Published in final edited form as:

Cell Calcium. 2012 December ; 52(6): 488–500. doi:10.1016/j.ceca.2012.10.001.

Deletion of Orai1 Alters Expression of Multiple Genes during Osteoclast and Osteoblast Maturation

Sung-Yong Hwang¹, Julie Foley, Takuro Numaga-Tomita, John G. Petranka, Gary S. Bird, and James W. Putney Jr.²

Laboratory of Signal Transduction, National Institute of Environmental Health Sciences – NIH, Department of Health and Human Services, Research Triangle Park, NC 27709, USA

Summary

Store-operated Ca^{2+} entry (SOCE) is a major Ca^{2+} influx pathway in most non-excitabile cell types and Orai1 was recently identified as an essential pore-subunit of SOCE channels. Here we investigate the physiological role of Orai1 in bone homeostasis using Orai1-deficient mice (Orai1^{-/-}). Orai1^{-/-} mice developed osteopenia with decreased bone mineral density and trabecular bone volume. To identify the nature and origin of the bone defect, bone-resorbing osteoclasts and bone-forming osteoblasts from Orai1^{-/-} mice were examined. Orai1-mediated SOCE was completely abolished in Orai1^{-/-} osteoclast precursor cells and osteoclastogenesis in vitro from Orai1^{-/-} mice was impaired due to a defect in cell fusion of pre-osteoclasts. Also, resorption activity in vitro was comparable but the size of pits formed by Orai1^{-/-} osteoclasts was smaller. We next assessed the role of Orai1 in osteoblast differentiation and function by using a pre-osteoblast cell line, as well as primary osteoblasts from wild-type and Orai1^{-/-} mice. SOCE in MC3T3-E1 pre-osteoblastic cells was inactivated by lentiviral overexpression of a pore-dead Orai1 mutant. Lack of SOCE in MC3T3-E1 had no effect on alkaline phosphatase staining and expression but substantially inhibited mineralized nodule formation. Consistent with this finding, Orai1-mediated SOCE was markedly reduced in Orai1^{-/-} osteoblast precursor cells and osteoblastogenesis in vitro from Orai1^{-/-} stromal cells showed impaired mineral deposition but no change in differentiation. This indicates that Orai1 is involved in the function but not in the differentiation of osteoblasts. Together, these results suggest that Orai1 plays a critical role in bone homeostasis by regulating both osteoblasts and osteoclasts.

Bone is a highly dynamic tissue that undergoes constant remodeling throughout life. This bone homeostasis is maintained by balanced activities of bone-forming osteoblasts and bone-resorbing osteoclasts [1–3]. Bone resorption and formation are determined by proliferation and differentiation of progenitors of the two bone cells. Osteoclasts are differentiated from hematopoietic cells within the monocyte/macrophage lineage. Osteoblasts originate from mesenchymal stem cells. Although these two bone cells originate from different progenitors, they interact to regulate differentiation and function of one another. Osteoblasts express two cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of NF κ B ligand (RANKL, also known as TRANCE/OPGL/ODF), both of which are essential for osteoclastogenesis [1;3]. RANKL from osteoblasts drives

²To whom correspondence should be addressed: James W. Putney, National Institute of Environmental Health Sciences – NIH, Research Triangle Park, NC 27709, USA, Tel.: (919) 541-1420; Fax: (919) 541-1898; putney@niehs.nih.gov.

¹Present address: Department of Pathology and Immunology, Washington University in St Louis, School of Medicine, St. Louis, MO 63110, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

osteoclastogenesis by providing an essential signal to osteoclast progenitors through the membrane-anchored receptor RANK in osteoclasts. Osteoblasts also synthesize and secrete osteoprotegerin (OPG), a decoy receptor of RANKL, which blocks the interaction between RANKL and RANK [4;5]. Many bone diseases such as postmenopausal osteoporosis, metastatic osteolytic lesions and bone destruction in rheumatoid arthritis are caused by excessive osteoclastogenesis [6–9], whereas senile osteoporosis mainly results from defects in osteoblastogenesis [10;11]. Therefore, it is critical to define molecular mechanisms underlying differentiation and activation of these cells to develop new therapeutic approaches for the diseases.

A number of signaling pathways are involved in the differentiation of these two major bone cell types. For osteoclast differentiation, binding of RANKL to the RANK receptor leads to recruitment of TNF receptor-associated factor 6 followed by activation of various downstream signaling targets. These include NF κ B, MAP kinases (Erk1/2, p38, and c-Jun N-terminal kinase), and c-Fos [3;12]. For osteoblast differentiation, multiple transcription factors including Runx2, Osterix, and several members of activation protein-1 (AP-1) group of transcription factors such as JunB, Fra-1, and Δ FosB [13–16] were shown to play a pivotal role.

A rise in intracellular Ca^{2+} levels serves as a versatile signal in regulating multiple cellular functions including exocytosis, proliferation, differentiation, growth, and cell death [17;18]. It has long been known that Ca^{2+} signaling plays a critical role in the functions of bone cells [19–25]. Importantly, Takayanagi et al. [21] showed that RANKL evokes Ca^{2+} oscillations in pre-osteoclasts. These Ca^{2+} oscillations result in robust induction of nuclear factor-activated T cells c1 (NFATc1), a Ca^{2+} /calcineurin-dependent master regulator of osteoclastogenesis. Extracellular stimuli such as neurotransmitters or hormones activate cell membrane receptors coupled to phospholipase C, resulting in intracellular Ca^{2+} release from the endoplasmic reticulum (ER) followed by extracellular Ca^{2+} influx through store-operated Ca^{2+} entry (SOCE) [26]. SOCE not only refills the depleted ER Ca^{2+} stores but also provides a direct Ca^{2+} signal to activate downstream responses [27]. SOCE channels are comprised of Orai subunits, primarily Orai1. Either deletion of the *Orai1* gene or overexpression of a pore-dead Orai1 mutant (E106Q) markedly reduces SOCE [28;29]. Clinically, T cells from patients with a severe combined immune deficiency (SCID) syndrome showed lack of SOCE, which is attributed to mutations in the *Orai1* gene [30]. We previously demonstrated by a gene silencing approach that Orai1-mediated SOCE plays a critical role in osteoclast differentiation from RAW264.7 cells [31]. In addition, several studies reported the presence of SOCE in osteoblasts [32;33]. Labelle et al. [33] showed that SOCE is involved in osteoblast proliferation, and Robinson et al. [34] recently reported that pharmacological inhibition of SOCE impairs differentiation of human osteoblastic cells. However, the role of SOCE in differentiation and function of primary osteoblasts and osteoclasts lacking an *Orai1* gene, and in regulation of the genes associated with their differentiation and function, has not been investigated.

Here we have investigated the physiological role of Orai1 in the differentiation and function of the two major bone cell types by using *Orai1*^{−/−} mice. We observed that the ablation of Orai1 in mice results in osteopenia with decreased bone mineral density and trabecular bone volume. This phenotype cannot be due to increased activity of osteoclasts, because we found that osteoclastogenesis in vitro is actually impaired in cells from *Orai1*^{−/−} mice. On the other hand, although osteoblast differentiation is not altered, mineralization is substantially impaired in *Orai1*^{−/−} osteoblasts due to a defect in expression of collagen type 1. In addition, the regulated expression of a number of key genes involved in the maturation of these two major bone cell types is altered in the absence of Orai1. Thus, Orai1 appears to play a

critical role in bone remodeling by regulating intracellular Ca^{2+} signaling that leads to specific gene expression in osteoblasts and osteoclasts.

Materials and Methods

Reagents and antibodies

M-CSF and mouse soluble RANKL were obtained from R & D Systems (Minneapolis, MN, USA) and PeproTech EC (London, UK), respectively. Anti-ATP6v0d2 antibody (ab87059) was purchased from Abcam (Cambridge, MA, USA).

Mice

Orai1^{-/-} mice were obtained from Dr. Jean-Pierre Kinet (Harvard Medical School) and described previously [35]. Orai1^{-/-} mice in C57BL/6 background were then outbred for at least two generations with ICR mice in order to improve the survival of knockout animals. All mice used in these experiments were 6–13 weeks old and genotyping was performed on genomic DNA extracted from tails. Mice were housed in the animal facility of the National Institute of Environmental Health Sciences (NIEHS) according to the Association for the Assessment and Accreditation of Laboratory Animal Care Guidelines. All studies were approved by the Animal Care and Use Committee at the NIEHS.

Histology and microcomputed tomography (μCT)

Long bones were fixed, decalcified, and paraffin-embedded and stained with either H&E or for TRAP. μCT ($\mu\text{CT}40$, Scanco medical, Bassersdorf, Switzerland) was performed on femurs (15 μm isotropic voxel resolution). Cortical analysis was completed on an area 0.5 mm thick that was 5.25 mm proximal to the distal growth plate. For trabecular analysis, a three-dimensional cylindrical volume 0.750 mm in the z direction (axial) was selected and 0.98 mm in diameter that was 0.750 mm proximal from the distal growth plate was selected.

Bone mineral density

Bone mineral density of femurs from 6–13 week-old mice was determined using a Lunar PIXImus II densitometer (GE Healthcare, Piscataway, NJ, USA). The PIXImus was calibrated using a phantom of known BMD before use.

Cell culture

MC3T3-E1 cells (subclone 14) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in α -MEM (no ascorbic acid, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin G and 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% CO_2 . Osteoblastic differentiation of MC3T3-E1 cells was induced by 50 $\mu\text{g}/\text{ml}$ ascorbic acid (Sigma, St. Louis, MO, USA) and 10 mM β -glycerophosphate (Sigma, St. Louis, MO, USA).

Lentiviral transfection

Recombinant lentiviruses were produced as previously described [31]. Briefly, either pCDH511B-1 pCDH-CMV-MCS-EF1-copGFP (System Biosciences, CA, USA) or pCDH-CMV-human Orai1 E106Q-EGFP-EF1-puro lentiviral plasmids were co-transfected with psPAX2 and pMD2.G into HEK293T/17 cells (ATCC, VA, USA) using LipofectAMINE Plus (Invitrogen, Carlsbad, CA, USA). The medium was replaced after 24 h, and viral supernatants were harvested 2 d post-transfection and stored at -80°C until use. MC3T3-E1 cells were plated at a density of 200,000 cells/well of a 6-well plate and infected with 120

MOI of viral supernatant containing 5 µg/ml polybrene (Sigma, St. Louis, MO, USA). The media was replaced the following day and cultured for further experiments.

Osteoclast differentiation in vitro

Bone marrow cells were flushed out from femurs and tibia and cultured in α -MEM containing 10% FBS and M-CSF (30 ng/ml) overnight. Nonadherent cells were harvested and cultured in the presence of M-CSF for 3 d. After 3 d, adherent cells were used as bone marrow-derived macrophages (BMMs). The BMMs were plated on a 48-well plate at a density of 15,000 cells/well and cultured in the presence of 100 ng/ml RANKL and M-CSF (30 ng/ml) for 4 d. The medium was replaced every 2 d. The cultured cells were stained for tartrate-resistant acid phosphatase (TRAP) staining using an Acid Phosphatase Leukocyte kit (#387A, Sigma, St. Louis, MO USA). TRAP staining was performed in triplicate wells for each condition according to the manufacturer's instruction. In order to measure secreted TRAP during osteoclastogenesis, cells were plated on a 96-well plate at a density of 6,000 cells/well and cultured in the presence or absence of RANKL for 4 d (media was replaced on day 2). On day 4, 25 µl of the media were mixed with 75 µl of TRAP solution (Acid Phosphatase Leukocyte kit #387, Sigma, St. Louis, MO, USA). The mixture was incubated at 37°C for 2 h and the absorbance was read at 540 nm (adapted from a protocol of TRAP staining kit, B-Bridge international, Inc., Cupertino, CA, USA).

In vitro resorption pit assay

BMMs were seeded on the 16-well BD BioCoat® Osteologic® calcium phosphate-coated slides (BD Biosciences, San Jose, CA, USA) at a density of 6,000 cells/well and osteoclastogenesis was induced by 100 ng/ml RANKL for 7 d. The medium was replaced every 2 d. Cells were detached with sodium hypochlorite solution (Sigma, St. Louis, MO, USA) and rinsed with distilled water. The plates were stained with 5% silver nitrate as instructed in Technical Bulletin #444 of BD Biosciences (http://www.bdbiosciences.com/external_files/dl/doc/tech_bulletin/live/web_enabled/TB444.pdf). The number of pits per well was determined in quadruplicate wells. For measurement of pit size, regions of interest (ROIs) for each pit were drawn and analyzed by MetaMorph imaging software (Molecular Devices, Inc., Sunnyvale, CA, USA).

Osteoblast differentiation in vitro

For osteoblast differentiation from mesenchymal stromal cells, bone marrow cells from long bones were cultured in α -MEM (no ascorbic acid, Invitrogen, Carlsbad, CA, USA) containing 10% FBS for 9 d [36]. Cells were replated and cultured until they became confluent. Osteoblast differentiation was induced in osteogenic medium (50 µg/ml ascorbic acid, 10 mM β -glycerophosphate, and 10 nM dexamethasone). After 7 d, cells were fixed with 4% paraformaldehyde and stained for alkaline phosphatase (ALP, Sigma, St. Louis, MO, USA). Bone nodule formation was identified with 2% Alizarin Red S (Sigma, St. Louis, MO, USA) staining on d 14. For quantification, Alizarin Red staining from the cell matrix was released with 10% cetylpyridinium chloride in 10 mM sodium phosphate for 15 m. The concentration of released Alizarin Red was determined by measuring the absorbance at 562 nm [37;38]. Mineral deposition was alternatively measured by von Kossa staining (5% silver nitrate, Sigma, St. Louis, MO, USA) on d 21.

Optical imaging of intracellular Ca²⁺ concentrations

Cells were seeded on coverslips and cultured overnight. BMMs and bone marrow stromal cells (BMSCs) were loaded with 5 µM Fura-2/AM Ca²⁺ indicator dyes (Invitrogen, Carlsbad, CA, USA) for 45 m at room temperature (MC3T3-E1 at 37 °C). Relative intracellular Ca²⁺ concentrations are reported as the ratio of fluorescence emitted when cells

were excited alternately at 340 and 380 nm wavelengths of light. Peak ratio values of Fura-2 from BMMs, BMSCs, and MC3T3-E1 cells in response to thapsigargin (2 μ M) were consistently between 1 and 2 while $R_{\max}$, determined *in situ*, averaged about 5 for BMMs and MC3T3-E1, and 3.5 for BMSC, indicating that the indicator was less than half-saturated and thus yielded ratios that are proportional to Ca^{2+} concentrations.

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

BMMs from mouse long bones were plated at a density of 250,000 cells/well of a 6-well plate. After culture for 24 h, cells were treated with 100 ng/ml RANKL for 3 d. MC3T3-E1 cells were plated at a density of 200,000 cells/well of a 6-well plate and cultured in osteogenic medium for 7 d. BMSCs were plated at a density of 100,000 cells/well of a 6-well plate and cultured in osteogenic medium for 7 d. Total RNA was isolated and reverse transcribed to cDNA using an Omniscript RT kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. qRT-PCR was performed using a SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) with the ABI Prism 7000 Instrument (Applied Biosystems, Carlsbad, CA, USA). The mRNA levels were normalized to GAPDH. The sequence of primer pairs for murine ATP6v0d2, cathepsin K, DC-STAMP, GAPDH, NFATc1, and TRAP was previously described [31]. Sequences of other primer pairs are as follows.

ALP sense 5'-ATCTTTGGTCTGGCTCCCATG-3' and antisense 5'-TTTCCCGTTCACCGTCCAC-3' [39];

cFms sense 5'-CCTCCTCTGGTCTGCTGCTGG-3' and antisense 5'-GCTCACACATCGCAGGGTCACC-3' [40];

Colla1 sense 5'-CCTGGTAAAGATGGTGCC-3' and antisense 5'-CACCAGGTTCACCTTTCGCACC-3' [41];

Colla2 sense 5'-TGGTCTCTGGGCATCTCAGGC-3' and antisense 5'-GGTGAACCTGCTGTTGCCCTCA-3' [41];

Fra-1 sense 5'-CCCCCGCAAGCTCAGGCACAGAC-3' and antisense 5'-GCAGATGGGGCGATGGGCTTCC-3' [16];

NFATc2 sense 5'-TGGCCCGCGACATCTACCCT-3' and antisense 5'-TGGTAGAAGGCGTGCGGCTT-3' [42];

OPG sense 5'-GCACATTTGGCCTCCTGCTAATTC-3' and antisense 5'-ACTCTCGGCATTCACTTTGGTCCC-3' [43];

Orai1 5'-CTCAAAGCTTCCAGCCGGAC-3' and antisense 5'-GGGAGCGGTAGAAGTGAACA-3';

Orai2 primer pairs were purchased from SABiosciences (PPM39942B)

Osteocalcin sense 5'-GCAATAAGGTAGTGAACAGACTCC-3' and antisense 5'-GTTTGTAGGCGGTCTTCAAGC-3' [39];

Osterix sense 5'-CTGGGGAAAGGAGGCACAAAGAAG-3' and antisense 5'-GGGTAAAGGGGAGCAAAGTCAGAT-3' [43];

RANK sense 5'-TCATCTCTGTGGTAGTAGTGGCTG-3' and antisense 5'-TTAGGAGCAGTGAACCAGTCGAAG-3' [43];

RANKL sense 5'-CAGCCATTTGCACACCTCACCATC-3' and antisense 5'-TTTCGTGCTCCCTCCTTTCATCAG-3' [43];

Runx2 sense 5'-GAACCAAGAAGGCACAGACA-3' and antisense 5'-AACTGCCTGGGGTCTGAAAA-3' [43].

Western blot analysis

Cells were plated on a 6-well plate at the same density used in RNA isolation. Cells were lysed in RIPA buffer and protein concentrations were measured using a Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA, USA). Thirty μ g of proteins were separated on 4 – 20% gradient SDS-PAGE (Bio-Rad, Hercules, CA, USA) and transferred to a PVDF membrane. The membrane was incubated with primary antibodies at 4 °C overnight and incubated with a HRP-conjugated secondary antibody for 45 m, and developed using an enhanced chemiluminescence system.

CFU-F and CFU-ALP assay

Bone marrow cells were plated at a density of 1×10^7 cells/well in a 6-well plate and cultured for 14 d in α -MEM (no ascorbic acid, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. Cells were fixed with 4% formaldehyde and stained with H&E. Colonies containing more than 30 or more cells were counted as CFU-F. For CFU-ALP, colonies were further cultured in osteogenic medium for 7 d and subjected to ALP staining.

Cell viability

Cell viability was determined using a cell proliferation reagent WST-1 for osteoclasts (Roche Applied Science, Bavaria, Germany) and a MTT kit for osteoblasts (Cayman Chemical Company, Ann Arbor, MI, USA) according to manufacturer's protocol.

Statistical analyses

Statistical significance was determined by use of ANOVA or Student's *t* test. All statistical analyses were carried out using GraphPad Prism Software (GraphPad, La Jolla, CA, USA). *P* values less than 0.05 were considered statistically significant.

RESULTS

Orai1^{-/-} mice are osteopenic

To investigate the possible role of Orai1 in skeletal formation, we first analyzed the bone characteristics of Orai1^{-/-} mice. Section of femurs from Orai1^{-/-} mice stained with H&E revealed reduced number of trabeculae compared to wild-type (WT) littermates (Fig. 1A). A Lunar PIXImus densitometer demonstrated significantly lower bone mineral density (BMD) of femurs from both male and female Orai1^{-/-} mice (Fig. 1B). Consistently, a microcomputed tomography (μ CT) study revealed substantial decrease in bone mass of Orai1^{-/-} femurs [Fig. 1C, axial (left panel) and coronal section (right panel)]. The number and thickness of trabeculae were significantly reduced whereas trabecular separation was increased in Orai1^{-/-} femurs compared to that of WT littermates (Fig. 1D). In addition, μ CT scan exhibited a consistent slight decrease in cortical bone volume over total bone volume in Orai1^{-/-} femurs compared to WT littermates, but it was not statistically significant (data not shown). These observations indicate that deletion of *Orai1* gene in mice results in osteopenia with decreased bone mineral density and trabecular bone volume. A similar conclusion was reached by Robinson et al. [34] with a different Orai1^{-/-} mouse model, although the functions of isolated osteoclasts and osteoblasts from these mice were not investigated.

We considered that the osteopenia in Orai1^{-/-} mice could result from either increased activity of bone-resorbing osteoclasts or decreased mineralization from osteoblasts. We first

investigated the number and function of osteoclasts. Bone sections of femurs stained with tartrate-resistant acid phosphatase (TRAP, an osteoclast biomarker) showed that the number of TRAP-positive (TRAP⁺) cells was comparable in WT and *Orai1*^{-/-} mice (Fig. 2A). Subsequently we determined whether deletion of *Orai1* affects osteoclastogenesis in vitro using BMMs isolated from long bones of *Orai1*^{-/-} mice. We confirmed deletion of *Orai1* gene in *Orai1*^{-/-} BMMs by quantitative real-time PCR (qRT-PCR) analysis (Supplemental Figure 1A). We have so far been unable to reliably detect murine *Orai1* protein by Western blot analysis. However, we found by Ca²⁺ imaging analysis that SOCE was completely abolished in *Orai1*^{-/-} BMMs (Supplemental Figure 1B), consistent with a lack of functional *Orai1* protein. This also indicates that other *Orai* forms (*Orai2* and 3) do not contribute significantly to SOCE in these cells.

Osteoclastogenesis in vitro of *Orai1*^{-/-} BMMs is impaired

We next investigated the differentiation potential of BMMs via an in vitro osteoclastogenesis assay. BMMs were cultured in the presence of M-CSF and RANKL for 4 d. The differentiated WT osteoclasts were stained for TRAP (stained in dark purple, Fig. 2B). Osteoclastogenesis in vitro was impaired in BMMs from *Orai1*^{-/-} mice (Fig. 2B). TRAP⁺-multinucleated cells (MNCs) were counted under a light microscope. The number of TRAP⁺ MNCs per well was significantly smaller in BMMs from *Orai1*^{-/-} mice compared to WT controls (WT 85.3 ± 10.6, KO 21.0 ± 2.6, *** p<0.001). In addition, the size of *Orai1*^{-/-} osteoclasts is markedly smaller than WT osteoclasts (data not shown), suggesting defective multinucleation of *Orai1*^{-/-} BMMs during osteoclastogenesis. We also measured TRAP proteins secreted from osteoclasts into the media during osteoclastogenesis. RANKL highly induced the secretion of TRAP from WT osteoclasts (Fig. 2C). Consistent with the defective osteoclast differentiation as shown in Fig. 2B, the amount of secreted TRAP during RANKL-induced osteoclastogenesis was dramatically decreased from *Orai1*^{-/-} osteoclasts (Fig. 2C). We next examined whether *Orai1* deficiency affects proliferation or survival of osteoclasts using a WST-1 assay. BMMs were cultured with either M-CSF only or combination of M-CSF and RANKL for 4 d. We found that treatment of RANKL reduced the proliferation of both WT and *Orai1*^{-/-} BMMs, implying that RANKL induces differentiation rather than proliferation of BMMs at 4 d (Fig. 2D). However, the slower rate of proliferation during RANKL-induced osteoclastogenesis was not significantly different between WT and *Orai1*^{-/-} osteoclasts, indicating that the inhibition of osteoclast formation from *Orai1*^{-/-} BMMs is not caused by a difference in cell proliferation or survival (Fig. 2D). We also examined the resorptive activity of *Orai1*^{-/-} BMMs by using Ca²⁺-phosphate coated plates (Fig. 2E). The total area of pits (pixels per plate) formed by *Orai1*^{-/-} osteoclasts was comparable to that for WT osteoclasts (WT 136,061 ± 7,237, KO 136,489 ± 17,705) whereas the average size of pits (pixels per pit) was significantly smaller for *Orai1*^{-/-} osteoclasts (WT 241.1 ± 13.6, KO 173.0 ± 10.9, ** p<0.01). These observations suggest that once osteoclasts were formed from *Orai1*^{-/-} BMMs, the rate of resorption is similar to WT osteoclasts. However, it may indicate defects in fusion of pre-osteoclasts lacking *Orai1*-mediated SOCE.

Changes in osteoclastic genes in *Orai1*^{-/-} osteoclasts

To determine possible molecular players that lead to the defect in osteoclastogenesis, we assessed the expression of osteoclast-specific markers in *Orai1*^{-/-} osteoclasts by qRT-PCR. RANKL-mediated induction of TRAP, an early osteoclast marker, was significantly reduced in *Orai1*^{-/-} osteoclasts (Fig. 3A), consistent with previous observations of defective formation of TRAP⁺-MNC and defective secretion of TRAP from *Orai1*^{-/-} BMMs (Fig. 2B and 2C, respectively). Induction of cathepsin K, a late osteoclast marker, was also markedly decreased in *Orai1*^{-/-} osteoclasts (Fig. 3A). Two osteoclastic fusion genes, *d2 isoform of vacuolar ATPase Vo domain* (ATP6v0d2) and *dendritic cell-specific transmembrane protein*

(DC-STAMP), were investigated. Both genes are highly induced during RANKL-mediated osteoclastogenesis and are essential for cell fusion of pre-osteoclasts [44;45]. As shown in Fig. 3A, RANKL-mediated induction of the two osteoclastic fusion genes was markedly abrogated in *Orai1*^{-/-} osteoclasts, reflecting the observed smaller size of TRAP⁺ MNCs and pits formed by *Orai1*^{-/-} osteoclasts. Western blot analyses confirmed that the induction of ATP6v0d2 proteins in response to RANKL was substantially reduced in *Orai1*^{-/-} osteoclasts (Fig. 3B). These observations are consistent with our previous study with RAW264.7 cells, which showed that *Orai1*-mediated Ca²⁺ entry plays a critical role in the cell-cell fusion of pre-osteoclasts [31]. qRT-PCR analysis revealed that expression levels of both RANK and M-CSF receptors (cFms) in *Orai1*^{-/-} osteoclasts were comparable to that of WT osteoclasts, suggesting that responsiveness of pre-osteoclasts to the two major cytokines is not altered in *Orai1*^{-/-} osteoclasts (Fig. 3A). However, expression of cFms was significantly elevated in unstimulated *Orai1*^{-/-} cells, such that RANKL caused a decrease in expression. The basis for this unexpected result is not known. Also unexpectedly, and in contrast to our earlier finding with RAW264.7 cells [31], the induction of NFATc1 message (Fig. 3A) as well as protein (not shown) in response to RANKL was not suppressed in *Orai1*^{-/-} osteoclasts.

Orai1-mediated SOCE is critical for mineralization of osteoblasts

These findings provide new information on the role of *Orai1* and by inference SOCE in the differentiation and function of bone-resorbing osteoclasts. However, our data also indicate that defects in osteoclastogenesis in vitro and resorption activity in *Orai1*^{-/-} osteoclasts are unlikely to explain the osteopenia of *Orai1*^{-/-} mice. Thus we next investigated the possibility that osteoblast function may be impaired in *Orai1*^{-/-} mice. In order to determine whether lack of *Orai1*-mediated SOCE affects differentiation and function of osteoblasts, we first used a murine pre-osteoblast cell line, MC3T3-E1. Cells were infected with lentivirus expressing a pore-dead *Orai1* mutant (*Orai1* E106Q) to completely suppress *Orai1*-mediated SOCE [28;29]. Ca²⁺ imaging analysis confirmed essentially complete inhibition of SOCE in MC3T3-E1 cells infected with *Orai1* E106Q lentivirus compared to that of GFP control (Fig. 4A). Differentiation of these cells was induced by ascorbic acid and β -glycerophosphate for 7 d at which time they were stained for alkaline phosphatase (ALP), an early osteoblast differentiation marker. Intensity of ALP staining in osteoblasts infected with *Orai1* E106Q lentivirus was comparable to that of cells infected with GFP lentivirus (Fig. 4B), suggesting that SOCE may not be involved in early differentiation of osteoblasts. In order to determine whether the lack of SOCE affects the activity of osteoblasts, extracellular mineral deposition was analyzed. Alizarin Red (AR) staining after 14 d of induction revealed a substantial decrease in mineralized extracellular matrix formed by osteoblasts expressing *Orai1* E106Q compared to control (Fig. 4C, left panel). Subsequently, the AR staining was released by 10 % cetylpyridinium chloride and quantified by spectrophotometry. Consistent with the AR staining, the amount of released dye was significantly less in osteoblasts infected with *Orai1* E106Q lentivirus compared to control, indicating impaired mineral deposition by osteoblasts when SOCE is abolished (Fig. 4C, right panel). The infected cells were further cultured for 21 d and bone nodule formation was determined by von Kossa staining. Consistently, bone nodule formation by osteoblasts infected with *Orai1* E106Q lentivirus was markedly reduced compared to GFP control (Fig. 4D). Taken together, these results indicate that *Orai1*-mediated SOCE plays a critical role in the mineral deposition of osteoblasts. To determine whether the impaired function of osteoblasts infected with *Orai1* E106Q lentivirus is not due to a reduction in proliferative potential, cell proliferation (or survival) was measured at 7 d (Fig. 4E, left panel) and 14 d (Fig. 4E, right panel) of induction using a MTT assay. Osteogenic induction of MC3T3-E1 cells with ascorbic acid and β -glycerophosphate enhanced cell proliferation (or survival) at 7 d (Fig. 4E, left panel) and 14 d (Fig. 4E, right panel) compared to vehicle controls. However, the enhancement in cell proliferation at both 7 d and 14 d was similar in cells

infected with either GFP or Orai1 E106Q lentivirus, suggesting the defective mineralization in Orai1 E106Q-infected osteoblasts was not caused by a difference in cell proliferation (or survival). In an attempt to identify possible mechanisms underlying the reduced bone-forming activity of Orai1 E106Q-infected osteoblasts, we analyzed the expression levels of osteoblast marker genes by qRT-PCR after 7 d of osteogenic induction. As expected, we found that osteogenic medium significantly induced the expression of most osteoblastic marker genes. Notably, the induced expression of bone matrix markers, osteocalcin, collagen type 1 alpha1 and 2 (coll α 1 and coll α 2), was significantly reduced in osteoblasts infected with Orai1 E106Q lentivirus, whereas that of ALP and runx2 (early differentiation markers) in osteoblasts lacking SOCE was comparable to GFP controls (Fig. 5). Interestingly, the expression of Fra-1 was markedly reduced in osteoblasts lacking SOCE (Fig. 5). Fra-1 has been shown to be critical for bone matrix formation [16]. We also examined the expression levels of NFATc1 and c2, Ca²⁺-sensitive transcript factors known to be involved in osteoblast differentiation [46]. However, there was no difference in the expression levels of these factors (Fig. 5). In addition, expression of osterix, an osteoblastic transcription factor that is known to interact with NFATc1 [46], was not changed in osteoblasts derived from MC3T3-E1 lacking SOCE (Fig. 5). These results indicate that early differentiation is not altered but bone matrix formation is impaired in osteoblasts expressing the Orai1 pore-dead mutant.

Defective mineralization of Orai1^{-/-} osteoblasts

To investigate the role of Orai1 in the differentiation and function of primary osteoblasts derived from bone marrow stromal cells (BMSCs), BMSCs were obtained from 9 d culture of whole bone marrow cells in the absence of osteogenic medium [36]. qRT-PCR analysis confirmed ablation of *Orai1* gene in Orai1^{-/-} BMSC (Fig. 6A). To confirm a lack of SOCE in Orai1^{-/-} BMSC, Ca²⁺ imaging analysis was performed, which showed a substantial but not complete abolishment of SOCE in Orai1^{-/-} BMSCs compared to WT BMSCs (Fig. 6B). Low concentration of Gd³⁺ inhibited not only SOCE in WT BMSCs, but also residual SOCE in Orai1^{-/-} BMSCs, indicating contribution of other isoforms of Orai (Orai2 and/or Orai3) in the activation of SOCE in Orai1^{-/-} BMSCs (Fig. 6B). Leak control with no depletion of ER Ca²⁺ stores confirmed the presence of residual SOCE in Orai1^{-/-} stromal cells (Fig. 6B). Indeed, qRT-PCR showed substantial levels of Orai2 and 3 mRNA in Orai1^{-/-} BMSC (Orai2 in Fig. 7 and data not shown for Orai3). We next examined the osteoblastic differentiation potential of Orai1^{-/-} BMSCs in vitro. The BMSCs were cultured in osteogenic medium (50 μ M ascorbic acid, 10 mM β -glycerophosphate, and 10 nM dexamethasone) for 7 d and subjected to ALP staining. Intensity of ALP staining of Orai1^{-/-} osteoblasts was similar to that of WT osteoblasts (Fig. 6C) which is consistent with the lack of difference in ALP staining of MC3T3-E1 cells infected with Orai1 E106Q lentivirus (Fig. 4B). Cells were further cultured in osteogenic medium for 14 d and analyzed for the effect of Orai1 deficiency on mineral deposition by AR staining. We confirmed that osteogenic medium highly induced mineral deposition of WT stromal cells evidenced by strong AR staining (Fig. 6D, bottom panel) compared to vehicle controls (Fig. 6D, top panel). Importantly, the intensity of AR staining was significantly lower in Orai1^{-/-} osteoblasts (Fig. 6D). Spectrophotometric analysis of released dye from the AR-positive mineral deposition consistently showed a significant decrease in mineral deposition in Orai1^{-/-} osteoblasts compared to WT osteoblasts (Fig. 6E). In addition, von Kossa staining at 21 d of induction revealed substantial reduction in bone nodule formation of Orai1^{-/-} osteoblasts (Fig. 6F). Consistent with the observed defect in mineral deposition in MC3T3-E1 cells infected with a dominant-negative Orai1 mutant, these results indicate that Orai1-mediated SOCE also plays a critical role in mineral deposition of primary osteoblasts derived from BMSCs. We next performed a colony-forming unit fibroblast (CFU-F) assay to measure any difference in the number of bone marrow-derived mesenchymal progenitors in the mutant

mice [47;48]. We observed no significant difference in the number of CFU-F between WT and KO mice (Fig. 6G, Supplemental Fig. 3A). Also the number of CFU-ALP was comparable between WT and *Orai1*^{-/-} mice (Supplemental Fig. 3B), suggesting the number of mesenchymal progenitors is similar in WT and mutant mice. To determine whether the loss of *Orai1* affects the proliferative potential of primary osteoblasts, we measured cell proliferation (or survival) of BMSCs using a MTT assay. We found no difference in cell proliferation between WT and *Orai1*^{-/-} osteoblasts at both 7 d (Fig. 6H, left panel) and 21 d (Fig. 6H, right panel) of induction by osteogenic medium. To identify candidate molecules responsible for the observed defects in primary *Orai1*^{-/-} osteoblasts, the expression levels of key osteoblastic marker genes at 7 d of induction was investigated by qRT-PCR (Fig. 7). We confirmed the loss of *Orai1* gene and unaffected expression of *Orai2* in differentiated *Orai1*^{-/-} osteoblasts (Fig. 7). Consistent with the observation from MC3T3-E1 lacking SOCE, the expression levels of *collα2* and *Fra-1* were decreased in *Orai1*^{-/-} osteoblasts compared to WT osteoblasts (Fig. 7). Both OPG and RANKL expression were also decreased in mutant osteoblasts. Consistent with MC3T3-E1 lacking SOCE, we found no difference in the expression levels of NFATc1 between WT and *Orai1*^{-/-} osteoblasts (Fig. 7). The expression of ALP and osteocalcin was increased in mutant osteoblasts (Fig. 7), likely as a compensatory action of cells against defective mineralization. However, there was no difference in *runx2* expression between two groups (Fig. 7), again consistent with the data from MC3T3-E1 infected with *Orai1* E106Q mutant virus as shown in Fig. 5. Since the effect of dexamethasone on the differentiation of osteoblasts is controversial [49–51], we cultured BMSCs in osteogenic medium without dexamethasone for 7 d, and ALP staining and qRT-PCR of the osteoblast marker genes were assessed (Supplemental Fig. 2). Although dexamethasone slightly suppressed the intensity of ALP staining (Supplemental Fig. 2A), changes in the expression levels of osteoblastic markers were consistent with those for cells cultured with dexamethasone (Supplemental Fig. 2B). The expression levels of *Collα2* and *Fra-1* were significantly reduced in *Orai1*^{-/-} osteoblasts cultured in osteogenic medium without dexamethasone (Supplemental Fig. 2B). In addition, von Kossa staining of osteoblasts cultured in the absence of dexamethasone also showed decreased mineral deposition of *Orai1*^{-/-} osteoblasts (Supplemental Fig. 2C). Taken together, these observations demonstrate that *Orai1* plays a pivotal role in the mineralization function of osteoblasts by regulating expression of bone matrix proteins.

DISCUSSION

Intracellular Ca^{2+} signaling has long been known to involve differentiation and function of the two major bone cell types [20;23–25]. Recent studies utilizing pharmacological inhibition of SOCE or gene silencing of *Orai1* revealed an important role for SOCE in osteoclastogenesis [31;52]. We previously showed that knockdown of *Orai1* in RAW264.7 cells impairs osteoclastogenesis by inhibiting transactivation of NFATc1, a Ca^{2+} -dependent transcription factor [31]. In the present study, osteoclastogenesis in vitro is also impaired in *Orai1*^{-/-} BMMs. However, induction of NFATc1 by RANKL in *Orai1*^{-/-} osteoclasts was comparable to that of WT osteoclasts (Fig. 3A). Since TG-induced SOCE was completely abolished in *Orai1*^{-/-} BMMs, these results suggest that one or more Ca^{2+} channels other than store-operated channels are also likely involved in osteoclastogenesis induced by RANKL. Indeed, previous studies have suggested that a role for other Ca^{2+} influx channels in osteoclastogenesis [53–56].

The decrease in bone mass in *Orai1*^{-/-} mice cannot result from decreased differentiation of osteoclasts, which would result in an increase in bone mass due to a defect in bone resorption and so we investigated the possible role of the bone-forming osteoblasts in the osteopenic phenotype of *Orai1*^{-/-} mice. The presence of SOCE has previously been shown in primary and osteoblastic cell lines [32;33;57]. Robinson et al. [34] recently reported that a

pharmacological inhibitor of SOCE reduced the differentiation and function of human osteoblastic cells. However, our results, utilizing both an osteoblastic cell line and Orai1-deficient primary osteoblast cells, indicate that Orai1-mediated SOCE is involved in the function but not in the differentiation of osteoblasts. This discrepancy may be due to the use of different species (human vs. mouse), or different strategies to interfere with SOCE (pharmacological inhibition vs. gene deletion). MC3T3-E1 cells expressing a pore-dead dominant-negative Orai1 mutant showed defects in mineralization despite the fact that osteoblast differentiation was not affected. Consistently, osteoblasts differentiated from Orai1^{-/-} BMSCs showed impaired mineralization accompanied by reduced expression of *col1a2*. Orai1 deficiency also did not affect osteoblast differentiation. Interestingly, in both MC3T3-E1 and primary osteoblasts, the expression of Fra-1 is reduced by inhibition of Orai1. Fra-1 is a component of the dimeric transcription factor, AP-1 and conditional knockout of Fra-1 (Fra-1^{+/-}) in mice results in low-turnover osteopenia [16]. Similar to Orai1^{-/-} mice, osteoclasts in vivo were normal but expression of *col2* was decreased in long bones of Fra-1^{+/-} mice. This suggests that Orai1-mediated Ca²⁺ signaling in osteoblasts may regulate downstream targets including transcription factors. Further study is needed to identify those targets. However, we cannot rule out the possibility that Orai1-mediated SOCE may play a role in the secretion of collagen via exocytosis. Synaptotagmin (Syt) is a Ca²⁺-dependent regulator of exocytosis, and Syt VII-deficient mice showed osteopenia with defects in bone resorption and formation [58]. Secretion of cathepsin K from osteoclasts and bone matrix proteins from osteoblasts was impaired in Syt VII-deficient mice. Orai1-mediated Ca²⁺ signaling may be involved in the activation of synaptotagmin to control exocytosis in bone cells. However, further work is needed to clarify the role of Orai1 in exocytosis of bone cells. Since other isoforms of Orai appear to be expressed in osteoblasts, and since some residual SOCE was seen in Orai1^{-/-} stromal cells (Fig. 7 and Fig. 6B, respectively), it is also possible that other isoforms of Orai (Orai2 and 3) and/or other Ca²⁺ entry channels may contribute to osteoblastogenesis. Baldi et al showed that TRPC3-like proteins contributed to SOCE in an osteoblastic cell [59]. Several studies have reported that L-type voltage-sensitive channels can regulate intracellular Ca²⁺ signaling in osteoblasts [23;60;61].

The results of this study strongly implicate Orai1-mediated Ca²⁺ entry as a major signaling mechanism regulating the differentiation and/or functions of the two bone cell types. Two independently generated Orai1^{-/-} mouse models result in small homozygous pups [35;62], a phenotype largely attributed to impaired skeletal muscle development [63]. The results of the current study, and those of Robinson et al. [34] suggest that effects of Orai1 deficiency on bone may contribute as well. It appears that Orai1^{-/-} mice develop low turnover osteopenia due to defects in osteoblasts and osteoclasts. It has been demonstrated that deletion of specific genes often results in low-turnover osteopenia due to impaired function of both osteoblasts and osteoclasts. Genetic ablation of *Abl*, *CCR1*, *JunB*, or *Sh3bp2* in mice results in low-turnover osteopenia [13;64–66]. Similar to Orai1^{-/-} mice, *Abl*-deficient mice developed osteopenia due to defects in osteoblast maturation [64].

However, identifying the precise underlying molecular mechanisms will require further study. Several key questions remain. What are the in vivo stimuli for Orai1 activation in osteoclasts and osteoblasts? What are the immediate downstream targets of Orai1 activation that leads to specific gene expression in bone cells? Is either the expression or activation of Orai1 changed in pathological conditions of bone homeostasis including postmenopausal osteoporosis and rheumatoid arthritis? Do other isoforms of Orai (Orai2 and/or Orai3) contribute to bone remodeling? From the clinical perspective, do patients with the SCID syndrome in which Orai1 gene is mutated have a bone phenotype [30]? Although a defect in dental enamel calcification was reported in patients with Orai1 mutation [67], to our knowledge the skeletal phenotype of SCID patients has not yet been extensively

investigated. In order to unravel the precise role of Orai1 in each bone cell type, lineage-specific conditional knockout animals will be necessary.

In summary, we have demonstrated an osteopenic phenotype of Orai1^{-/-} mice and we have delineated a physiological role of Orai1 that controls both osteoblasts and osteoclasts in this mouse model. We elucidated that SOCE is important for cell fusion of pre-osteoclasts and show that Orai1-mediated SOCE plays a crucial role in the mineralization of osteoblasts by regulating expression of bone matrix protein. The implications of these findings to the management of bone diseases must also await future research. As a key regulator of bone remodeling and mineralization, one or more of the Orai channels could be potential therapeutic targets for treatment of bone diseases. Additionally, blockers of store-operated channels are currently in development for use as immune suppressants (www.calcimedica.com). It will be important to be aware of the potential long-term effects of such agents on bone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences. We thank Jeff Tucker, Drs. Agnes Janoshazi, and Shilan Wu for technical assistance. We thank Drs. Charles Romeo and Negin Martin (Viral Vector Core, NIEHS) for technical assistance in lentivirus preparations. We also thank Drs. Xiaoling Li and Robert Oakley for helpful comments.

References

1. Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. *Dev Cell*. 2002; 2:389–406. [PubMed: 11970890]
2. Zaidi M. Skeletal remodeling in health and disease. *Nat Med*. 2007; 13:791–801. [PubMed: 17618270]
3. Novack DV, Teitelbaum SL. The osteoclast: friend or foe? *Annu Rev Pathol*. 2008; 3:457–484. [PubMed: 18039135]
4. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell*. 1997; 89:309–319. [PubMed: 9108485]
5. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A*. 1998; 95:3597–3602. [PubMed: 9520411]
6. Rodan GA, Martin TJ. Therapeutic approaches to bone diseases. *Science*. 2000; 289:1508–1514. [PubMed: 10968781]
7. Romas E, Gillespie MT, Martin TJ. Involvement of receptor activator of NFkappaB ligand and tumor necrosis factor-alpha in bone destruction in rheumatoid arthritis. *Bone*. 2002; 30:340–346. [PubMed: 11856640]
8. Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *J Clin Invest*. 2005; 115:3318–3325. [PubMed: 16322775]
9. Takayanagi H. Osteoimmunology and the effects of the immune system on bone. *Nat Rev Rheumatol*. 2009; 5:667–676. [PubMed: 19884898]
10. Chan GK, Duque G. Age-related bone loss: old bone, new facts. *Gerontology*. 2002; 48:62–71. [PubMed: 11867927]

11. Rosen CJ, Bouxsein ML. Mechanisms of disease: is osteoporosis the obesity of bone? *Nat Clin Pract Rheumatol*. 2006; 2:35–43. [PubMed: 16932650]
12. Yavropoulou MP, Yovos JG. Osteoclastogenesis--current knowledge and future perspectives. *J Musculoskelet Neuronal Interact*. 2008; 8:204–216. [PubMed: 18799853]
13. Kenner L, Hoeberitz A, Beil FT, Keon N, Karreth F, Eferl R, Scheuch H, Szremska A, Amling M, Schorpp-Kistner M, Angel P, Wagner EF. Mice lacking JunB are osteopenic due to cell-autonomous osteoblast and osteoclast defects. *J Cell Biol*. 2004; 164:613–623. [PubMed: 14769860]
14. Sabatakos G, Sims NA, Chen J, Aoki K, Kelz MB, Amling M, Bouali Y, Mukhopadhyay K, Ford K, Nestler EJ, Baron R. Overexpression of DeltaFosB transcription factor(s) increases bone formation and inhibits adipogenesis. *Nat Med*. 2000; 6:985–990. [PubMed: 10973317]
15. Neve A, Corrado A, Cantatore FP. Osteoblast physiology in normal and pathological conditions. *Cell Tissue Res*. 2011; 343:289–302. [PubMed: 21120535]
16. Eferl R, Hoeberitz A, Schilling AF, Rath M, Karreth F, Kenner L, Amling M, Wagner EF. The Fos-related antigen Fra-1 is an activator of bone matrix formation. *EMBO J*. 2004; 23:2789–2799. [PubMed: 15229648]
17. Berridge MJ. Inositol trisphosphate and calcium signalling. *Nature*. 1993; 361:315–325. [PubMed: 8381210]
18. Carafoli E. Calcium signaling: A tale for all seasons. *Proc Nat Acad Sci USA*. 2002; 99:1115–1122. [PubMed: 11830654]
19. Xia SL, Ferrier J. Localized calcium signaling in multinucleated osteoclasts. *J Cell Physiol*. 1996; 167:148–155. [PubMed: 8698832]
20. Duncan RL, Akanbi KA, Farach-Carson MC. Calcium signals and calcium channels in osteoblastic cells. *Semin Nephrol*. 1998; 18:178–190. [PubMed: 9541272]
21. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T, Taniguchi T. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell*. 2002; 3:889–901. [PubMed: 12479813]
22. Jorgensen NR, Geist ST, Civitelli R, Steinberg TH. ATP- and gap junction-dependent intercellular calcium signaling in osteoblastic cells. *J Cell Biol*. 1997; 139:497–506. [PubMed: 9334351]
23. Bergh JJ, Xu Y, Farach-Carson MC. Osteoprotegerin expression and secretion are regulated by calcium influx through the L-type voltage-sensitive calcium channel. *e*. 2004; 145:426–436.
24. Blair HC, Schlesinger PH, Huang CL, Zaidi M. Calcium signalling and calcium transport in bone disease. *Subcell Biochem*. 2007; 45:539–562. [PubMed: 18193652]
25. Hwang SY, Putney JW Jr. Calcium signaling in osteoclasts. *Biochim Biophys Acta*. 2011; 1813:979–983. [PubMed: 21075150]
26. Parekh AB, Putney JW. Store-operated calcium channels. *Physiol Rev*. 2005; 85:757–810. [PubMed: 15788710]
27. Parekh AB. Functional consequences of activating store-operated CRAC channels. *Cell Calcium*. 2007; 42:111–121. [PubMed: 17445883]
28. Vig M, Beck A, Billingsley JM, Lis A, Parvez S, Peinelt C, Koomoa DL, Soboloff J, Gill DL, Fleig A, Kinet JP, Penner R. CRACM1 multimers form the ion-selective pore of the CRAC channel. *Curr Biol*. 2006; 16:2073–2079. [PubMed: 16978865]
29. Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG. Orai1 is an essential pore subunit of the CRAC channel. *Nature*. 2006; 443:230–233. [PubMed: 16921383]
30. Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature*. 2006; 441:179–185. [PubMed: 16582901]
31. Hwang SY, Putney JW. Orai1-mediated calcium entry plays a critical role in osteoclast differentiation and function by regulating activation of the transcription factor NFATc1. *FASEB J*. 2012; 26:1484–1492. [PubMed: 22198385]
32. Wiemann M, Busselberg D, Schirmmacher K, Bingmann D. A calcium release activated calcium influx in primary cultures of rat osteoblast-like cells. *Calcif Tissue Int*. 1998; 63:154–159. [PubMed: 9685522]

33. Labelle D, Jumarie C, Moreau R. Capacitative calcium entry and proliferation of human osteoblast-like MG-63 cells. *Cell Prolif.* 2007; 40:866–884. [PubMed: 18021176]
34. Robinson LJ, Mancarella S, Songsawad D, Tourkova IL, Barnett JB, Gill DL, Soboloff J, Blair HC. Gene disruption of the calcium channel *Orai1* results in inhibition of osteoclast and osteoblast differentiation and impairs skeletal development. *Lab Invest.* 2012
35. Vig M, DeHaven WI, Bird GS, Billingsley JM, Wang H, Rao PE, Hutchings AB, Jouvin MH, Putney JW, Kinet JP. Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels. *Nat Immunol.* 2008; 9:89–96. [PubMed: 18059270]
36. Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. *e.* 1994; 134:277–286.
37. Gregory CA, Gunn WG, Peister A, Prockop DJ. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem.* 2004; 329:77–84. [PubMed: 15136169]
38. Maeda T, Matsunuma A, Kurahashi I, Yanagawa T, Yoshida H, Horiuchi N. Induction of osteoblast differentiation indices by statins in MC3T3-E1 cells. *J Cell Biochem.* 2004; 92:458–471. [PubMed: 15156558]
39. Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H, Aburatani H, Nishimura R, Yoneda T. BMP2 regulates Osterix through *Msx2* and *Runx2* during osteoblast differentiation. *J Biol Chem.* 2008; 283:29119–29125. [PubMed: 18703512]
40. Cao H, Yu S, Yao Z, Galson DL, Jiang Y, Zhang X, Fan J, Lu B, Guan Y, Luo M, Lai Y, Zhu Y, Kurihara N, Patrene K, Roodman GD, Xiao G. Activating transcription factor 4 regulates osteoclast differentiation in mice. *J Clin Invest.* 2010; 120:2755–2766. [PubMed: 20628199]
41. Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M, Karsenty G. A *Cbfa1*-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev.* 1999; 13:1025–1036. [PubMed: 10215629]
42. Wilkins BJ, De Windt LJ, Bueno OF, Braz JC, Glascock BJ, Kimball TF, Molkentin JD. Targeted disruption of *NFATc3*, but not *NFATc4*, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth. *Mol Cell Biol.* 2002; 22:7603–7613. [PubMed: 12370307]
43. Salingcarnboriboon R, Tsuji K, Komori T, Nakashima K, Ezura Y, Noda M. *Runx2* is a target of mechanical unloading to alter osteoblastic activity and bone formation in vivo. *e.* 2006; 147:2296–2305.
44. Yagi M, Miyamoto T, Sawatani Y, Iwamoto K, Hosogane N, Fujita N, Morita K, Ninomiya K, Suzuki T, Miyamoto K, Oike Y, Takeya M, Toyama Y, Suda T. DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. *J Exp Med.* 2005; 202:345–351. [PubMed: 16061724]
45. Lee SH, Rho J, Jeong D, Sul JY, Kim T, Kim N, Kang JS, Miyamoto T, Suda T, Lee SK, Pignolo RJ, Koczon-Jaremko B, Lorenzo J, Choi Y. v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. *Nat Med.* 2006; 12:1403–1409. [PubMed: 17128270]
46. Koga T, Matsui Y, Asagiri M, Kodama T, de CB, Nakashima K, Takayanagi H. NFAT and Osterix cooperatively regulate bone formation. *Nat Med.* 2005; 11:880–885. [PubMed: 16041384]
47. Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiarieri D, McKenzie S, Broxmeyer HE, Moore MA. Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood.* 1980; 56:289–301. [PubMed: 6994839]
48. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem.* 1997; 64:295–312. [PubMed: 9027589]
49. Wong MM, Rao LG, Ly H, Hamilton L, Tong J, Sturtridge W, McBroom R, Aubin JE, Murray TM. Long-term effects of physiologic concentrations of dexamethasone on human bone-derived cells. *J Bone Miner Res.* 1990; 5:803–813. [PubMed: 2173356]
50. Kasugai S, Todescan R Jr, Nagata T, Yao KL, Butler WT, Sodek J. Expression of bone matrix proteins associated with mineralized tissue formation by adult rat bone marrow cells in vitro:

- inductive effects of dexamethasone on the osteoblastic phenotype. *J Cell Physiol.* 1991; 147:111–120. [PubMed: 2037618]
51. Fernandez M, Minguell JJ. Hydrocortisone regulates types I and III collagen gene expression and collagen synthesis in human marrow stromal cells. *Biol Res.* 1997; 30:85–90. [PubMed: 9283621]
  52. Zhou Y, Lewis TL, Robinson LJ, Brundage KM, Schafer R, Martin KH, Blair HC, Soboloff J, Barnett JB. The role of calcium release activated calcium channels in osteoclast differentiation. *J Cell Physiol.* 2010; 226:1082–1089. [PubMed: 20839232]
  53. Hoenderop JG, van Leeuwen JP, van der Eerden BC, Kersten FF, van der Kemp AW, Merillat AM, Waarsing JH, Rossier BC, Vallon V, Hummler E, Bindels RJ. Renal Ca²⁺ wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. *J Clin Invest.* 2003; 112:1906–1914. [PubMed: 14679186]
  54. van der Eerden BC, Hoenderop JG, de Vries TJ, Schoenmaker T, Buurman CJ, Uitterlinden AG, Pols HA, Bindels RJ, van Leeuwen JP. The epithelial Ca²⁺ channel TRPV5 is essential for proper osteoclastic bone resorption. *Proc Natl Acad Sci U S A.* 2005; 102:17507–17512. [PubMed: 16291808]
  55. Masuyama R, Vriens J, Voets T, Karashima Y, Owsianik G, Vennekens R, Lieben L, Torrekens S, Moermans K, Vanden BA, Bouillon R, Nilius B, Carmeliet G. TRPV4-mediated calcium influx regulates terminal differentiation of osteoclasts. *Cell Metab.* 2008; 8:257–265. [PubMed: 18762026]
  56. Kajiya H, Okamoto F, Nemoto T, Kimachi K, Toh-Goto K, Nakayama S, Okabe K. RANKL-induced TRPV2 expression regulates osteoclastogenesis via calcium oscillations. *Cell Calcium.* 2010; 48:260–269. [PubMed: 20980052]
  57. Baldi C, Vazquez G, Boland R. Characterization of a 1,25(OH)₂-vitamin D₃-responsive capacitative Ca²⁺ entry pathway in rat osteoblast-like cells. *J Cell Biochem.* 2002; 86:678–687. [PubMed: 12210734]
  58. Zhao H, Ito Y, Chappel J, Andrews NW, Teitelbaum SL, Ross FP. Synaptotagmin VII regulates bone remodeling by modulating osteoclast and osteoblast secretion. *Dev Cell.* 2008; 14:914–925. [PubMed: 18539119]
  59. Baldi C, Vazquez G, Calvo JC, Boland R. TRPC3-like protein is involved in the capacitative cation entry induced by 1 α ,25-dihydroxy-vitamin D₃ in ROS 17/2.8 osteoblastic cells. *J Cell Biochem.* 2003; 90:197–205. [PubMed: 12938168]
  60. Duncan R, Misler S. Voltage-activated and stretch-activated Ba²⁺ conducting channels in an osteoblast-like cell line (UMR 106). *Febs Lett.* 1989; 251:17–21. [PubMed: 2473926]
  61. Jorgensen NR, Teilmann SC, Henriksen Z, Civitelli R, Sorensen OH, Steinberg TH. Activation of L-type calcium channels is required for gap junction-mediated intercellular calcium signaling in osteoblastic cells. *J Biol Chem.* 2003; 278:4082–4086. [PubMed: 12446698]
  62. Gwack Y, Srikanth S, Oh-hora M, Hogan PG, Lamperti ED, Yamashita M, Gelinas C, Neems DS, Sasaki Y, Feske S, Prakriya M, Rajewsky K, Rao A. Hair Loss and Defective T- and B-Cell Function in Mice Lacking ORAI1. *Mol Cell Biol.* 2008; 28:5209–5222. [PubMed: 18591248]
  63. Stiber J, Hawkins A, Zhang ZS, Wang S, Burch J, Graham V, Ward CC, Seth M, Finch E, Malouf N, Williams RS, Eu JP, Rosenberg P. STIM1 signalling controls store-operated calcium entry required for development and contractile function in skeletal muscle. *Nat Cell Biol.* 2008; 10:688–697. [PubMed: 18488020]
  64. Li B, Boast S, de los SK, Schieren I, Quiroz M, Teitelbaum SL, Tondravi MM, Goff SP. Mice deficient in Abl are osteoporotic and have defects in osteoblast maturation. *Nat Genet.* 2000; 24:304–308. [PubMed: 10700189]
  65. Hoshino A, Iimura T, Ueha S, Hanada S, Maruoka Y, Mayahara M, Suzuki K, Imai T, Ito M, Manome Y, Yasuhara M, Kirino T, Yamaguchi A, Matsushima K, Yamamoto K. Deficiency of chemokine receptor CCR1 causes osteopenia due to impaired functions of osteoclasts and osteoblasts. *J Biol Chem.* 2010; 285:28826–28837. [PubMed: 20571024]
  66. Levaot N, Simoncic PD, Dimitriou ID, Scotter A, La RJ, Ng AH, Willett TL, Wang CJ, Janmohamed S, Grynepas M, Reichenberger E, Rottapel R. 3BP2-deficient mice are osteoporotic with impaired osteoblast and osteoclast functions. *J Clin Invest.* 2011; 121:3244–3257. [PubMed: 21765218]

67. McCarl CA, Picard C, Khalil S, Kawasaki T, Rother J, Papolos A, Kutok J, Hivroz C, Ledeist F, Plogmann K, Ehl S, Notheis G, Albert MH, Belohradsky BH, Kirschner J, Rao A, Fischer A, Feske S. ORAI1 deficiency and lack of store-operated Ca^{2+} entry cause immunodeficiency, myopathy, and ectodermal dysplasia. *J Allergy Clin Immunol.* 2009; 124:1311–1318. [PubMed: 20004786]

\$watermark-text

\$watermark-text

\$watermark-text

Figure 1.

Ablation of *Orai1* gene in mice results in osteopenia. (A) Histological sections of the femurs from wild-type (WT) and *Orai1*^{-/-} mice (7-week females). The sections were stained with haematoxylin and eosin (H&E). The *Orai1*^{-/-} femurs show reduced volume of trabeculae (magnification, x4). (B) Bone mineral density (BMD) of the femurs of WT and *Orai1*^{-/-} mice was measured using a Lunar PIXImus II densitometer. The BMD from both males (6–13 weeks) and females (7–12 weeks) is significantly reduced in *Orai1*^{-/-} mice (*** $p < 0.001$). For males, both WT and *Orai1*^{-/-} groups contained mice of the following ages: one 6 week, two 7 week, two 8 week, two 9 week, and one each 10, 11, 12, 13 week. For females, both groups contained: one 7 week, one 8 week, one 10 week, two 11 week and one 12 week. (C) Microcomputed tomography of the femurs from WT and *Orai1*^{-/-} mice (8-week males). Both axial (left panel) and coronal section (right panel) revealed lower bone mass in the femur of *Orai1*^{-/-} mice compared to WT littermates. (D) Three-dimensional trabecular structural parameters in the distal femur obtained from data illustrated by the example in Panel C. Data represent means $\pm$ SE. $n = 3$ (* $p < 0.05$). WT group included 8 week male, 7 week male, and 7 week female. Mutant group included 8 week male, and two 7 week females.

Figure 2.

Deletion of *Orai1* leads to defective osteoclastogenesis in vitro. (A) TRAP staining of the femurs from WT and *Orai1*^{-/-} mice (7-week old) showed no significant difference in the number of TRAP-positive osteoclasts. (B) Bone marrow derived macrophages/monocytes (BMMs) isolated from long bones (femurs and tibiae) were differentiated into mature multinucleated osteoclasts on plastic dishes in the presence of M-CSF and RANKL for 4 d and stained for TRAP, an osteoclast marker. Note that the size of osteoclasts generated from *Orai1*^{-/-} BMMs is substantially smaller than that of WT BMMs. (C) TRAP secreted in the media during osteoclastogenesis in vitro was measured after 4 d of differentiation. Data are representative of three independent experiments with similar results. Statistical significance was evaluated by an ANOVA followed by Newman-Keuls Multiple Comparison Test. Error bars denote standard error between samples performed in triplicate (*** p < 0.001). (D) WST-1 cell proliferation assay of osteoclasts cultured with or without RANKL for 4 d. Data are representative of two independent experiments with similar results. Error bars denote standard error between samples performed in triplicate (* < 0.05, ** p < 0.01, n.s.: non-significant). (E) BMMs were grown on the Ca²⁺-phosphate coated plate in the presence of M-CSF and RANKL for 7 d. The medium was replaced every 2 d. Cells were detached, stained with 5% silver nitrate, and photographed under a light microscope.

Figure 3.

Changes in osteoclastic genes in *Orai1*^{-/-} osteoclasts. (A) BMMs were cultured in the presence or absence of RANKL for 3 d. Total RNA was isolated and qRT-PCR analysis of osteoclast differentiation markers was performed. The relative expression levels were normalized to GAPDH. Note that whereas the expression of NFATc1 is not changed, the induction of two cell fusion factors, ATP6v0d2 and DC-STAMP, is significantly suppressed in *Orai1*^{-/-} osteoclasts. Statistical significance was evaluated by an ANOVA followed by Newman-Keuls Multiple Comparison Test. Data are means ± SE of triplicate samples from three independent experiments with similar results (**P < 0.01, ***P < 0.001, n.s.: non-significant). (B) Western blot analysis of ATP6v0d2. Cell lysates were harvested at 3 d of differentiation in the presence of RANKL. Data are representative of three independent experiments with similar results.

Figure 4.

Inhibition of Orai1-mediated SOCE leads to defective osteoblast function. (A) MC3T3-E1 cells were transduced with lentiviruses that overexpress either GFP or a pore- dead mutant Orai1 (Orai1 E106Q-GFP). Optical imaging of intracellular Ca^{2+} concentrations showed that SOCE is completely abolished in cells expressing the Orai1 E106Q mutant compared to the GFP control. (B) Infected MC3T3-E1 cells were differentiated in the presence of ascorbic acid (AA) and β -glycerophosphate (GP) for 7 d and stained for alkaline phosphatase (ALP). ALP activity is unchanged in cells infected with the Orai1 E106Q mutant compared to control. (C) MC3T3-E1 cells infected with either GFP or Orai1 E106Q-GFP lentivirus were differentiated for 14 d and deposition of extracellular matrix was analyzed by alizarin red staining (shown in dark red). The stained matrix was photographed (left panel) and the alizarin red staining was quantified as described in Materials and

Methods (right panel). Data are representative of three independent experiments with similar results (**P < 0.01, ***P < 0.001). (D) Mineralized nodule formation was assessed by von Kossa staining after 21 d of induction. Data are representative of three independent experiments with similar results. (E) MTT cell proliferation assay of osteoblasts cultured for 7 d (left panel) and 14 d (right panel). Data are representative of three independent experiments with similar results. Error bars denote standard error between samples performed in triplicate (**p < 0.001, n.s.: non-significant).

\$watermark-text

\$watermark-text

\$watermark-text

Figure 5.

Transcriptional regulation is altered in osteoblasts lacking SOCE. MC3T3-E1 cells infected with either GFP or Orail E106Q-GFP lentivirus were cultured in the presence of ascorbic acid (AA) and β-glycerophosphate (GP) for 7 d. Total RNA was isolated and qRT-PCR analysis of osteoblast differentiation markers was performed. Expression levels were normalized to GAPDH. Data are representative of three independent experiments with similar results. Error bars denote standard error between samples performed in triplicate (* < 0.05, ** < 0.01, *** p < 0.001, n.s.: non-significant).

Figure 6.

Mineralization is impaired in Orai1^{-/-} osteoblasts. (A) Total RNA was isolated from bone-marrow-derived stromal cells (BMSCs) and qRT-PCR analysis was performed to measure the levels of murine *Orai1* mRNA. (B) Optical imaging of intracellular Ca²⁺ concentrations showed markedly reduced SOCE in Orai1^{-/-} BMSCs compared to that of WT. Leak control without ER store depletion showed further reduction of SOCE in Orai1^{-/-} BMSC, indicating involvement of other SOCE channels such as Orai2 and/or Orai3. (C) Osteoblast differentiation of BMSCs was induced by osteogenic medium (50 µg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone) and stained for ALP after 7 d of induction. (D) BMSCs were cultured in either vehicle (Veh) or osteogenic medium (OSM) for 14 d and deposition of extracellular matrix was analyzed by alizarin red staining (shown in dark red). The stained matrix was photographed and the alizarin red staining was quantified as described in Materials and Methods (E). Data are representative of three independent experiments with similar results. (F) Mineralized nodule formation was determined by von Kossa staining after 21 d of differentiation of BMSCs. Data are representative of three independent experiments with similar results. (G) CFU-F of BMSCs was measured. (H) MTT cell proliferation assay of osteoblasts at 7 d (left panel) and 21 d

(right panel) of differentiation. Error bars denote standard error between samples performed in triplicate (** $p < 0.001$, n.s.: non-significant).

Watermark-text

Watermark-text

Watermark-text

Figure 7.

Deletion of Orai1 alters expression of multiple genes during osteoblast maturation. BMSCs were cultured in the presence of osteogenic medium for 7 d. Total RNA was isolated and qRT-PCR analysis of osteoblast differentiation markers was performed. Note that Orai1^{-/-} osteoblasts culture in osteogenic medium without dexamethasone showed similar transcriptional profile of the markers (Supplemental Fig. 2). Student's *t* test was used for statistical analysis. Data are representative of two independent experiments with similar results. Error bars denote standard error between samples performed in triplicate (* < 0.05, ** < 0.01, *** *p* < 0.001, n.s.: non-significant).