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Original Paper

Knockout of TRPV6 Causes Osteopenia in Mice by Increasing Osteoclastic Differentiation and Activity

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Key Words
TRPV6 • Ca^{2+} • Osteoclastogenesis • Bone resorption

Abstract
Background: Calcium ion (Ca^{2+}) signals are required for osteoclast differentiation. Previous study showed that transient receptor potential vanilloid 5 (TRPV5) is an essential Ca^{2+} transporter in osteoclastogenesis and bone resorption. TRPV5 and TRPV6 represent two highly homologous members within the transient receptor potential (TRP) superfamily. However, the role of TRPV6 in bone metabolism is still controversial and little is known about the involvement of TRPV6 in receptor activator of nuclear factor κ-B ligand (RANKL)-induced osteoclastogenesis. ***Methods:*** In our study, gene knockout mice, RNA interference, western blot, quantitative real-time PCR, tartrate-resistant acid phosphatase (TRAP) staining, pit formation assay, histomorphometry and measurement of serum parameters were employed to investigate the role of TRPV6 in bone homeostasis, osteoclastogenesis and bone resorption. ***Results:*** We found that TRPV6 depletion results in noticeable destruction of bone microarchitecture in TRPV6 knockout mice ($TRPV6^{-/-}$), suggesting that TRPV6 is a critical regulator in bone homeostasis. Inactivation of Trpv6 had no effect on osteoblastic bone formation. However, quantification of the TRAP staining showed a significantly increased osteoclast number and surface area in the metaphyseal area of femurs bone sections derived from $TRPV6^{-/-}$ mice. In agreement with our observations from $TRPV6^{-/-}$ mice, TRPV6 depletion *in vitro* significantly increased osteoclasts differentiation and bone resorption activity. ***Conclusion:*** Based on these results above, we can draw conclusions that TRPV6 plays an essential role in bone metabolism and is a critical regulator in osteoclasts differentiation and bone resorption.

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Introduction

Osteoclasts are multinucleated bone-resorbing cells that are unique in their ability to degrade mineralized matrices, such as bone and calcified cartilage [1]. Deficiency of osteoclasts leads to osteopetrosis, resulting in high bone mass with poor bone quality and increased fracture frequency due to defective bone remodeling. On the other hand, increased number and activity of osteoclasts under certain pathologic conditions causes accelerated bone resorption and may lead to osteoporosis and osteolytic diseases [2]. Therefore, it is necessary to elucidate the regulatory mechanisms of osteoclast signaling, which is the basis for understanding the mechanisms of osteoclast-related diseases.

Previous studies have made advances about the molecular mechanisms of osteoclast formation and function [3-7]. Although many systemic hormones and local cytokines participate in regulating osteoclast differentiation, the receptor activator of NF-κB (RANK) ligand (RANKL) is the most critical molecular for osteoclastogenesis in cooperation with macrophage colony-stimulating factor (M-CSF) in the interaction between stromal cells and cells of the osteoclast lineage [3-7]. It is well known that the induction of osteoclast formation by RANKL requires the activation of NF-κB and Jun N-terminal kinase (JNK) pathways via tumor necrosis factor receptor associated factor (TRAF) family proteins from RANK, the RANKL receptor [8]. Extensive studies indicate that calcium ion (Ca^{2+}) signals are also required for osteoclast differentiation [9, 10]. RANKL signaling induces oscillatory changes in intracellular Ca^{2+} concentrations, resulting in Ca^{2+} /calcineurin-dependent dephosphorylation and activation of nuclear factor of activated T cells c1 (NFATc1), which translocates to the nucleus and induces osteoclast-specific gene transcription to allow differentiation of osteoclasts [11-14]. Takayanagi et al reported that sustained $[Ca^{2+}]_i$ oscillations, rather than transient activation of a Ca^{2+} spike, is necessary for the sustained NFATc1 activation during osteoclastogenesis [15]. However, it is not yet fully understood how RANKL activates calcium signals and $[Ca^{2+}]_i$ oscillations leading to the induction and nuclear localization of NFATc1.

The transient receptor potential (TRP) family is a large protein family consisting of several subfamilies, of which the transient receptor potential vanilloid (TRPV) is an example. The TRPV family can be divided into 4 groups: TRPV1/2, TRPV3, TRPV4, and TRPV5/6. Osteoclasts express different families of TRPV channels, including TRPV1/2, TRPV4, and TRPV5/6 [16]. Previous studies showed that TRPV1-TRPV4 are all heat-activated channels that are nonselective for cations and modestly permeable to Ca^{2+} [17]. TRPV1/2 are likely involved in the Ca^{2+}-permeable pathway, which mediates osteoclastic Ca^{2+} oscillations [18]. Ca^{2+} oscillations disappear during osteoclast differentiation and are replaced by a sustained Ca^{2+} influx via members of the TRP family, such as TRPV4 [19]. However, the properties of the two other members of TRP subfamily, TRPV5 and TRPV6, are quite different from those of TRPV1-TRPV4. They are the only highly Ca^{2+}-selective channels in the TRP family, and both are tightly regulated by $[Ca^{2+}]_i$. Previous studies showed that TRPV5 and TRPV6 play a crucial role as gatekeepers in epithelial Ca^{2+} transport, and as selective Ca^{2+} influx pathways in nonexcitable cells [16]. As TRPV6 and TRPV5 share 75% homology at the aminoacid level, therefore, TRPV6 and TRPV5 represent two highly homologous members within the TRP superfamily [20]. It has been confirmed that TRPV5 is an essential Ca^{2+} transporter in osteoclastic differentiation and bone resorption [10]. However, the regulation effects of TRPV6 on bone metabolism are still controversial and little is known about the involvement of TRPV6 in RANKL-induced osteoclastogenesis.

In the present study, we observed the effects of TRPV6 on bone metabolism, osteoclasts differentiation and bone resorption activity. We found that the depletion of TRPV6 results in noticeable destruction of bone microarchitecture in TRPV6 knockout mice ($TRPV6^{-/-}$). Inactivation of Trpv6 had no effect on osteoblastic bone formation. However, osteoclasts formation are significantly increased in the femurs of $TRPV6^{-/-}$ mice compared with that in $TRPV6^{+/+}$ mice. Moreover, we assessed the involvement of TRPV6 in osteoclast formation and function by culturing bone marrow cells from $TRPV6^{+/+}$ and $TRPV6^{-/-}$ mice with

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osteoclast-inducing cytokines M-CSF and RANKL. We found that TRPV6 deficiency produced a significant increase in the number of osteoclasts formation and bone resorbing activity. To further confirm these resutlts *in vitro*, RNA interference was used to silence TRPV6 gene in osteoclasts precursors. In agreement with the results from *in vivo*, we found that the formation and bone resorption of osteoclasts are significantly increased after blocking TRPV6 expression in osteoclasts precursors. Based on these results, we can draw conclusion that TRPV6 plays an essential role in bone metabolism and is a critical regulator in osteoclasts differentiation and bone resorption.

Materials and Methods

Chemicals

"Culture medium" for osteoclastogenesis comprised Eagle's minimum essential medium with penicillin, streptomycin, and glutamine (both from GibcoBRL, UK) and 10% heat inactivated fetal calf serum (Autogen Bioclear, UK). RANKL and M-CSF were purchased from (PeproTech, NJ). Tartrate-resistant acid phosphatase (TRAP) kit was from (Sigma, MO). Fura-4-acetoxymethyl ester (Fura-4/AM) was purchased from Molecular Probes (Eugene, OR). Mouse intact PTH ELISA kit was from (Immutopics, USA). 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) enzyme immunoassay kit was from (Immunodiagnostic Systems Ltd, UK). Anti-TRPV6 antibody, anti-TRPV4 antibody, anti-TRPV5 antibody and anti-GAPDH antibody were from (Santa Cruz, USA). Anti-TRPV2 antibody was purchased from (Alomone Labs, Israel). Other conventional agents used in the study were from (Sigma-Aldrich, USA).

Cell immunofluorescence

Bone marrow-derived monocytes (BMMs) were grown on 6 cm dishes and induced by RANKL and M-CSF for 7 days. Then they were fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 20 minutes, washed with PBS three times, incubated in 0.2% Triton X-100 for fifteen minutes, and blocked for one hour with 10% normal goat serum in PBS. Additionally, cells were incubated in the primary antibody TRPV6, diluted in 1% normal serum in PBS overnight at 4°C, washed three times with PBS for 5 minutes, and incubated with secondary antibody. Cells were then washed with PBS and mounted with anti-fade mountingmedium. Observations were performed by epifluorescence in a confocal laser scanning microscope (Leica, Germany). The experiments were set in triplicate on three independent occasions.

Generation of Trpv6 gene knockout mouse model

All procedures involving mice were approved by The Second Military Medical University Animal Study Committee and were carried out in accordance with the guide for the humane use and care of laboratory animals. Mouse genomic DNA sequence of Trpv6 gene was obtained from Ensembl database.Trpv6 gene knockout vector (pBR322-MK-Trpv6) was constructed. Trpv6 knockout vector was transferred into the embryonic stem (ES) cells by electroporation and screening of both G418 and Ganciclovoir resistant clones were performed routinely. The homologous recombined ES cell clones were identified by PCR. The correct homologously recombined ES cells were micro injected into C57BL/6J mouse blastocysts to obtain chimera mouse. Male mice with a chimera rate of 50 were mated with C57BL/6J female mice: the off springs with gray fur were obtained, which were identified as heterozygote mice by PCR. Heterozygote mice were intercrossed to generate homozygote mice. Mice were sacrificed at 8-weeks; serum and bones were isolated and processed as described [21]. For dynamic histomorphometry, animals received injection of tetracycline (30 mg/kg) 9 and 1 days prior to sacrifice.

In Vitro osteoclastogenesis

Mice preosteoclasts, and osteoclasts were generated as previous description [22]. Briefly, isolated bone marrow-derived monocytes (BMMs) from mice were cultured in alpha-MEM containing 10% FBS added 50 ng/mL recombinant M-CSF for three days to generate preosteoclasts. After three days, 20 ng/mL recombinant M-CSF and 40 ng/mL recombinant RANKL were added for 72-96 hr to generate mature osteoclasts. The cells were stained for TRAP according to the manufacturer's suggestions. TRAP-positive cells containing more than three nuclei were counted as osteoclasts under microscopic examination.

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Skeletal phenotyping

The distal end of intact femur from TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice were scanned using micro-CT (GE, UK) and X ray to assess bone mass, density, and trabecular microarchitecture. Parameters computed from these data include bone mineral density (BMD) and trabecular number (Tb.N) Serial tomographs, reconstructed from raw data using the cone-beam reconstruction software (NRecon, v.1.4.4.0; Skyscan), were used to compute trabecular and cortical parameters, respectively from the metaphyseal and mid-diaphyseal area.

TRAP staining

TRAP staining was used as a marker for mature osteoclasts. Cells were fixed and stained for TRAP activity using a Leukocyte acid phosphatase kit. Pre-osteoclasts and mature multinucleated osteoclasts (more than three nuclei) appeared dark red and were counted by light microscopy.

Pit formation assay

Bone resorption activity was assessed by pit formation assay according to previous reports [23] with slight modification. BMMs were cultured on bovine cortical bone slices in 96-well plates and induced by RANKL and MCSF. After 7-9 days, the slices were placed for 10 minutes in 1 M NH4OH and were sonicated to remove the cells. The cell-free slices were stained in 1% toluidine blue in 1% sodium borate for three minutes. The experiment was repeated three times. The resorption pits appeared dark blue and were viewed by light microscopy. The percentage of pit area to a "random field of view" was counted.

Selection of siRNA and preparation of lentivirus

The sequence of small interference RNA (siRNA) specifically targeting mRNA of TRPV6 is 5′-AACTTGAG CAGCTTGCTCAGAGCCT-3′. The negative control siRNA targeting LacZ (si-LacZ) is 5′-CTCGGCGT TTCATCTGTGG-3′. The short hairpin RNA (shRNA) oligos were annealed and ligated into the BglII/HindIII-site of pSUPER. The H1 promoter shRNA expression cassettes were subcloned into the lentivirus transfer vector pLB. This was co-transfected with the packaging plasmids, pCMV-Dr8.2 and pCMV-VSV-G, into HEK293T cells using a calcium phosphate co-precipitation method. The medium was replaced with fresh DMEM after co-transfection for 8 hours. The lentiviral supernatant was harvested after 48-72 hours and titers were determined by infecting HEK293T cells with serial dilutions of concentrated lentivirus in the presence of 4 μg/ml polybrene. For depletion of TRPV6 in osteoclasts, BMMs were induced with M-CSF for three days and then transduced with lentiviral supernatant for 8 hours. The medium was replaced with fresh α-MEM containing 20 ng/ml RANKL and 40 ng/ml M-CSF for primary culture.

Western blot analysis

Western blotting experiments were carried out using standard technique with modifications. Briefly, cells were gently washed twice with PBS and scraped into SDS sample buffer. Equal amounts of total protein were resolved on 8% SDS-PAGE gels and transferred onto polyvinyl difluoride membranes using the semi-dry transfer technique. The primary antibodies against TRPV6, TRPV2, TRPV4 and TRPV5 were used and GAPDH was used as an internal control.

$[Ca^{2+}]_i$ oscillation measurement

$[Ca^{2+}]_i$ oscillation measurements were performed as described previously [15]. The cells were incubated with M-CSF for 72 hr and then with 5 μM fluo-4 AM, 5 μM fura red AM, and 0.05% pluronic F127 for 30 min. The cells were post-incubated in DMEM medium with 20 ng/mL M-CSF for 20 min and were mounted on a confocal microscope (Leica, Germany). To estimate intracellular Ca^{2+} concentration in single cells, the ratio of the fluorescence intensity of fluo-4 to fura red was calculated.

Histology and histomorphometry

Histology and histomorphometry were performed as described previously [24] . Left tibiae were fixed in Burkhardt's solution, embedded undecalcified in methyl methacrylate, and sectioned at 4 μm. The sections were stained according to Goldner to assess mineralized bone and unmineralized bone matrix respectively. Unstained sections were used to analyze tetracycline labeling. Histomorphometric analysis was done as described [25], using a Zeiss Axiovert microscope (Zeiss, Germany) and an Axiovision image analyzing system.

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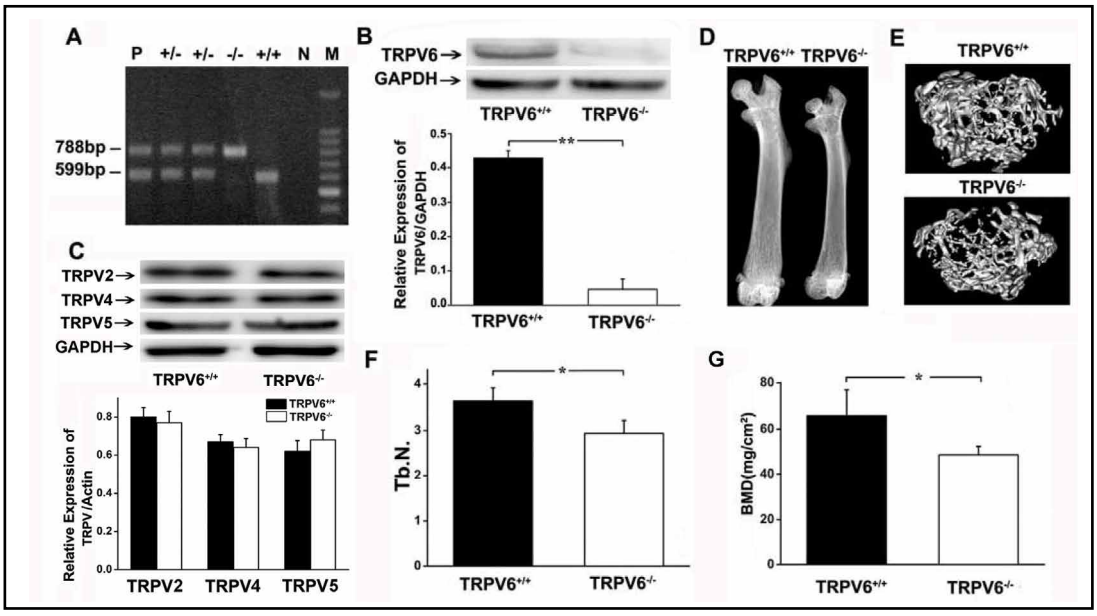

Fig. 1. The generation and identification of TRPV6$^{-/-}$ mice. A: Genotype analysis of mice by PCR. 599 bp fragment could be amplified in wild type mice, 788 bp fragment in homozygous mice, 599 bp and 788 bp fragments in heterozygous mice. M: MBI Gene Ruler 100bp marker; N: Negative Control; P: Positive Control (genome DNA in ES cell); +/-: Heterozygous mice; -/-: Homozygous mice; +/+: Wild type mice. B: Western blot analysis of TRPV6 expression in osteoclasts precursors from TRPV6$^{+/+}$ mice and TRPV6$^{-/-}$ mice. Summarized data showed that TRPV6 expression was significantly decreased in osteoclasts precursors from TRPV6$^{-/-}$ mice. *n = 3, **P <0.01.* C: Western blot analysis of TRPV2, TRPV4 and TRPV5 expressions in osteoclasts from TRPV6$^{+/+}$ mice and TRPV6$^{-/-}$ mice. Summarized data showed that TRPV2, TRPV4 and TRPV5 expressions were not significantly changed in osteoclasts from TRPV6$^{-/-}$ mice. *n = 3.* D: Radiographic analysis of intact femurs from TRPV6$^{+/+}$ mice and TRPV6$^{-/-}$ mice. *n = 4.* E. Representative figures of micro-CT analysis of the distal end of intact femurs from TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice. *n = 4.* F: Trabecular number (Tb.N) in the distal end of intact femurs from TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice. *n = 4, *P < 0.05.* G: Bone mineral density (BMD) in the distal end of intact femurs from TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice. *n = 4, *P < 0.05.*

Measurement of plasma calcium, serum PTH and 1,25(OH)2D3

Plasma ionized calcium was measured using a NOVA-8 electrolyte analyzer (Nova Biomedical, USA), while serum PTH and $1,25(OH)_2D_3$ levels were measured using a commercial, immunoassay kit, according to the manufacturer's instructions.

Statistics

The composite data are expressed as means ± s.e.m. Statistical analysis was performed with one-way ANOVA. Differences were considered to be significant at $P < 0.05$ and very significant at $P < 0.01$.

Results

The generation and identification of TRPV6$^{-/-}$ mice

To gain insights into the biological function of TRPV6 in bone metabolism and osteoclastogenesis *in vivo*, mice with depletion of TRPV6 were generated by homologous recombination. A targeting vector for deletion of TRPV6 was constructed and described in Materials and Methods. The null mutation of TRPV6 was genotyped by PCR (Fig. 1A). To identify whether the TRPV6$^{-/-}$ mice model was successfully constructed, we compared the expression of TRPV6 in osteoclasts precursors from TRPV6$^{+/+}$ mice and TRPV6$^{-/-}$ mice (Fig. 1B). We found that TRPV6 expression is significantly depleted in TRPV6$^{-/-}$ mice compared

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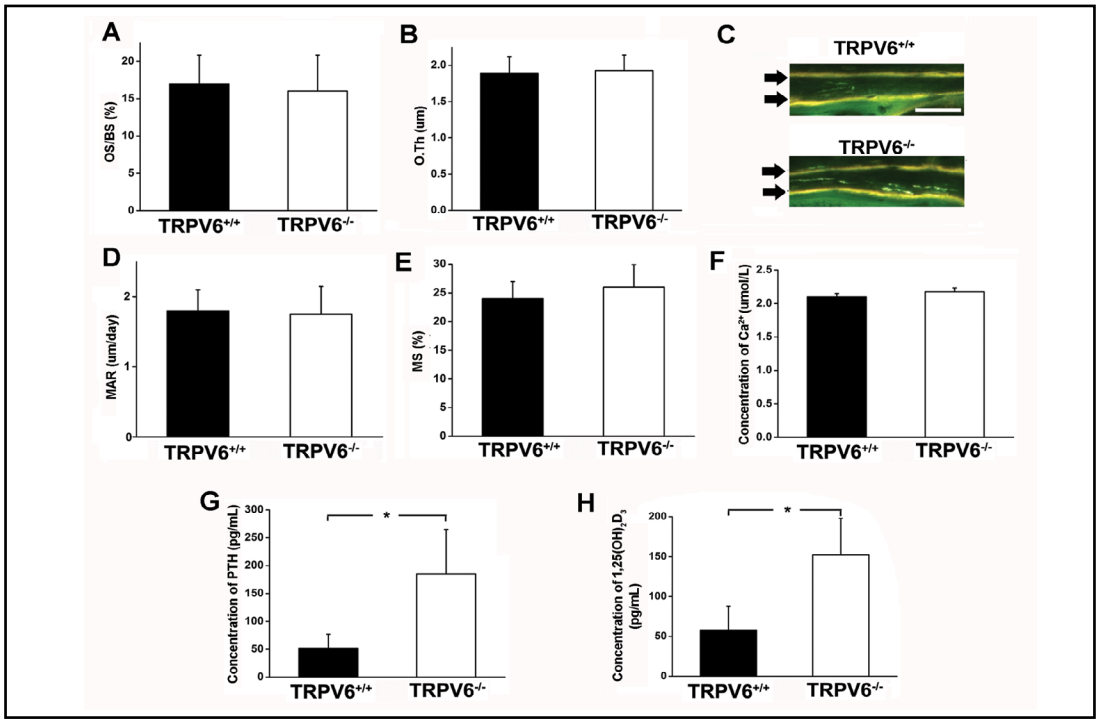

Fig. 2. Bone formation is not affected by Trpv6 inactivation. A: Quantification of the osteoid surface (OS/BS) from Goldner-stained tibia sections of TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice. *n = 4.* B: Quantification of the thickness (O.Th) from Goldner-stained tibia sections of TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice. *n = 4.* C: Tetracycline labeling demonstrating comparable distance between double labels in both genotypes. *n = 4.* D: Quantification of the mineral apposition rate (MAR) in both genotypes. *n = 4.* E: Quantification of the mineralizing surface (MS) in both genotypes. *n = 4.* F: Plasma calcium level in TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice. *n = 4.* G: Serum PTH level in TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice. *n = 4, **P <0.01.* H: Serum 1,25(OH)$_2$D$_3$ level in TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice. *n = 4, **P <0.01.*

with that in TRPV6$^{+/+}$ mice, suggesting that TRPV6 gene knockout mouse model is generated successfully. Furthermore, we examined the expression of TRPV2, TRPV4 and TRPV5 in osteoclasts precursors from TRPV6$^{+/+}$ mice and TRPV6$^{-/-}$ mice (Fig. 1C). No significant differences were found, suggesting that the TRPV6$^{-/-}$ mice is specificity.

To verify the function of TRPV6 in bone metabolism, we studied femurs radio graphs of TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice. X-ray analysis suggested femurs from TRPV6$^{-/-}$ mice show decreased bone length and density of both cortical and trabecular bones (Fig. 1D). There results were confirmed by Micro-CT analysis (Fig. 1E). We compared the trabecular number (Tb.N.) and bone mineral density (BMD) between TRPV6$^{+/+}$ mice and TRPV6$^{-/-}$ mice (Fig. 1F, G). We found that depletion of TRPV6 result in noticeable decline in Tb.N. and BMD, suggesting that TRPV6 is a critical regulator in bone metabolism.

Bone formation remained unchanged in TRPV6$^{-/-}$ mice

BMD was decreased in TRPV6$^{-/-}$ mice, which may be caused by decreasing of osteoblastic bone formation or/and increasing of osteoclastic bone resorption in long bone. Therefore, we first observed the effect of TRPV6 deficiency on osteoblastic bone formation. Histological analysis of Goldner stained tibia sections of Trpv6$^{+/+}$ and Trpv6$^{-/-}$ mice revealed that osteoid abundance (OS/BS; Fig. 2A) and thickness (O.Th; Fig. 2B) were similar in the two genotypes. Dynamic bone formation parameters were assessed by repeated injection of tetracycline, respectively nine and one day before sacrifice (Fig. 2C). The mineral apposition rate (MAR; Fig. 2D) and mineralizing surface (MS; Fig. 2E) was comparable between genotypes,

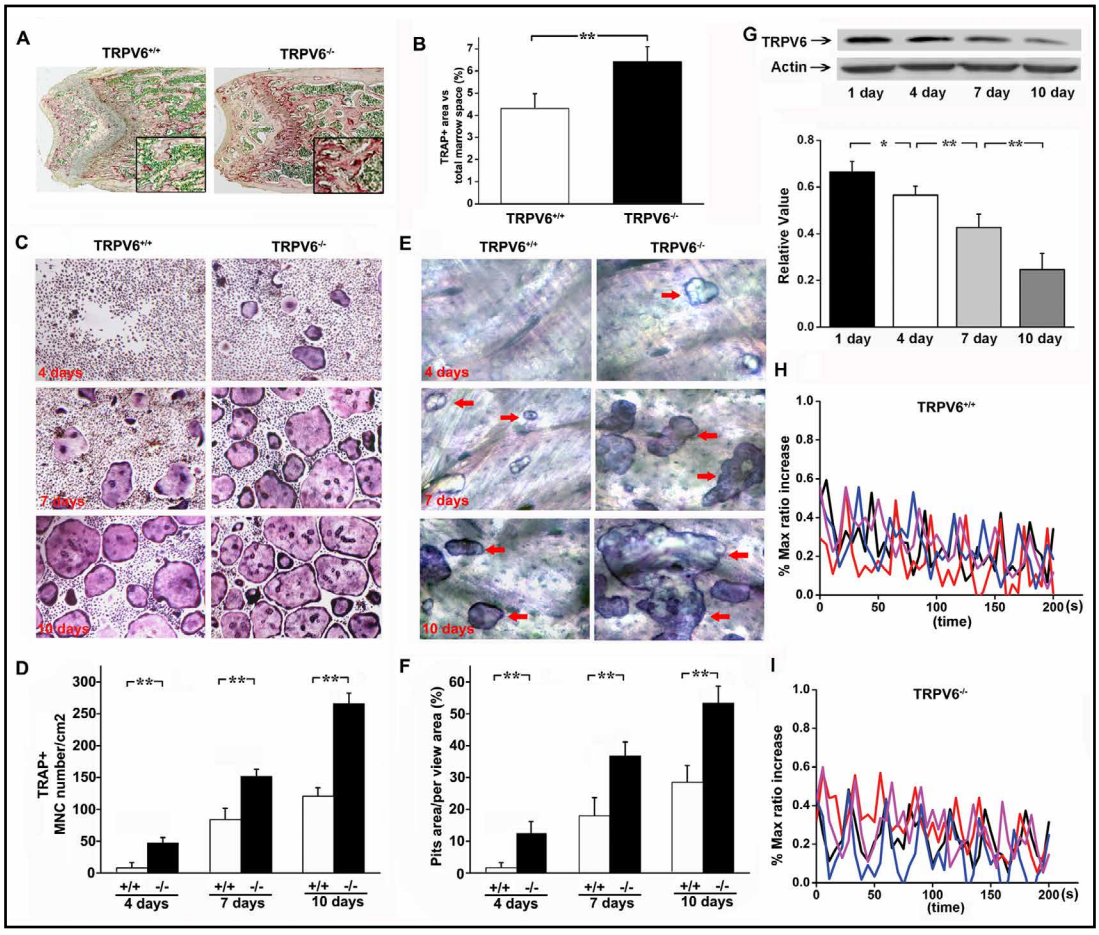

Fig. 3. The effects of TRPV6 depletion on osteoclastogenesis and bone resorption *in vivo*. A: Histologic sections of femurs were stained for TRAP activity. The results showed increased osteoclasts number. and strong TRAP activity in TRPV6$^{-/-}$ mice. B: Quantitative analysis of TRAP^{+}-stained area in femurs sections of TRPV6$^{+/+}$ mice and TRPV6$^{-/-}$ mice. *n = 4, **P <0.01.* C and D: BMMs from TRPV6$^{+/+}$ and TRPV6$^{-/-}$ mice were incubated with RANKL/M-CSF as described in Materials and Methods. TRAP^{+} multinucleated cells (MNCs) could be detected at different time points by TRAP staining analysis. *n = 3, **P <0.01.* E: Patterns of resorption pits on bovine cortical bone slices. F: Quantification of resorption area per view area. *n = 3, **P <0.01.* G: Western blot analysis of TRPV6 expression during osteoclast differentiation. Summarized data showed that the time-dependent decrease in TRPV6 expression during osteoclast differentiation. *n = 3, *P < 0.05, **P <0.01.* H and I: $[Ca^{2+}]_i$ changes were traced in TRPV6$^{+/+}$ and TRPV6$^{-/-}$ BMMs treated with M-CSF for 72 hr $[Ca^{2+}]_i$. Changes were estimated as the ratio of fluorescence intensity of fluo-4 to fura red, plotted at 5-sec intervals. *n = 4.*

suggesting normal osteoblast function and number. Taken together, inactivation of Trpv6 had no effect on osteoblastic bone formation.

Next, we observed the effect of TRPV6 deficiency on plasma calcium level. We found that plasma calcium concentration remained normal in Trpv6$^{-/-}$ mice (Fig. 2F). However, serum PTH and 1,25$(OH)_2D_3$ levels are significantly increased in Trpv6$^{-/-}$ mice (Fig. 2G, H), suggesting that compensatory mechanisms, acting at the skeleton and/or kidney, contribute to preserve normal plasma calcium level.

Osteoclastogenesis and bone resorption are enhanced in TRPV6$^{-/-}$ mice

To further explore the effect of TRPV6 depletion on osteoclastogenesis *in vivo*, we analyzed TRAP staining in the metaphyseal area of femurs bone sections derived from

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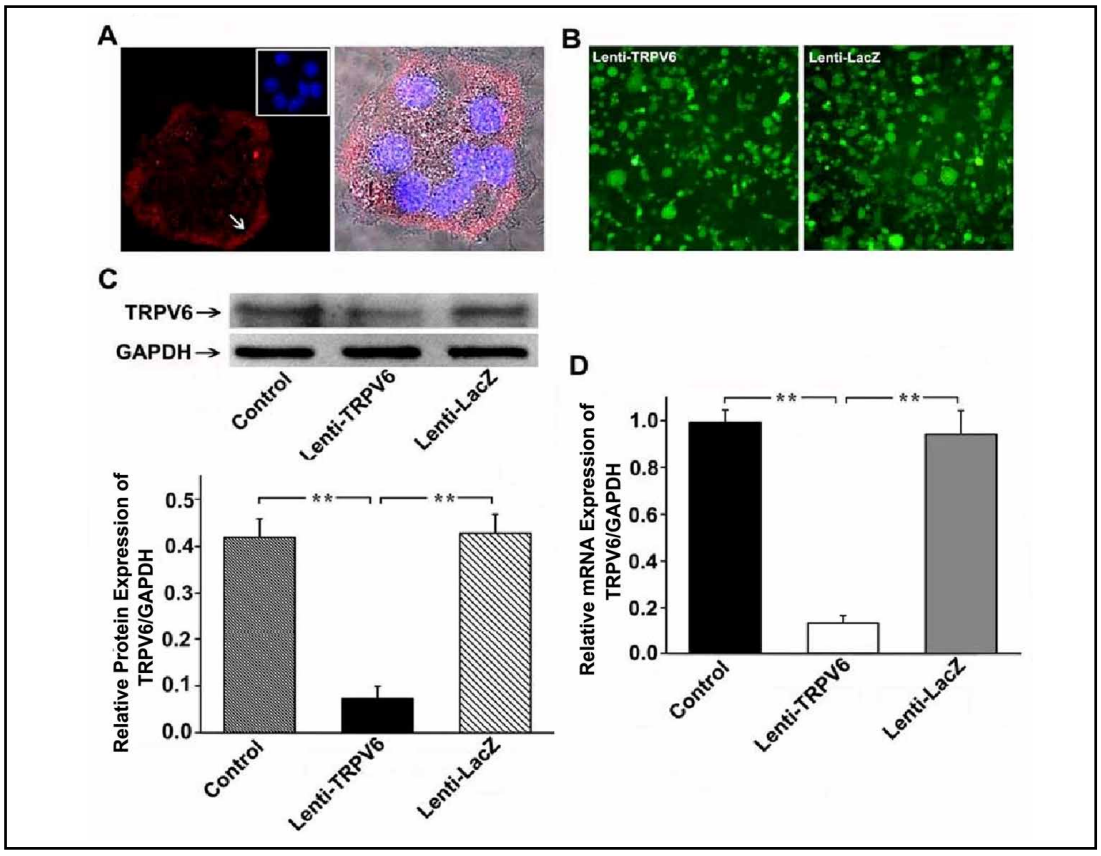

Fig. 4. Effective depletion of TRPV6 expression by lentiviral siRNA in osteoclasts precursors. A. TRPV6 staining (red) was demonstrated in osteoclasts. Confocal laser scanning microscopy showed TRPV6 staining (arrow head) predominantly at the cell membrane. Nuclei are stained with DAPI (blue). B. All cells expressed GFP, showing that cells were infected by lentivirus and also expressed siRNA. C and D. Verified TRPV6 knockdown effect by lentivirus-mediated transduction of primary culture osteoclasts precursors. SiRNAs was showed to deplete 85% of the expression of TRPV6 in osteoclasts precursors by western blot and real-time RT-PCR. ***P <0.01.*

$TRPV6^{+/+}$ and $TRPV6^{-/-}$ mice. The number of TRAP positive cells was increased in the $TRPV6^{-/-}$ mice compared with $TRPV6^{+/+}$ mice (Fig. 3A). Quantification of the TRAP staining showed a significantly increased osteoclast number and surface area (Fig. 3B) in the metaphysis of $TRPV6^{-/-}$ femurs. Furthermore, we further assessed the involvement of TRPV6 in osteoclast formation and function by culturing bone marrow cells from $TRPV6^{+/+}$ and $TRPV6^{-/-}$ mice with osteoclast-inducing cytokines M-CSF and RANKL in different time course. In cultures derived from $TRPV6^{-/-}$ mice, the number of osteoclasts was significantly higher compared with $TRPV6^{+/+}$ cultures (Fig. 3C, D). These data strongly supported our *in vivo* findings that osteoclastogenesis is enhanced in $TRPV6^{-/-}$ mice. Subsequently, bone resorption was measured by a resorption pit assay based on osteoclasts derived from bone marrow cultures and seeded on cortical bone slices. In cultures of $TRPV6^{-/-}$ osteoclasts, resorption pits were significantly enhanced (Fig. 3E, F). To understand the time-related function of TRPV6 in osteoclastogenesis, we quantified TRPV6 expression at several time points during osteoclast differentiation. We found that the time-dependent decrease in TRPV6 expression during osteoclast differentiation (Fig. 3G). However, RANKL-induced $[Ca^{2+}]_i$ oscillation response was not significantly affected by TRPV6 inhibition (Fig. 3H, I).

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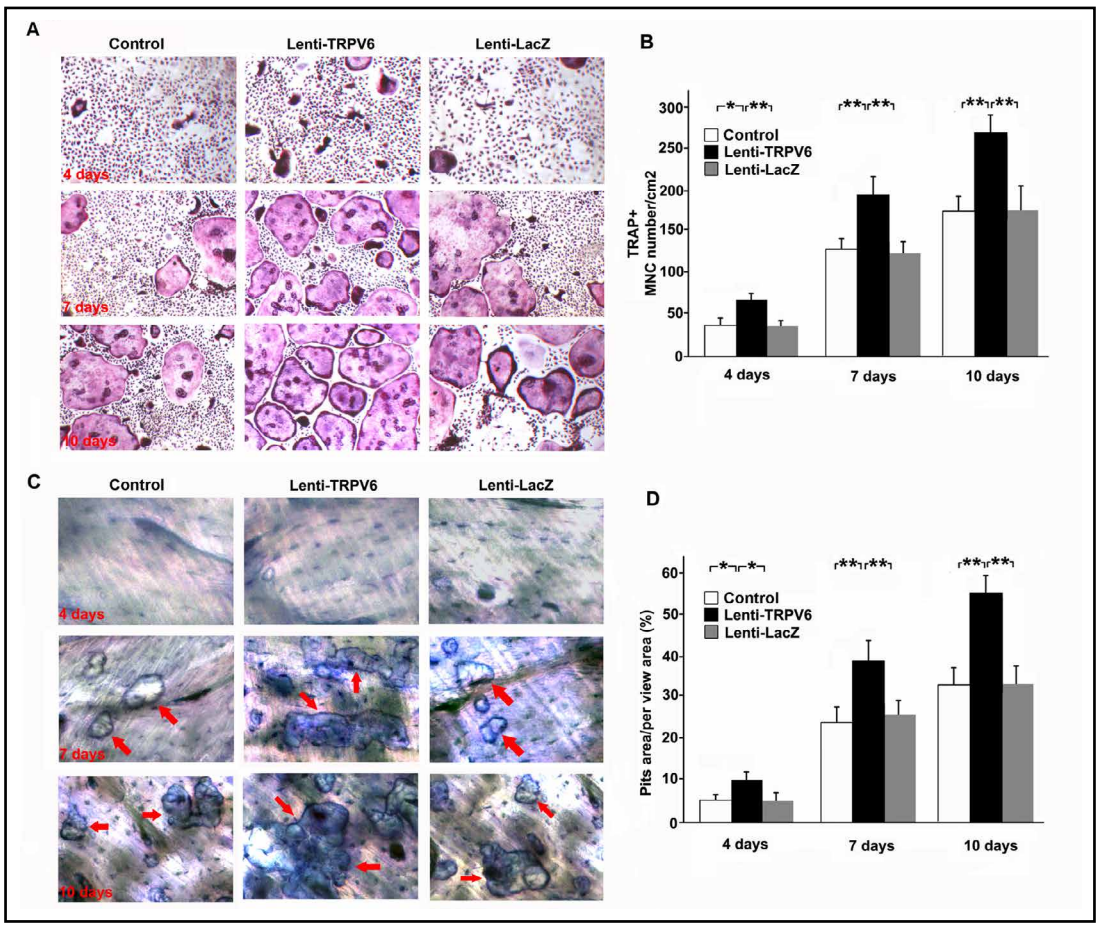

Fig. 5. The effects of TRPV6 depletion on osteoclasts differentiation and bone resorption activity *in vitro.* A and B: TRAP stain of osteoclasts precursors with lentivirus-siRNA targeting TRPV6 or lentivirus-LacZ at different time points. Summarized data showed that the depletion of TRPV6 enhanced the osteoclastogenesis. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$. C and D: Pit formation assay of osteoclasts precursors with lentivirus-siRNA targeting TRPV6 or lentivirus-LacZ at different time points. Summarized data showed that the depletion of TRPV6 enhanced the bone resorption activity. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.

Effective depletion of TRPV6 expression by lentiviral siRNA in osteoclasts precursors

We observed the distribution of TRPV6 in mature osteoclasts by immunocytochemical fluorescence. The results showed that the distribution of TRPV6 in the cytoplasm and nucleus are very few, which mainly distribute in the cell membrane (Fig. 4A). To further confirm the effect of TRPV6 on osteoclasts differentiation and bone resorption *in vitro*, we used lentiviral constructs encoding siRNAs that target TRPV6 to infect osteoclasts precursors and deplete TRPV6 expression. For lentivirus-mediated TRPV6 knockdown, optimal viral particle numbers for infection were based on infection efficiency, determined from the percentage of target cells with green fluorescent protein (GFP), which is expressed independently from the RNAi sequence. We used 300 µl lentivirus supernatant to infect 2×10^4 BMMs after 72-hour induction by 50 ng/ml M-CSF. After another 48-hour period, infected GFP^+ cells were viewed under fluorescence microscopy. All cells expressed GFP, showing that cells were infected by lentivirus and also expressed siRNA (Fig. 4B). Furthermore, siRNAs was showed to deplete 85% of the expression of TRPV6 in osteoclasts precursors by western blot and real-time RT-PCR (Fig. 4C, D).

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Depletion of TRPV6 stimulates the osteoclasts differentiation and bone resorption activity in vitro

We assessed the differentiation and maturation of TRPV6-depleted osteoclast precursors by TRAP staining assay after infection. The number of stained multinuclear $TRAP^+$ osteoclasts showed more obviously time-dependent increases in TRPV6-depleted group compared with the two control group (control and Lenti-LacZ) (Fig. 5A, B). Furthermore, to examine potential functions of TRPV6 in bone resorption, we then assessed resorption pit formation by TRPV6-knockdown osteoclasts on bovine cortical bone slices. As shown in Fig. 5C, D , we found more significantly increased pit formation in osteoclasts depleted of TRPV6 compared with that in two control group (control and Lenti-LacZ). Taken together, these results strongly suggest that TRPV6 is an essential regulator of osteoclasts differentiation and function.

Discussion

In terrestrial vertebrates, the osteoclast performs the vital tasks of dissolving bone for calcium or pH homeostasis and removing bone for growth or to replace bone [26]. It has been established that in the presence of M-CSF sufficient to maintain cell growth and survival, RANKL, via its tumor necrosis factor family receptor RANK, is sufficient to induce complete osteoclastic differentiation from hematopoietic precursors and that knockout mice with defects in the RANKL system cannot form osteoclasts [27]. It is also established that the Ca^{2+} oscillations followed by the sustained Ca^{2+} influx are both needed for NFATc1 activation and proper osteoclast differentiation [28]. In the present study, we identified that TRPV6 is a negative regulator of RANKL induced osteoclasts differentiation and activity. This is the study linking a TRPV6 with osteoclasts differentiation and activity, which provides key insights into the mechanisms of bone metabolism.

It is well known that Ca^{2+} signaling controls multiple cellular functions, including proliferation, apoptosis, and differentiation [29]. Recent studies have highlighted the importance of intracellular Ca^{2+} signaling for osteoclast differentiation and activity. RANKL-induced $[Ca^{2+}]_i$ increase has been reported to inhibit cell motility and the resorption of cytoskeletal structures in mature osteoclasts, resulting in suppression of bone-resorption activity [30]. Further studies confirmed that TRPV5 as an epithelial Ca^{2+} channel which is essential for osteoclastic bone resorption and differentiation [10]. TRPV5 and TRPV6 share 75% homology at the aminoacid level. However, to our knowledge, the regulation effects of TRPV6 in bone metabolism are still controversial and there have been no reports to directly observe the effect of TRPV6 on osteoclasts differentiation and function. Our study is the first effort to elucidate the direct effect of TRPV6 on RANKL-induced osteoclasts differentiation and bone resorption function.

In our study, mice with depletion of TRPV6 were generated to verify the effect of TRPV6 on bone metabolism. Our results showed that TRPV6 depletion results in noticeable decline in BMD and Tb.N, indicating that TRPV6 is a critical regulator in bone homeostasis. Furthermore, the number of TRAP positive cells in the metaphyseal area of femurs bone sections was increased in the $TRPV6^{-/-}$ mice compared with $TRPV6^{+/+}$ mice. Therefore, based on above findings, we believed that TRPV6 contributes to bone homeostasis through the regulation of osteoclasts differentiation and activity. The involvement of TRPV6 in bone metabolism is still controversial. Bianco et al found that the phenotype of mice with targeted disruption of the Trpv6 (Trpv6 KO) epithelial calcium channel exhibit disordered Ca^{2+} homeostasis, including defective intestinal Ca^{2+} absorption, decreased BMD, deficient weight gain, and reduced fertility [31], which are coincidence with our results. Lieben et al study showed that restricting dietary calcium result in a comparable reduction in bone mass accrual in $Trpv6^{+/+}$ and $Trpv6^{-/-}$ mice (-35% and 45% respectively) [24]. This decrease in bone mass was associated with a similar increase in bone resorption, whereas serum osteocalcin levels and the amount of unmineralized bone matrix were only significantly increased in $Trpv6^{-/-}$ mice

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[24]. However, van der Eerden et al found that bone microarchitecture and mineralization were unaffected in Trpv6D541A/D541A mice in which aspartate 541 in the pore region was replaced with alanine to render TRPV6 channels non-functional [32]. Based on these reports above, we speculated the different animal models used result in the different results. In our study, the TRPV6$^{-/-}$ mice were generated by deleting the second exons, which lead to subsequent mRNA reading frame shift and cannot continue coding protein. Phenotype generated in our study can be completely attributed to the deletion of Trpv6 gene, without any ambiguity. Van der Eerden et al used a knock-in mouse with replacement of one amino acid (D541A), which impairs the permeability of TRPV6 channels for Ca^{2+} without affecting channel architecture and interaction with associated proteins. The reasons of different results from different animal model need to be further explored.

Previous studies indicated that both Ca^{2+} oscillation/calcineurin-dependent and -independent signaling pathways contribute to NFATc1 activation, leading to efficient osteoclastogenesis. The Ca^{2+} oscillations turn on a number of Ca^{2+}/calmodulin-activated proteins including calcineurin, calmodulin-dependent protein kinases (CaMK) and CaMK-mediated CREB (cAMP response element-binding protein) [8, 33, 34]. Upon activation of the phosphatase calcineurin, the transcription factor NFATc1 becomes phosphorylated, translocates to the nucleus, and increases osteoclast-specific gene transcription. Even though sustained Ca^{2+} oscillation induced by RANKL is reportedly necessary for osteoclastogenesis, a Ca^{2+} oscillation/calcineurin -independent mechanism of osteoclastogenesis has been also demonstrated [35]. In our study, RANKL-induced calcium oscillation response was not significantly affected by TRPV6 inhibition. Therefore, we speculated that TRPV6 may be not an essential regulator of Ca^{2+} oscillations in osteoclasts and Ca^{2+} oscillation/calcineurin -independent signaling pathways may contributes to TRPV6 deficiency-induced osteoclastogenesis.

An important finding reported here is that the knockout of TRPV6 increased the osteoclastic differentiation and function. Based on our results and previous studies, we speculated about the property of true nature TRPV6 in physiological condition during osteoclastogenesis. First, TRPV6 channels are known to be involved in Ca^{2+} homeostasis, regulating Ca^{2+} flux through intestine epithelial cells [36]. Our observation that TRPV6 knock-out mice reduced bone thickness, and TRPV6 has an inhibitory effect on the process of bone resorption in osteoclasts also relates TRPV6 to bone homeostasis. This is consistent with the close relationships known to exist between intestine and bone in calcium and skeletal homeostasis. Second, TRPV6 channels are highly expressed in osteoclasts and located on the plasma membrane of osteoclasts. We speculated that TRPV6 may regulate bone resorption directly from the ruffled border. Third, TRPV6 might have a direct influence on the bone resorption function of osteoclasts by activating signaling pathway in osteoclasts mediated by Ca^{2+} entry. However, the roles of TRPV6 in physiological condition during osteoclastogenesis need to be further explored.

Previous studies showed that the increase in the expression of TRPV channels family, such as TRPV2 and TRPV4 promotes the osteoclastic differentiation and resorption [18]. However, the present data controversially shows the deletion and silencing of TRPV6 channels facilitates the osteoclastic function. Some reasons may be accounted for this phenomenon. Firstly, it is likely that depletion of TRPV6 has positive effect on osteoclast precursor cells, such as promoting cells survival and inhibiting apoptosis, leading to an increase in the amount of osteoclasts. Similar speculation also was presented in van der Eerden et al study [10]. They found that TRPV5 deficiency leads to an increase in the osteoclast size and number. Furthermore, as Chamoux et al reports, they showed that suppressing of TRPV5 expression increases bone resorption in osteoclasts [37]. They believed that TRPV5 might have a direct influence on the bone resorption function of osteoclasts by activating a stop signal in the resorbing osteoclasts mediated by Ca^{2+} entry. Therefore, we speculated it may be one of mechanisms that depletion of TRPV6 promotes bone resorption function of osteoclasts. Moreover, it is possible that impaired osteoclast differentiation in TRPV6$^{-/-}$ mice results from deficiencies in other signaling pathways evoked by RANKL and M-CSF, such as

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NF-κB pathway. However, the precise molecular mechanisms of TRPV6 in RANKL-induced osteoclastogenesis need to be further elucidated in the future study.

In summary, this study showed an animal model depleting the epithelial calcium channel TRPV6, which lead to destruction of bone microarchitecture. In addition, depletion of TRPV6 *in vitro* significantly increased osteoclasts differentiation and bone resorption activity. This study is an effort to establish a regulation mechanism of bone homeostasis, and to provide insights into the potential contribution of TRPV6 in the regulation of osteoclasts differentiation and activity.

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Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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