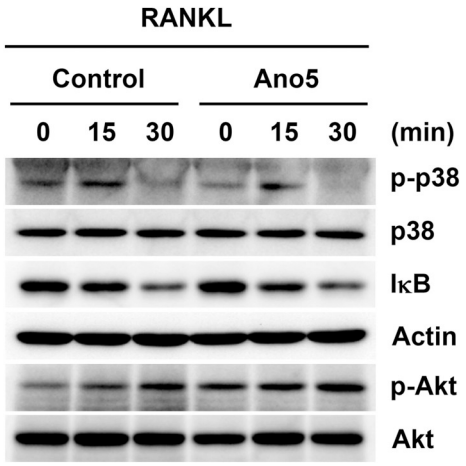

Fig. 4. Ano5 increases Akt phosphorylation. BMMs were transduced with pMSCV-IRES-EGFP (control) or Ano5 retrovirus and stimulated with RANKL for the indicated times. Whole cell lysates were analyzed via western blotting using specific antibodies, as indicated.

triggering receptor expressed on myeloid cells 2 (TREM2)- or osteoclast-associated receptor (OSCAR)-mediated co-stimulated signaling pathways to induce osteoclast differentiation [18–21].

Bone morphogenetic protein 2 (BMP2) is the most important growth factor for osteoblast differentiation of mesenchymal stem cells [22]. The binding of BMP2 to type II BMP receptors induces phosphorylation of BMP-specific Smad1/5/8. Activated Smads are translocated to the nucleus to activate the transcription of osteogenic genes, including distal-less homeobox 5 (Dlx5) and runt-related transcription factor 2 (Runx2). Runx2 then directly stimulates the transcription of osteoblastic genes, including alkaline phosphatase (ALP), bone sialoprotein, and osteocalcin [23–27].

In this study, we evaluated whether Ano5 directly regulates osteoclast or osteoblast differentiation.

2. Materials and methods

2.1. Reagents and antibodies

Antibodies against actin were purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies against phospho-Akt, Akt, phospho-p38, p38, and IκB were from Cell Signaling Technology (Beverly, MA, USA). LY294002 was purchased from Merck Millipore (Billerica, MA, USA). Recombinant human M-CSF was a gift from Dr. Daved Fremont (Washington University, St. Louis, MO) and recombinant human sRANKL was purified from bacteria. Recombinant human BMP2 was purchased from Cowellmedi (Seoul, Republic of Korea). Alizarin red and β-glycerophosphate were obtained from Sigma-Aldrich. Ascorbic acid was purchased from Junsei Chemical (Nihonbashi-honcho, Japan).

2.2. Osteoblast differentiation

Primary osteoblast precursor cells were isolated from newborn mouse calvaria by enzymatic digestion with 0.1% collagenase (Life Technologies, Calsbad, CA, USA) and 0.2% dispase II (Roche Diagnostics GmbH, Mannheim, Germany). For osteoblast differentiation, primary osteoblast precursor cells were cultured in osteogenic medium (OGM) containing BMP2 (100 ng/mL), ascorbic acid (50 μg/mL), and β-glycerophosphate (100 mM) for 3 days. To assess osteoblast differentiation, cells were subjected to ALP activity assay. Cells were lysed using osteoblast lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA]. The cell lysates were incubated with *p*-nitrophenyl phosphate substrate (Sigma-Aldrich), and ALP activity was measured with a spectrophotometer by measuring the absorbance at 405 nm. For mineralization assay, cells were cultured for 9–12 days, fixed with 70% ethanol, and then stained with 40 mM alizarin red (pH 4.2). After non-specific staining was removed with phosphate buffered saline wash, alizarin red staining was visualized with a CanoScan 4400F (Canon Inc., Japan). To quantify matrix calcification, alizarin red-stained cells were incubated with 10% cetylpyridinium chloride solution for 30 min at room temperature, and the absorbance was measured at 570 nm.

2.3. Osteoclast differentiation

Mouse bone marrow cells were isolated from the tibiae and femurs of 6-week-old ICR (Institute for Cancer Research) mice by flushing the bone marrow with α-minimum essential medium (α-MEM), and cultured in α-MEM containing 10% fetal bovine serum in the presence of M-CSF (30 ng/mL) for 3 days. Adherent bone marrow-derived macrophage-like cells (BMMs) were further cultured in the presence of M-CSF (30 ng/mL) and RANKL (20–150 ng/mL) for 1–4 days. Cultured cells were fixed with 10% formalin and stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells (MNCs) were counted as osteoclasts. Cells were observed under a Leica DM IRB microscope equipped with an N Plan 310 (0.25 numerical aperture) objective lens (Leica, Wetzlar, Germany). Images were captured with a ProgRes CF scan camera (Jenoptik, Jena, Germany) using ProgRes Capture Pro software (Jenoptik).

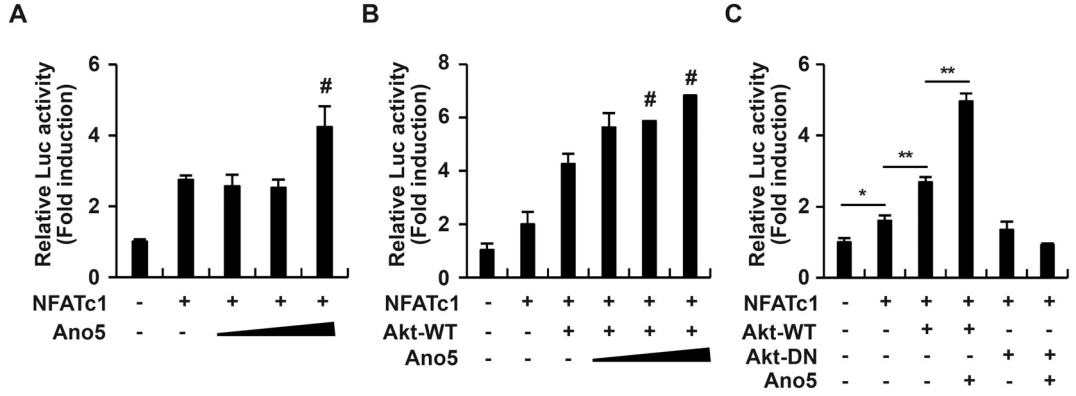

Fig. 5. Ano5 enhances Akt-dependent NFATc1 transcriptional activity. (A–C) 293T cells were transfected with an OSCAR reporter construct, NFATc1, Ano5, Akt-WT, and Akt-DN, as indicated. Luciferase activity was measured using a dual-luciferase reporter assay system. Data are expressed as the mean ± SD of triplicate samples. $^{\#}p < 0.05$, $^{*}p < 0.01$, $^{**}p < 0.01$ versus control.

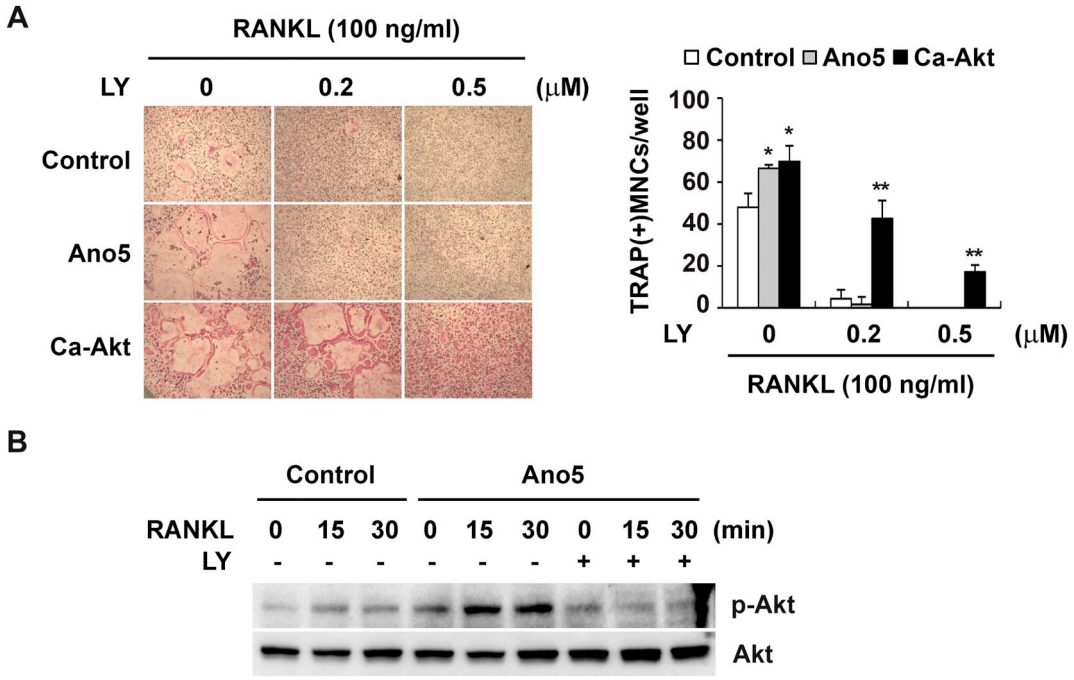

Fig. 6. Inhibition of Akt blocks Ano5-mediated enhancement of osteoclast differentiation. (A) BMMs were transduced with pMSVC-IRES-EGFP (control), Ano5, or Ca-Akt retrovirus, and then cultured with M-CSF and RANKL in the absence or presence of LY294002. Cultured cells were stained for TRAP (left panel). Numbers of $TRAP^+$ MNCs were counted (right panel). Data are expressed as the mean ± SD of triplicate samples. $*p < 0.01$, $**p < 0.01$ versus control. (B) BMMs were transduced with pMSCV-IRES-EGFP (control) or Ano5 retrovirus. Transduced BMMs were pretreated with LY294002 for 1 h and stimulated with RANKL for the indicated times. Whole cell lysates were analyzed via western blotting using specific antibodies, as indicated.

2.4. Retroviral gene transduction

Retroviral vectors were transfected into the packaging cell line (Plat E) using FuGENE 6 (Promega, Madison, WI, USA) according to the manufacturer's protocol. Viral supernatants were collected from the culture medium at 48 h after transfection. BMMs or osteoblasts were incubated with viral supernatants for 6 h in the presence of 10 μg/mL polybrene (Sigma-Aldrich).

2.5. Small interfering RNA (siRNA) transfection

Control and Ano5 siRNAs were purchased from Dharmacon (Lafayette, CO, USA). The siRNAs were transfected into BMMs using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol.

2.6. Quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time PCR analyses were performed in triplicate on the Rotor-Gene Q (Qiagen, Hilden, Germany) using the SYBR Green PCR Master Mix (Qiagen). All values were normalized to the level of an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative quantitation value of each target gene compared to the calibrator for that target was expressed as $2^{-(Ct-Cc)}$, in which Ct and Cc are the mean threshold cycle differences after normalization to GAPDH. The relative expression levels of the samples are presented as a semi-log plot. The primer sequences were as follows: c-fos, 5′-ATG GGC TCT CCT GTC AAC ACA-3′ and 5′-TGG CAA TCT CAG TCT GCA ACG CAG-3′; NFATc1, 5′-CTC GAA AGA CAG CAC TGG AGC AT-3′ and 5′-CGG CTG CCT TCC GTC TCA TAG-3′; TRAP, 5′-CTG GAG TGC ACG ATG CCA GCG ACA-3′ and 5′-TCC GTG CTC GGC GAT GGA CCA GA-3′; OSCAR, 5′-TGC TGG TAA CGG ATC AGC TCC CCA GA-3′ and 5′-CCA AGG AGC CAG AAC CTT CGA AAC T-3′; ALP, 5′-CAA GGA TAT CGA CGT GAT CAT G-3′ and 5′-GTC AGT CAG GTT GTT CCG ATT C-3′; Ano5, 5′-CGT GGA GGA TTT GAA GAA AGA T-3′ and 5′-TGT TGA GGA TGG AAA GAA AGT G-3′; GAPDH, 5′-TGA CCA CAG TCC ATG CCA TCA CTG-3′ and 5′-CAG GAG ACA ACC TGG TCC TCA GTG-3′.

2.7. Western blotting

Cells were harvested in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, and protease inhibitor cocktail] and centrifuged at 13,000 × *g* for 15 min at 4 °C. Protein concentrations in the supernatants were determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Merck Millipore). Membranes were incubated with the appropriate antibodies. Signals were detected using an enhanced chemiluminescence reagent (Merck Millipore) and analyzed with an LAS-3000 luminescent image analyzer (GE Healthcare, Piscataway, NJ, USA).

2.8. Luciferase assay

293T cells were plated in 24-well plates at a density of 2×10^4 cells/well 24 h prior to transfection. The reporter plasmid and various expression vectors were co-transfected into 293T cells using FuGENE 6, according to the manufacturer's protocol. After 48 h of transfection, cells were lysed in passive lysis buffer (Promega). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

2.9. Statistical analysis

Statistical analyses were performed using an unpaired Student's *t*-test. All data are presented as the mean ± standard deviation (SD). *p* values < 0.05 were considered statistically significant.

3. Results

3.1. Ano5 does not affect osteoblast differentiation and function

Ano5 is highly expressed in the human skeletal muscle, cardiac muscle, chondrocytes, and osteoblasts [13]. As expected, Ano5 was highly expressed during osteoblast differentiation (Fig. 1A). Although the level of expression was relatively low compared to that in osteoblasts, Ano5 was also expressed in osteoclasts but not osteoclast precursors cells (Fig. 1B). Since Ano5 was highly expressed in osteoblasts, we used siRNAs targeting Ano5 to identify the role of Ano5 in osteoblasts through loss-of-function experiments. When osteoblasts were cultured in OGM containing ascorbic acid, β-glycerophosphate, and BMP2, ALP activity was increased and bone nodule formation was induced (Fig. 2). Downregulation of Ano5 expression did not affect ALP activity and bone nodule formation (Fig. 2). These results suggested that Ano5 does not regulate osteoblast differentiation and function.

3.2. Ano5 enhances osteoclast differentiation

Since Ano5 was not expressed in osteoclast precursor cells, we retrovirally overexpressed Ano5 in BMMs to identify its role in osteoclasts through gain-of-function experiments. Ano5 overexpression significantly enhanced RANKL-induced osteoclast differentiation (Fig. 3A). Interestingly, Ano5 overexpression increased RANKL-induced NFATc1 expression without altering c-fos expression (Fig. 3B). Therefore, these results indicated that Ano5 plays an important role in RANKL-induced osteoclast differentiation, probably through the regulation of NFATc1 expression.

3.3. Ano5 regulates osteoclast differentiation via activating the Akt signaling pathway

RANKL activates several early signaling pathways via RANK-TRAF6-dependent cascades, including Erk, JNK, p38, Akt, and NF-κB pathways to induce osteoclast differentiation [18,20,28,29]. Given that Ano5 significantly increased NFATc1 expression as well as osteoclast differentiation, we investigated whether Ano5 regulates RANKL-induced early signaling pathways that are involved in NFATc1 expression. As shown in Fig. 4, RANKL successfully activated the p38, NF-κB, and Akt pathways. Ano5 overexpression strongly increased RANKL-induced Akt phosphorylation, but did not affect activation of p38 and NF-κB (Fig. 4). We previously reported that the PI3K-Akt signaling pathway enhances NFATc1 expression and its transcriptional activity to regulate osteoclast differentiation [30,31]. Therefore, we further investigated whether Ano5 regulates NFATc1 transcriptional activity via regulating Akt activation. Consistent with previous results [31], when an OSCAR report construct was co-transfected with NFATc1, the promoter activity was significantly increased (Fig. 5A). This increased promoter activity was slightly more increased by Ano5 expression (Fig. 5A). As expected, co-transfection of wild-type Akt (Akt-WT) and NFATc1 synergistically enhanced the transcriptional activity of NFATc1 (Fig. 5B and C). Of note, Ano5 more efficiently increased the transcriptional activity of NFATc1 when Akt-WT was co-transfected with NFATc1, while Ano5 did not regulate OSCAR promoter activity when co-transfected with a dominant negative form of Akt (Akt-DN) (Fig. 5B and C). Collectively, these results indicated that Ano5 enhances the transcriptional activity of NFATc1 by promoting Akt activation. Finally, we tested whether Ano5 regulates RANKL-induced osteoclast differentiation through signaling pathways other than the Akt signaling pathway. As shown in Fig. 6A, RANKL-induced osteoclast differentiation was inhibited by LY294002 treatment. These inhibitory effects were significantly restored by a constitutively active form of Akt (Ca-Akt) overexpression (Fig. 6A). However, when Akt activation was blocked by LY294002 treatment, Ano5 overexpression enhanced neither RANKL-induced osteoclast differentiation nor RANKL-mediated Akt phosphorylation (Fig. 6A and B). These results suggested that Ano5 regulates osteoclast differentiation mainly through promoting Akt activation.

4. Discussion

Genetic mutations in Ano5 have been identified to be responsible for GDD [2,13,14,32–34]. GDD patients with a mutation in the Ano5 gene display skeletal abnormalities, such as an osteopetrosis-like sclerosis of the long bones and fibrous dysplasia-like cemento-osseous lesions of the jaw bone. Several clinical studies support an essential role of Ano5 in normal bone remodeling. However, it remains to be addressed whether Ano5 regulates osteoclast and osteoblast differentiation.

A high expression of Ano5 in osteoblasts led us to first investigate whether Ano5 affects osteoblast differentiation and function and regulates bone remodeling. Ano5 downregulation did not have any effect on osteoblast differentiation and bone nodule formation. Therefore, Ano5 does not seem to be involved in bone remodeling via regulating osteoblast differentiation and function. Recently, it has been reported that Ano6 is required for proper bone mineralization by activating phosphatidylserine scrambling in osteoblasts [35,36]. Ano6-deficient osteoblasts display reduced capacity to produce a calcified bone matrix in vivo and in vitro. Because Ano6 is the closest paralog of Ano5 among all other anoctamin family members, and shows similar intracellular localization to Ano5, we cannot entirely rule out a possibility that Ano6 may compensate for the loss of function of Ano5 in osteoblasts.

Osteopetrosis-like sclerosis of the long bones in patients with GDD may be a result of osteoclast dysfunction. In this study, ectopic expression of Ano5 in osteoclast precursor cells significantly enhanced RANKL-induced osteoclast differentiation by activating Akt signaling. Ano5-induced Akt activation contributed to the activation of NFATc1, which is a master regulator of osteoclast function as well as osteoclast differentiation and fusion [37]. Interestingly, Ano5 may potentially act as a phospholipid scramblase that can influence Akt activation and fusion [38,39]. Therefore, it is necessary to clarify whether the function of Ano5 as a phospholipid scramblase is closely related to Akt activation and osteoclast differentiation.

Recently, Rolvien et al. analyzed the bone architecture of a 13-year-old GDD patient with a missense mutation in the Ano5 gene [34]. They observed that trabecular bone mass in the long bones was increased with elevated serum markers of bone formation and bone resorption, whereas bone volume fraction in the iliac crest was normal. The pathological process of high bone turnover was confirmed by increased osteoblast and osteoclast indices through the analysis of an iliac crest biopsy. Osteopetrosis was ruled out because the iliac crest histology revealed an increase in not only the number of osteoblasts forming new osteoid but also in osteoclast numbers and eroded surfaces. However, we cannot completely exclude the possibility of osteoclast defects in patients with GDD. Our present results showed that Ano5 plays a more important role in osteoclasts than in osteoblasts. The study by Rolvien et al. confirmed an increase in osteoclast number only in the iliac crest but not in the long bones, which are the predominant sites of bone mass increase in patients with GDD. Of note, GDD involves an osteopetrosis-like sclerosis of the long bones despite increased expression of markers of bone resorption [34], and, in fact, increased serum concentrations of osteoclast markers, such as creatine kinase BB isozyme and TRAP, are often observed in osteopetrosis [16]. Hence, high bone mass in the long bones of patients with GDD might mediate osteoclast dysfunction caused by Ano5 mutations.

Previously, mutations in the Ano5 gene have been associated with two types of autosomal recessive muscular dystrophies, limb girdle muscular dystrophy type 2L (LGMD2L) and Miyoshi myopathy type 3, which have characteristics that resemble dysferlinopathies [40,41]. Most of the muscular dystrophy mutations in Ano5 gene cause Ano5 deficiency due to frameshift or truncation of the protein or splice site changes, representing loss-of-function phenotype, whereas GDD

mutations in the Ano5 gene lead to mutations in a single amino-acid position of the Ano5 protein, reflecting gain-of-function phenotype [32,42,43]. It is important to note that patients with LGMDL2L exhibit no pathogenic phenotype related to the bone, which is evident in patients with GDD [44]. This suggests that deficiency of Ano5 in bone cells may be sufficiently compensated by other factors in vivo. However, dominant missense mutations in the Ano5 gene in GDD may reflect a gain-of-function effect, possibly a dominant negative effect, predominantly in the skeletal tissues, which may result in reduced osteoclast differentiation and function [2,13,14]. The relatively low expression of Ano5 in osteoclasts compared to that in muscle tissues, and the different patterns of Ano5 mutations between GDD and LGMDL2L may explain how the loss-of-function recessive mutations in the Ano5 gene can only cause muscular dystrophy phenotype without any pathogenic phenotype related to the bone.

Collectively, increased bone mass in the long bones of patients with GDD may be involved in reduced osteoclast differentiation and function. Our results indicate that Ano5 expression can regulate osteoclast differentiation rather than osteoblast differentiation to contribute to normal bone remodeling. Although recently, Ano5 knockout mice were generated, their skeletal phenotype has not been analyzed. To clearly understand how Ano5 mutations in GDD cause abnormalities in the bone and whether Ano5 mutations affect osteoclast differentiation and function, further studies using both Ano5 knockout and knock-in mice with mutated residues responsible for GDD are needed.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgements

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