Deletion of Orai1 leads to bone loss aggravated with aging and impairs function of osteoblast lineage cells

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ABSTRACT

Osteoblast lineage cells, a group of cells including mesenchymal progenitors, osteoblasts, and osteocytes, are tightly controlled for differentiation, proliferation and stage-specific functions in processes of skeletal development, growth and maintenance. Recently, the plasma membrane calcium channel Orai1 was highlighted for its role in skeletal development and osteoblast differentiation. Yet the roles of Orai1 in osteoblast lineage cells at various stages of maturation have not been investigated. Herein we report the severe bone loss that occurred in Orai1−/− mice, aggravated by aging, as shown by the microcomputed tomography (mCT) and bone histomorphometry analysis of 8-week and 12-week old Orai1−/− mice and sex-matched WT littermates. We also report that Orai1 deficiency affected the differentiation, proliferation, and type I collagen secretion of primary calvarial osteoblasts, mesenchymal progenitors, and osteocytes in Orai1−/− mice; specifically, our study revealed a significant decrease in the expression of osteogenic genes Fgf23, DMP1 and Phex in the cortical long bone of Orai1−/− mice; a defective cellular and nuclear morphology of Orai1−/− osteocytes; and defective osteogenic differentiation of Orai1−/− primary calvarial osteoblasts (pOBs), including a decrease in extra- cellular-secretion of type I collagen. An increase in the mesenchymal progenitor population of Orai1−/− bone marrow cells was indicated by a colony forming unit-fibroblasts (CFU-F) assay, and the increased proliferation of Orai1−/− pOBs was indicated by an MTT assay. Notably, Orai1 deficiency reduced the nuclear localization and transcription activity of the Nuclear Factor of Activated T-cell c1 (NFATc1), a calcium-regulated transcription factor, in pOBs. Altogether, our study demonstrated the crucial role of Orai1 in bone development and maintenance, via its diverse effects on osteoblast lineage cells from mesenchymal progenitors to osteocytes.

1. Introduction

Throughout the life of an organism, bone is remodeled and maintained by tightly controlled bone resorption and bone formation. Bone formation has been known to be mediated primarily by osteoblasts, the main bone-forming cells of the osteoblast lineage (Kronenberg, 2016). Recent studies reveal the crucial roles of other osteoblast lineage cells such as mesenchymal progenitors and osteocytes in mineral and bone homeostasis (Dallas et al., 2013; Ono and Kronenberg, 2015). Thus a stringent regulation of the osteoblast lineage, from progenitors to osteocytes, is essential to ensure proper bone formation and homeostasis at local and systemic levels.

For the molecular machinery involved in osteoblast biology, a plasma membrane calcium channel, Orai1, was recently identified for its role in bone homeostasis (Robinson et al., 2012; Hwang and Putney, 2012; Hwang et al., 2012). Upon calcium storage depletion in the endoplasmic reticulum (ER), Orai1 is activated and allows a rapid and transient calcium influx, called store-operated calcium entry (SOCE), to...
the cytoplasm (Feske et al., 2006). Genetic disruption of Orai1 impaired the skeletal development of 3-week-old mice and resulted to the osteopenia with decreased bone mineral density in adult mice due to significantly defective osteoblastic bone formation overriding defective osteoclastic bone resorption (Robinson et al., 2012; Hwang et al., 2012). As a cellular mechanism underlying a decreased bone density of Orai1−/− mice, defective osteoblast differentiation was suggested based on the in vitro differentiation assay using Orai1−/− bone marrow stromal cells and the osteoblast cell line (Robinson et al., 2012; Hwang et al., 2012). Yet the impact of Orai1 deficiency on various osteoblast lineage cells and their cumulative contributions to bone homeostasis have not been fully investigated, limiting our understanding of Orai1 in bone biology.

Herein, we show that Orai1 is broadly involved in differentiation, proliferation, and function of various osteoblast lineage cells. Orai1 deficiency impacted differentiation of osteoblast lineage cells from progenitors to osteocytes, indicated by the increased progenitor population within Orai1−/− bone marrow cells and the morphologically defective osteocytes in Orai1−/− mice. Orai1 deficiency also affected the secretory function of primary calvarial osteoblasts (pOBs), leading to a decrease in the amount of extracellular mature type I collagen. Moreover, Orai1 deficiency in pOBs led to an increase in in vitro proliferation, which corroborates an increase in the number of osteoblasts per bone perimeter in Orai1−/− mice. Also, defective activation of Nuclear Factor of Activated T-cell 1 (NFATc1), a calcium-regulated transcription factor, was observed in Orai1−/− pOBs, suggesting that defective SOCE resulting from Orai1 deficiency may impact various calcium signaling pathways in osteoblasts. These diverse effects of Orai1 deficiency imply that Orai1 is a critical regulator of cellular functions of osteoblast lineage cells, emphasizing the importance of intracellular Ca2+-homeostasis in osteoblast biology, bone homeostasis, and other degenerative bone disorders.

2. Materials and methods

2.1. Mice

Orai1−/− mice were generated by Dr. Yousang Gwack (University of California, Los Angeles) as previously described (Kim et al., 2011). Mice were genotyped by PCR of tail DNA as previously described (Gwack et al., 2008). All mice were maintained in pathogen-free barrier facilities and used in accordance with the protocols approved by the Institutional Animal Care and Use Committee at the University of California, Los Angeles.

2.2. Microcomputed tomography (μCT) and bone histomorphometry analysis

μCT were performed on femur and vertebrae as previously described (16 μm resolution) (Aghaloo et al., n.d.-a), using a Scanco μCT40 scanner (Scanco Medical, Switzerland). Visualization, reconstruction, and volume analysis of the data were performed using the Metamorph Imaging system (Universal Imaging, USA). Histomorphometric analysis of femurs was performed at the histomorphometry core laboratory (UCLA), following previously described protocols (Hsu et al., 2008). For both analyses, samples were isolated from Orai1−/− mice and sex-matched WT littermates.

2.3. Scanning Electron Microscopy (SEM) analysis

The analysis was performed at UCLA department of materials science and engineering core facility using a Nova NanoSEM 230 scanning electron microscope (FEI, USA) with field emission gun and variable pressure capabilities equipped with backscattered electron detectors and an energy dispersive x-ray spectrometer (ThermoScientific, USA). Femur samples from 8-week old Orai1−/− male mice and sex-matched WT littermates were fixed in 4% Glutaraldehyde overnight at 4°C, non-decalcified, resin-casted and coronal-sectioned at the distal metaphyseal area, sputter-coated with gold palladium, and subsequently examined with SEM.

2.4. Primary cell isolation and culture

pOBs were isolated from fetal or neonatal Orai1−/− mice and WT littermates following the previously described protocol (Tetradis et al., 2001). Mice were individually marked, kept alive until the completion of PCR genotyping of tail DNA. Calvaria from Orai1−/− and WT mice were separated for cell isolation. Bone marrow stromal cells (BMSCs) were isolated from long bones of 8–12 week old Orai1−/− mice and WT littermates as previously described (Aghaloo et al., n.d.-b). Mesenchymal progenitors were isolated from BMSCs following the published protocol using frequent medium changes for progenitor separation (Soleiman and Nadri, 2009). For proliferation, cells were plated at the concentration of 40,000 cells/ml and cultured in DMEM (ThermoFisher scientific, Waltham, MA) with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. For osteoblastic differentiation, confluent pOBs and BMSCs cultured in proliferation medium were changed to osteogenic medium, which was α-MEM (Invitrogen, Carlsbad, CA) with 10% FBS, 100 units/ml penicillin,100 μg/ml streptomycin supplemented with 50 μg/ml ascorbic acid (Sigma, St. Louis, MO, USA) and 10 mM beta-glycerophosphate (Sigma, St. Louis, MO, USA). Media were replaced every 2–3 days.

2.5. In vitro osteogenic differentiation assays

pOBs and BMSCs cultured in osteogenic medium for designated days were fixed with 4% paraformaldehyde and stained for Alkaline phosphatase, Alizarin Red, and Von Kossa stainings as previously described (Aghaloo et al., n.d.-b).

2.6. RNA extraction and real-time quantitative PCR (qPCR)

RNA was extracted from cultured cells or compact long bones isolated from using triazol (Invitrogen, Carlsbad, CA) and prepared for qPCR as previously described (Pirih et al., 2008). The sequences of gene-specific primers for qPCR are listed in Supplementary Table 1.

2.7. Western blot analysis

Western blot with anti-type I collagen antibody (Santa Cruz Biotechnology) was performed using pepsin-digested extracts and total cell lysates prepared from pOBs cultured in osteogenic medium for 3 weeks, following the published protocol for intracellular and extra-cellular type I collagen (Zhao et al., 2008).

2.8. Colony-forming unit (CFU) assay

Cells were isolated from bone marrow cells following the published protocol (Soleiman and Nadri, 2009), plated at the very low density of 10,000 cells/well in 6 well plate, cultured in proliferation medium for 10 days and prepared for Giemsa staining to increase the visibility for counting CFUs. Colonies containing > 50 cells were determined under the microscope, considered as CFUs.

2.9. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cell counting assay

For MTT assay, pOBs were plated at 20,000 cells per well of 96-well plate in triplicates, cultured for 12 h, and subjected to the assay using an MTT kit (Cayman Chemical Company, Ann Arbor, MI) following the manufacturer’s protocol. For cell counting assay, pOBs were plated at 20,000 cells per well of 24-well plate in duplicates, cultured for...
designated days, harvested by brief trypsin treatment, centrifuged and re-suspended to stain with toluidine blue for viable cell counting.

2.10. Monitoring of intracellular free Ca\(^{2+}\) concentrations

Optical monitoring of intracellular Ca\(^{2+}\) concentration was performed as previously described (Gwack et al., 2008). pOBs were seeded onto #1 glass coverslips in 24 well plate and cultured overnight. For Ca\(^{2+}\) monitoring, the medium was changed into Ca\(^{2+}\) free HBSS solution and loaded with 5 μM Fura-2, a quantifiable fluorescent Ca\(^{2+}\) dye. Imaging was performed with the Metamorph/Metafluo system (Universal Imaging). Each trace is an average of at least 30–40 osteoblasts. To induce SOCE, intracellular Ca\(^{2+}\) storage was depleted by albumin, and incubated with 1.0 μM thapsigargin (Sigma, St. Louis, MO) following the manufacturer’s protocol. pOBs were plated at 40,000 cells/ml per chamber of poly-L-lysine-coated glass 4-chamber slides and cultured for 24 h. Subconfluent cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100/PBS for 5 min, saturated with 0.5% bovine serum albumin, and incubated with 1.0 μg/ml anti-NFATc1 mAb 7A6 (Santa Cruz Biotechnology) for 1 h at room temperature, then after washing with PBS stained with Alexa Fluor 488-conjugated goat anti-mouse IgG (Jackson ImmunoResearch), mounted with coverslips using Vectashield (Vector Laboratories), and observed under a Nikon Inverted Stage fluorescent microscope (Nikon).

2.11. NFAT immunocytochemistry

pOBs were at plated at 40,000 cells/ml per chamber of poly-L-lysine-coated glass 4-chamber slides and cultured for 24 h. Subconfluent cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100/PBS for 5 min, saturated with 0.5% bovine serum albumin, and incubated with 1.0 μg/ml anti-NFATc1 mAb 7A6 (Santa Cruz Biotechnology) for 1 h at room temperature, then after washing with PBS stained with Alexa Fluor 488-conjugated goat anti-mouse IgG (BDbioscience, San Jose) for 1 h at RT. After washing, cells were counterstained with the DNA-intercalating dye DAPI (4,6-diamidino-2-phenylindole), mounted with coverslips using Vectashield (Vector Laboratories), and observed under a Nikon Inverted Stage fluorescent microscope (Nikon).

2.12. Luciferase reporter assay

Transient transfection of luciferase plasmid to pOBs was performed using Lipofectin with Plus reagents (ThermoFisher Scientific, Waltham, MA) following the manufacturer’s protocol. pTL luciferase plasmid containing three tandem repeats of NFAT/AP-1 consensus binding sites in its promoter was provided by Dr. Yousang Gwack. Then at 48 h after transfection, cells were stimulated with 25 nM PMA and 1 μM Ionomycin for 6 h, collected in luciferase lysis buffer and subsequently processed for the luciferase assay (Promega, Madison, WI) following the manufacturer’s protocol.

2.13. Statistical analysis

All values are presented as mean ± standard error of the mean (SEM) from at least 3 independent experiments. Data were analyzed with the paired two-tailed Student’s t-tests as appropriate for the data set. A p-value < 0.05 was considered as statistically significant.

3. Results

3.1. Age-related bone loss in Orai1−/− mice

To evaluate the effect of Orai1 deficiency on the overall bone formation, we performed quantitative microcomputed tomography (μCT) analysis for femurs and lumbar vertebrae (L4) of 8 week-old Orai1−/− mice and sex-matched WT littermate controls. A severe osteoporotic bone phenotype of Orai1−/− mice was indicated, as Orai1−/− mice presented a lower trabecular bone mass in both femur and lumbar vertebrae (Fig. 1A and B), and a significant decrease in bone volume versus tissue volume (BV/TV), trabecular bone thickness (Tb.Th), and trabecular number (Tb.N.) (Fig. 1C). In addition to these quantitative defects, qualitative bone defects were also observed in Orai1−/− mice, shown as a significant decrease in bone mineral density (BMD) of both metaphyseal trabecular bone (Fig. 1C). The previous study of 3-week old Orai1−/− mice only showed a very mild effect of Orai1 deficiency on bone (Robinson et al., 2012). While perinatal Orai1−/− mice did not present obvious defects in cartilage and bone formation when examined with histological analysis and skeletal preparation (Sup Fig. 1), 8-week old Orai1−/− mice presented significant bone loss, indicated by μCT analysis. This led us to examine whether the effect of Orai1 deficiency on bone is age-related or not. Therefore, 8-week and 12-week old Orai1−/− mice with their WT littermate controls were analyzed with bone histomorphometry (Fig. 1D). Results indicated that bone loss in Orai1−/− mice was aggravated by aging, as deterioration of trabecular bone volume (BV/TV) in distal femoral metaphyseal area was significantly greater at 12-week postnatal than the one at 8-week. Consistent with the μCT findings, the static parameters of histomorphometry also indicated a severe osteoporotic bone phenotype of Orai1−/− mice, shown by a significant decrease in bone volume versus tissue volume (BV/TV), trabecular bone thickness (Tb.Th), and a significant increase in trabecular spacing (Tb.Sp.) (Fig. 1D).

3.2. Defective morphology of osteocytes in Orai1−/− mice

Previous studies indicated that decreased bone mass of Orai1−/− mice resulted from decreased osteoblastic bone formation, rather than increased osteoclastic bone resorption (Ono and Kronenberg, 2015; Hwang et al., 2012), yet it remained largely unidentified whether Orai1 affects osteoblastic lineage cells from mesenchymal progenitors to mature osteocytes. In bone, osteocytes consist > 90% of bone cells and play a major regulatory role in bone matrix mineralization and bone remodeling by cell-to-cell interactions and secretion of organic and inorganic factors (Dallas et al., 2013). To identify whether Orai1 deficiency affects osteocytes or not, we first examined the cellular and nuclei morphology of osteocytes in Orai1−/− mice.

Scanning Electron Microscope (SEM) analysis revealed that osteocytes in Orai1−/− mice were narrowed and ragged in shape, compared to WT cells that were oval or round (Fig. 2A&B). Osteocytes in Orai1−/− mice were also detached from its lacunae, exhibiting abnormal pericellular space between the cell and mineralized bone surface. This feature may be related to defective mineralization of Orai1−/− mice, as the peri-osteocytic space has been shown to contain mineralizing extracellular matrix (Wysolmerski, 2013; Alcobendas et al., 1991). For the nuclei morphology, osteocytes in Orai1−/− mice presented smaller nuclei compared to WT (Fig. 2C). Osteocytes in Orai1−/− mice also presented significantly less euchromatin, shown as the electron-lucent area in the nuclei. As euchromatin is the area of loose chromatin allowing active gene transcription, this suggested that osteocytes in Orai1−/− mice could be less active in gene transcription and protein synthesis compared to WT (Palumbo, 1986). Then, we further examined whether Orai1 deficiency affects gene expression in osteocytes. For qPCR analysis, RNAs were extracted from the cortical long bone of 12 week-old Orai1−/− and WT littermate control and prepared for the analysis. As expected, Orai1 expression was ablated in the samples from Orai1−/− mice (Fig. 2D). Osteogenic gene expression was also decreased in Orai1−/− mice, as Fgf23 and Phex were statistically significantly decreased and Dmp1 was slightly decreased (Fig. 2B). Together these data indicated the defective function of osteocytes in Orai1−/− mice.

3.3. Defective osteoblastic differentiation and increased proliferation of Orai1−/− osteoblasts and Orai1−/− bone marrow cells

To further investigate the role of Orai1 in osteoblast lineage cells at various differentiation stages, Orai1−/− primary calvarial osteoblasts (pOBs), which have never been examined in previous studies, and Orai1−/− bone marrow stromal cells (BMSCs) were cultured and
examined for their osteogenic potentials by in vitro differentiation assays. Orai1−/− pOBs presented significantly reduced Alkaline phosphatase (ALP) positivity, less calcium deposition and fewer nodule formation compared to WT controls, shown by ALP, alizarin red, and von Kossa stainings (Fig. 3A). Correspondingly, osteoblastic marker gene expression - ALP and Osteocalcin (OCN) - were significantly decreased and Orai1 gene expression was ablated in both Orai1−/− BMSCs and Orai1−/− pOBs, indicated by qPCR analysis (Fig. 3B).

Notably, for type I collagen, neither the mRNA expression level (Fig. 3C) nor the intracellular pro-α chain type I collagen protein level was significantly altered in Orai1−/− pOBs (Fig. 3D, middle panel), indicating that Orai1 deficiency did not affect the intracellular production of type I collagen protein. However, a significant decrease in extracellular type I collagen protein level in Orai1−/− pOBs indicated that Orai1 deficiency affected extracellular secretion of type I collagen in osteoblasts (Fig. 3D, top panel). As the type I collagen comprises 80% of the unmineralized organic matrix that undergoes mineralization to form bone (Landis and Jacquet, 2013), a significant decrease in extracellular type I collagen can lead to defective bone mineralization. Therefore, definitive secretion of type I collagen by Orai1−/− osteoblasts may, at least partly, account for significantly decreased bone mineral density and bone mass in Orai1−/− mice. Also similarly to Orai1−/− pOBs, Orai1−/− BMSCs presented decreased expression of osteogenic marker gene ALP and OCN throughout osteogenic differentiation (Fig. 3E). In addition to pOBs, the effect of Orai1 deficiency on BMSCs and mesenchymal progenitors was also examined. The proportional change in the number of mesenchymal progenitors in bone marrow cells of Orai1−/− mice was examined by colony-forming unit fibroblast (CFU-F). Interestingly, Orai1−/− cells yielded a significantly higher number of CFU-F, suggesting the increased progenitor population within Orai1−/− bone marrow cells (Fig 3F and G). Altogether our data suggested a crucial role of Orai1 throughout osteoblast differentiation from mesenchymal progenitors to mineralizing osteoblasts and osteocytes. Meanwhile, the effect of Orai1 deficiency on osteoblast proliferation was also examined by MTT assay and cell counting assay. Assays were performed between one to three days after plating, to access proliferative potential during linear growth prior to confluency. Notably, both MTT assay and cell counting assays indicated significantly upregulated in vitro proliferation of Orai1−/− pOBs compared to WT (Fig 4A and B). The absorbance value of MTT assay correlated well with the number of both WT and Orai1−/− pOBs, therefore MTT assay was valid to examine pOB proliferation (Sup Fig. 2). Taken altogether, these data suggested that Orai1 deficiency affected both differentiation and proliferation of osteoblast lineage cells.

3.4. Defective Ca2+-induced NFAT activation in Orai1−/− osteoblasts

Finally, to understand the underlying molecular mechanism of defective differentiation and proliferation of Orai1−/− osteoblasts...
Fig. 2. Orai1 deficiency affected osteocyte morphology and osteocytic gene expression in adult mice.

(A–C) Scanning electron micrograph (SEM) of the femoral compact bone from 8-week old male Orai1−/− mice (right panels) and male WT littermate control (left panels). In low magnification images (×800(A), ×4,000(B)), Orai1−/− osteocytes were narrowed and ragged in shape and detached from their lacunae, while WT cells were oval and exhibited no space between the cell and mineralized bone surface. In high magnification SEM (×10,000) images (C), nuclei of Orai1−/− osteocytes were smaller in size compared to WT. Euchromatin (indicated by white arrows), the electron-lucent areas in nuclei, was significantly less in Orai1−/− osteocytes, compared to WT.

(D) Osteocytic gene expression in cortical long bone. Changes in mRNA expression of osteoid osteocyte marker gene Phex, mineralizing osteocyte marker gene DMP1 and mature osteocyte marker gene Fgf23 in cortical long bone of 12 week-old Orai1−/− and WT mice were examined by qRT-PCR. Expression levels normalized to GAPDH are shown as relative fold expression to the WT (n = 4, mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001).
lineage cells, we aimed to investigate whether Orai1 deficiency affects SOCE and Ca\(^{2+}\)-signaling activation in osteoblasts, as defective SOCE and Ca\(^{2+}\) signaling are largely responsible for defective cellular phenotypes of other Orai1\(^{-/-}\) cell types (Gwack et al., 2008; Zhan et al., 2015; Somasundaram et al., 2014; Bikle and Mauro, 2014). Ca\(^{2+}\) imaging analysis showed a significant reduction of SOCE in Orai1\(^{-/-}\) pOBs, indicating a crucial regulatory role of Orai1 for SOCE in osteoblasts (Fig. 5A and Sup Fig. 3). A relatively mild reduction of SOCE in Orai1\(^{+/+}\) pOBs was shown, indicating a cumulative regulation of SOCE by Orai1 in osteoblasts (Fig. 5A). Also, an immediate attenuation of Ca\(^{2+}\) level in WT pOBs by the treatment of 2-aminoethoxydiphenyl borate (2-APB), a SOC channel blocker/Orai1 inhibitor, recapitulated a crucial role of Orai1 in the regulation of intracellular Ca\(^{2+}\) level in osteoblasts.

Then, to examine whether NFAT activation is defective in Orai1\(^{-/-}\) osteoblasts, immunocytochemistry using an antibody against NFATc1 was performed to visualize nuclear accumulation of NFAT, as activated NFAT translocates into the nucleus, where it interacts with other transcription factors and induces downstream gene expression (Winslow et al., 2006; Kawano et al., 2006; Koga et al., 2005). PMA and Ionomycin time-course immunocytochemistry indicated the highest level of nuclear NFAT in WT pOBs at 15 min of treatment (Sup Fig. 4). While > 80% of WT pOBs showed nuclear NFAT after treated with Phorbol myristate acetate (PMA) and Ionomycin for 15 min (Fig. 5B, top panels), only < 20% of Orai1\(^{-/-}\) pOBs showed nuclear NFAT (Fig. 5B, bottom panels). Furthermore, the effect of Orai1 deficiency on NFAT transcriptional activity was examined by luciferase reporter assay. Orai1\(^{-/-}\) and WT pOBs were transiently transfected with a luciferase reporter construct containing three tandem repeats of the NFAT-responsive activating protein-1 (AP-1) site in its promoter. PMA
Fig. 4. Orai1 deficiency led to an increase in proliferative potential of primary calvarial osteoblasts (pOBs).

(A) MTT assay of Orai1−/− and WT pOBs cultured for indicated days. Cells were plated in triplicates at 20,000 cells per well in 96-well plate. Representative result from three independent experiments with similar results is shown (n = 3, mean ± SEM, *p < 0.05, **p < 0.01).

(B) Cell counting assay of Orai1−/− and WT pOBs cultured for indicated days. Cells were plated at 10,000 cells per well in 12 well plate, cultured, trypsinized, trypan blue-stained for manual counting. Average results from three independent experiments are shown (n = 3, mean ± SEM, *p < 0.05).

Fig. 5. Orai1 deficiency resulted in defective NFATc1 activation in primary calvarial osteoblasts (pOBs).

(A) Optical imaging of intracellular free Ca2+ concentration ([Ca2+]i) in WT, Orai1+− and Orai1−/− pOBs. Left panel: The average intensity of Fura-2 fluorescence from 40 cells at different time points is graphically presented with the s.e.m. Cells were loaded with Fura-2 to visualize Ca2+, treated with thapsigargin (TG, 1 μM) in Ca2+-free HEPES-buffered saline solution to induce Store-operated Ca2+ entry (SOCE) (black arrow), and with SOC inhibitor 2-APB (100 μM) to block SOCE (gray arrow). Right panel: The bar graph shows the average fluorescence intensity from 40 cells with s.e.m. at 0 and 650 s. Statistically significant differences were noted between WT, Orai1+/− and Orai1−/− pOBs at 650 s.

(B) NFATc1 immunocytochemistry. Cells were treated with PMA (25 nM) and Ionomycin (1 μM) for 15 min to activate Ca2+ signaling, fixed, permeabilized, and stained with anti-NFAT antibody followed by FITC-conjugated secondary antibody (green). A decrease in NFATc1 nuclear localization (red arrowhead) was observed in Orai1−/− pOBs. Representative images from two independent experiments with similar results are shown.

(C) NFATc1 luciferase assay. NFAT transcription activity was evaluated by NFATc1 luciferase assay. WT and Orai1−/− pOBs were transiently transfected with a luciferase reporter construct containing NFAT-responsive elements in its promoter and treated with PMA (25 nM) and Ionomycin (1 μM) for 6 h. (n = 3, mean ± SEM, **p < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
and Ionomycin time-course luciferase assay indicated that the luciferase signal peaked at 6 h of treatment (Sup Fig. 5). Importantly, Orai1 −/− pOBs showed a significant decrease in the luciferase signal both at 0 and 6 h of PMA and Ionomycin treatment, indicating that Orai1 deficiency compromised NFAT transcriptional activity in osteoblasts (p-values 0.01 and 0.043, respectively) (Fig. 5C). Together these data suggested that Orai1 deficiency was associated defective Ca2+ entry and defective downstream activation of Ca2+ signaling pathways, such as NFAT signaling in osteoblasts.

4. Discussion

Our study highlights the role of Orai1 in bone formation, which occurs via the cumulative actions of various osteoblast lineage cells. Our μCT analysis revealed a severe bone loss when Orai1 −/− mice were compared with sex-matched WT littermate controls. As the previous study of adult Orai1 −/− mice reported only a slight bone loss indicating an osteopenic bone phenotype (Hwang et al., 2012), the sex-related differences in bone mass and the genetic variance of Orai1 −/− mice from outbreeding may account for this disparity between our results and theirs. In particular, our cellular analysis indicated the broad involvement of Orai1 in the function, proliferation, and differentiation of osteoblast lineage cells, reflecting the versatile role of SOCE including the activation of calcium signaling pathways.

The impact of Orai1 deficiency upon osteoblast differentiation has been previously demonstrated by in vitro osteogenic differentiation assays using human osteoblast cell line and Orai1 −/− bone marrow stromal cells (Robinson et al., 2012; Hwang et al., 2012; Lee et al., 2016). Herein, we examined various osteoblast lineage cells in Orai1 −/− mice, such as primary calvarial osteoblasts and osteocytes that have never been examined previously, and demonstrated that Orai1 deficiency broadly impacted the osteoblast lineage differentiation from mesenchymal progenitors to osteocytes. For osteocytes, our data indicated that the involvement of Orai1 in osteocyte biosynthesis, including Fgf23 and Phex gene expression. This corresponds well with a recent study of Zhang et al., which demonstrated the regulation of Fgf23 expression by SOCE and Orai1 in osteoblastic and perioseal cell lines (Zhang et al., 2016).

For osteoblasts, Orai1 affected the main function of osteoblasts, bone matrix deposition such as type I collagen-rich organic matrix. Interestingly, the intracellular protein level of type I collagen in Orai1 −/− osteoblasts was not affected, which indicates that the decrease in extracellular mature type I collagen may be due to defects in intracellular collagen trafficking from ER to plasma membrane or its secretion at the plasma membrane. Concurrent with our observation, Orai1 was recently shown to regulate the exocytosis/secretion of two types of microvesicles - secretory granules and cytotoxic granules in lymphocyte and natural killer cells (Lioudyno et al., 2008; Dickson et al., 2012; Barr et al., 2008; Maul-Pavicic et al., 2011). Although in different cell types, these reports together clearly indicate the involvement of Orai1 in cellular exocytosis/secretion.

While Orai1 has been shown to regulate the differentiation and function of numerous cell types, the role of Orai1 in cellular proliferation has not reached the consensus yet. Gwack et al. showed that Orai1 deficiency led to an increase in CD4+ T cell proliferation (Kim et al., 2011).

However, in keratinocytes, neural progenitor cells, cancer cell lines and proliferating mesenchymal stromal cells, gene deletion or pharmacological/viral inhibition of Orai1 was associated with a decrease in cellular proliferation (Zhan et al., 2015; Somasundaram et al., 2014; Bikle and Mauro, 2014; Maliske et al., n.d.), suggesting that cell-type context is important for the role of Orai1 in cellular proliferation. In vitro test results for osteoblast proliferation were inconsistent as well among groups. A previous study by Hwang et al., showed an insignificant impact of Orai1 upon proliferation of MC3T3 osteoblast cell line (Hwang et al., 2012). In that study, the authors used dominant-negative Orai1 expressing cells in comparison to WT controls and performed MTT assay one to two weeks after cell plating. Our MTT assay, however, clearly indicated an increase in in vitro proliferation of primary Orai1 −/− osteoblasts when their proliferative potential was accessed during the linear growth phase of cells, which was between one to three days after plating before cells reach the confluency. For in vivo proliferation, a study by Robinson et al. showed a decrease in the positive ALP labeled area on the vertebrae of Orai1 −/− mice (Robinson et al., 2012). Interestingly, our histomorphometry indicated an increase in osteoblast number per bone perimeter and an increase in osteoblast area per bone area in Orai1 −/− mice. As ALP-positivity is a marker of active/mature osteoblasts, the results of ours and Robinson et al. together may indicate the increased proliferation of ALP-negative premature osteoblasts and/or overall delayed differentiation of osteoblasts in Orai1 −/− mice.

The diverse effects of Orai1 deficiency on osteoblast secretion, proliferation, and differentiation are not surprising, given the versatile role of intracellular calcium in osteoblast biology. Orai1 can possibly mediate the activation of signaling pathways utilizing Ca2+, such as protein kinase C, BMP, Wnt, TGF-β1, calmodulin, and calcineurin/NFAT signaling pathways, all of which take crucial roles in various osteoblast functions, including differentiation and proliferation (Winslow et al., 2006; Kawano et al., 2006; Koga et al., 2005; Lee et al., 2016; Choo et al., 2009; Blair et al., 2007; Kohn and Moon, 2005). Moreover, defective Ca2+ entry resulted from defective Orai1 may disrupt intracellular calcium kinetics in ER and other calcium-mediating compartments, such as mitochondria and lysosomes, which mediate a variety of osteoblastic cellular functions such as metabolism, survival, apoptosis, secretion and differentiation (Zhao et al., 2008; Koizumi et al., 2003; Mahamid et al., n.d.; Sato et al., 2015; Cabral et al., 2016).

Altogether, our study addressed the importance of the calcium channel Orai1 and intracellular calcium homeostasis in osteoblast lineage cells, from mesenchymal progenitors to osteocytes. Further studies on how Orai1 affects the activation of various calcium signaling pathways and the calcium-mediating intracellular compartments in osteoblasts lineage cells, will allow us to better understand the molecular regulation mechanism of osteoblast functions of proliferation and differentiation as well as bone homeostasis.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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