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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 osteocytic 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 extracellular-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

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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 c1 (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 Ca^{2+}-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 littermate 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 (Soleimani 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 penicilin,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 extracellular 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 (Soleimani 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