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Gene Disruption of the Calcium Channel Orai1 Results in Inhibition of Osteoclast and Osteoblast Differentiation and Impairs Skeletal Development

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Abstract

Calcium signaling plays a central role in the regulation of bone cells, though uncertainty remains with regard to the channels involved. In previous studies, we determined that the calcium channel Orai1 was required for the formation of multinucleated osteoclasts in vitro. To define the skeletal functions of calcium release-activated calcium currents, we compared mice with targeted deletion of the calcium channel Orai1 to wild-type littermate controls, and examined differentiation and function of osteoblast and osteoclast precursors *in vitro* with and without Orai1 inhibition. Consistent with *in vitro* findings, Orai1$^{-/-}$ mice lacked multinucleated osteoclasts. Yet they did not develop osteopetrosis. Mononuclear cells expressing osteoclast products were found in Orai1$^{-/-}$ mice, and *in vitro* studies showed significantly reduced, but not absent, mineral resorption by the mononuclear osteoclast-like cells that form in culture from peripheral blood monocytic cells when Orai1 is inhibited. More prominent in Orai1$^{-/-}$ mice was a decrease in bone with retention of fetal cartilage. Micro-computed tomography showed reduced cortical ossification and thinned trabeculae in Orai1$^{-/-}$ animals compared to controls; bone deposition was markedly decreased in the knock-out. This suggested a previously unrecognized role for Orai1 within osteoblasts. Analysis of osteoblasts and precursors in Orai1$^{-/-}$ and control mice showed a significant decrease in alkaline phosphatase-expressing osteoblasts. *In vitro* studies confirmed that inhibiting Orai1 activity impaired differentiation and function of human osteoblasts, supporting a critical function for Orai1 in osteoblasts, in addition to its role as a regulator of osteoclast formation.

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Calcium signaling plays a central role in the regulation of osteoclast differentiation, but the mediators of calcium signals in bone cell development remain to be fully defined. In monocytic osteoclast precursors, calcium signals activate calmodulin-dependent kinases and phosphatases leading to activation of transcriptional regulators including the cAMP response element-binding protein (CREB) and nuclear factor of activated T cells 2 (NFAT2, also denoted NFATc1) (1, 2). Calcium channels from several families, including inositol-1,4,5-trisphosphate receptors (IP3R), ryanodine receptors, and transient receptor potential channels, have been implicated in osteoclast formation and function (2-4).

We showed recently that the cell membrane calcium channel Orai1, which is activated by endoplasmic reticulum (ER) calcium depletion, is required to support osteoclast development in vitro (5). Specifically, when Orai1 activity or expression is inhibited, formation of multinucleated osteoclasts from RANKL treated human or murine monocytes is inhibited (5). In contrast, little is known of the role of store operated calcium entry in bone formation or osteoblast differentiation (4), though calcium signals are also important regulators of osteoblasts (6).

In osteoclast precursors, as in many cell types, release of intracellular calcium stores triggers a subsequent influx of extracellular calcium that is necessary for sustained calcium signaling (1,2). Our previous work showed that depletion of intracellular calcium stores activates STIM1-Orai1 mediated calcium influx (4). STIM1 is a transmembrane calcium sensing protein in the endoplasmic reticulum (ER) that undergoes a conformational change when ER calcium is depleted, resulting in aggregation and interaction with Orai1 in "puncta" in the plasmalemma, Orai channel opening and calcium influx (7,8). Coupling of STIM1 and Orai1 produce highly selective calcium currents (7,9). Consistent with our findings, Kim et al. demonstrated recently that knockdown of STIM1 expression or inhibition of IP3Rs with xestospongin C eliminated calcium oscillations required for osteoclastic differentiation in bone marrow macrophages treated with RANKL (10).

A small number of patients with Orai1 gene defects have been reported. Two of these cases have been classified as Immune Dysfunction with T-cell Inactivation Due to Calcium Entry Defect 1 (On-line Mendelian Inheritance in Man, file 612782). The predominant phenotype is that of immunodeficiency, and most patients die by one year of age as a result of infection (11,12). Early mortality has limited assessment of the skeletal consequences of the Orai mutations, but the patients are reported to show developmental delay compatible with the mouse phenotype. In addition, some patients are reported to show amelogenesis imperfecta, and skeletal dysmorphism has also been reported (11). We hypothesized that the developmental delay observed in these patients might reflect impaired skeletal development in the absence of Orai1, potentially linked to the in vitro defect we observed in osteoclastogenesis (5). If so, specific calcium channel inhibitors might be useful to prevent osteoclastic bone damage in clinical settings such as inflammatory arthritis.

We investigated the role of Orai1 in bone development using an Orai1$^{-/-}$ (KO) mouse. We compared the skeletal features of the knock-out mice to litter-matched controls with normal Orai1 (WT). This model is useful but poor survival of Orai1$^{-/-}$ animals is a limiting factor. Our results confirm the importance of Orai1 in osteoclast development: formation of multinucleated osteoclasts and resorption of mineralized tissue were impaired in KO mice *in vivo*. Yet osteopetrosis did not occur, apparently reflecting the presence of small osteoclast-like cells that retain a limited capacity for resorption of mineralized tissue as well as an unexpected defect in bone formation, reproduced by Orai1 inhibition *in vitro*. These findings together imply that Orai1 is necessary for normal bone formation as well as normal osteoclast differentiation and is a critical regulator of the skeleton.

Materials and Methods

Animals and genotyping

Orai1$^{-/-}$mice were originally generated by Gwack et al. as previously described in detail (13); briefly, the Orai1 gene was disrupted by homologous recombination using B6/3 embryonic stem cells derived from C57BL/6 mice (TaconicArtemis, Koln, Germany); chimeric mice were generated by blastocyst injection and founders bred to C57BL/6 mice (TaconicArtemis) (13). In the C57BL/6 background, homozygous Orai1 deficiency resulted in neonatal death. We therefore generated Orai1$^{-/-}$mice in a mixed genetic background: heterozygous Orai1$^{+/-}$ C57BL/6 mice were intercrossed for one to six generations with mice of the outbred ICR strain (Taconic), after which ICR Orai1$^{+/-}$ heterozygous were mated to generate Orai1$^{-/-}$mice, which survive for ~4 weeks. All animals were maintained in pathogen-free barrier facilities and used in accordance with IACUC approved protocols. Mice were genotyped by PCR of tail DNA with primers: forward 5′-GGGTGTGGCGTATGCAAATAACCT-3′, reverse (WT) 5′-ACTCGAGCCGGTCTCCC-3′, and reverse (KO) 5′-TCGTACCACCTTCTTGGGACTTGA-3′. Products were separated on 2% agarose to show the 200 bp WT and 300 bp targeted allele products. Because Orai1$^{-/-}$ animals die by ~ 4 weeks, mice were sacrificed at 3 weeks.

Skeletal radiography and whole mounts

Skeletons were fixed overnight in 5% formaldehyde and stored until needed in 70% ethanol at −20°C. Whole animal radiography used a Faxitron MX20 (Lincolnshire, IL); images were recorded on film. μCT was performed on a Viva CT40 (Scanco, Bassersdorf, Switzerland) with reconstruction by instrument-specific software. The scan slice increment was 10 μm. Reconstructions used a density cutoff of 150 mg/cm3. After removal of skin and viscera, cartilage was stained with alcian blue, 0.03% in ethanol and 20% acetic acid, overnight, followed by alizarin red 0.03% in 1% KOH, for 30 days at 4 °C. Skeletons were then washed and clarified in 20% glycerol with 1% KOH.

Histology, antibodies, and microscopy

Decalcified paraffin embedded tissue was cut at 6 μm, deparaffinized, and stained with hematoxylin and eosin or used for antibody labeling. For alkaline phosphatase, after citrate-EDTA antigen retrieval and blocking in PBS and 2% BSA, sections were incubated with

rabbit monoclonal anti-alkaline phosphatase antibody (Epitomics, Burlingame, CA) at 1:100, washed, and incubated with Cy3-labeled goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at 1:500. Frozen sections were cut with carbide blades and sections captured using the CryoJane transfer system (Leica Microsystems, Bannockburn, IL). To evaluate bone formation, mice received intraperitoneal calcein, 1 mg/kg, 48 hours before sacrifice, and frozen sections were examined by fluorescence microscopy. Tartrate resistant acid phosphatase (TRAP) activity was assayed in frozen sections fixed in citrate at pH 5.4 with 40% acetone. Acid phosphatase was then determined in 670 mM tartrate at pH 5.6 using naphthol AS-BI phosphate substrate and fast garnet GBC to visualize the product as a red precipitate (Sigma, St Louis, MO, USA). Calcified tissue in sections was labeled by 1% AgNO3 under ultraviolet light (von Kossa stain) until contrast of mineralized (black) with non-mineralized tissue was adequate (~30 minutes) followed by eosin counterstain. Western blots were performed as described (14). Anti-Orai1 was O8264 rabbit polyclonal (Sigma) produced using a peptide immunogen corresponding to amino acids 288-301 of human Orai1. Imaging used a Nikon TE2000 inverted microscope with a 14-bit 2048 × 2048 pixel monochrome charge coupled device and RGB filters for color reconstruction (Spot, Sterling Heights, MI, USA). Green fluorescence (calcein) used excitation 450-490 nm, a 510 nm dichroic mirror, and a 500-570 nm emission filter. For red fluorescence, excitation was 530-560 nm, a 575 nm dichroic mirror and a 580-650 nm emission filter.

Cell culture

Human CD14 positive monocytic cells were isolated and differentiated by RANKL treatment (14). Orai1 activity was inhibited with 50 μM 3,4-dichloropropionanilide (ChemServices, West Chester, PA) (5) using equivalent solvent (ethanol) in controls. To assay resorption, osteoclasts were grown on slides coated with mineralized matrix (BioCoat, BD Biosciences, San Jose, CA). After 12 days, cells were removed and the matrix stained using AgNO3 to differentiate resorbed areas from intact matrix. Area resorbed was determined by computer aided analysis (Fovea Pro, Reindeer Graphics, Asheville, NC). Human osteoprogenitor cells (CC-2538) were from Lonza (Walkersville, MD, USA) and cultured in growth medium: Dulbecco's modified Eagle's medium with 10% fetal bovine serum. For osteoblast differentiation and matrix mineralization, confluent cultures were changed to medium supplemented with 200 nM hydrocortisone, 10 mM glycerol-2-phosphate, 50 ∞g/ml ascorbic acid, 2 mM CaCl2 and 10 nM 1,25-dihydroxyvitamin D (Sigma). Media were replaced every 2-3 days. Alkaline phosphatase activity was assayed using naphthol AS-MX phosphate substrate in citrate buffer pH 8 with fast blue RR to visualize the product as a blue precipitate (Sigma). To stain matrix mineral, cultures fixed in 5% formaldehyde at pH 8 were incubated in 2% Alizarin Red S (Sigma) for 20 minutes at room temperature.

Quantitative PCR

Total RNA was isolated using phenol/guanidine isothiocyanate (Trizol, Invitrogen); RNA was quantified by A260. Reverse transcription used 500 ng of total RNA, random hexamer primers and MMLV reverse transcriptase (SuperScript III, Invitrogen). The 20 μl reactions contained 20 mM Tris, pH 8.3, 50 mM KCl, 5 mM MgCl2, 10 mM dithiothreitol, 0.5 mM of

each dNTP, and RNase inhibitor (40 U, RNaseOUT, Invitrogen). Primer extension was performed at 42 °C for 1 hour. One μl aliquots were used for PCR reactions in a prepared polymerase mixture (Brilliant III SYBR Green master mix, Agilent, Santa Clara, CA) with 2.5 mM MgCl2 and 400 nM oligonucleotide primers. After 10 min at 95°C, 15 second 95 °C and 1 minute 60 °C cycles were done on a MX3000P (Stratagene). Relative mRNA quantity was calculated by comparative cycle threshold (14), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. PCR product sizes were verified by agarose gel electrophoresis. Primers: GAPDH (GenBank: AF261085) forward GAGTCAACGGATTTGGTCGT, reverse TTGATTTTGGAGGGATCTCG; RUNX2 (GenBank AF001450) forward CCTCGGAGAGGTACCAGATG, reverse TTCCCGAGGTCCATCTACTG; collagen type I alpha 1 (COL1A1, GenBank Z74615) forward AGGGCCAAGACGAAGACATCCC, reverse TGTCGCAGACGCAGATCCG; alkaline phosphatase *(*ALPL, GenBank BC021289) forward CCTTGCTCACTCACTCACTCC, reverse TTTTTTTTGCCGTTCCAAAC; RANKL (TNFSF11, GenBank AF013171) forward ATCGTTGGATCACAGCACAT, reverse AGACTCACTTTATGGGAACCAGA; Osteoprotegerin (TNFRSF11B, GenBank U94332) forward AACGCCAACACAGCTCACAAGAAC, reverse TGCTCGAAGGTGAGGTTAGCATGT; and ORAI calcium release-activated calcium modulator 1 (ORAI1, GenBank AK027372) forward AGGTGATGAGCCTCAACGAGCA, reverse AGTCGTGGTCAGCGTCCAGCT.

Statistics

Numerical data are shown as mean ± standard deviation and represent comparisons of three independent determinations unless specified. Student's t-test was used for individual comparisons; analysis of variance was performed for comparisons of multiple samples.

Results

Effects of Orai1 Deletion on Skeletal Modeling, Bone Density and Structure

Orai1$^{-/-}$ mice (13) were identified by PCR of genomic DNA (Fig 1A). Orai1$^{-/-}$ mice were consistently smaller and showed less post-natal growth than WT littermates. Heterozygotes showed no prominent abnormalities. For the WT and heterozygote groups, variation in weight and size within groups exceeded intergroup differences (data not shown); this heterogeneity likely reflects the outbreeding of carriers. Since our purpose was to determine the major effects of Orai1 deficiency, heterozygotes were not further evaluated. Mouse skeletons were examined at 3 weeks, near the limit of survival for Orai1$^{-/-}$ mice (~4 weeks). The skeleton of the KO is proportionately smaller than the WT (Fig 1B). However, the modeling of the skeleton showed no gross defects: individual bones were scaled to the animal size. This suggested that Orai1 deficiency did not affect the modeling of the cartilaginous skeleton and that growth plate activity, while diminished, is properly regulated in the knockout. Analysis of radiographs, with comparison of ribs and vertebrae from WT and KO mice (Fig 1C), suggested decreased bone density in the KO (Fig 1C). Tooth eruption and gross morphology in Orai1$^{-/-}$ mice were similar to that of WT, except that the teeth in KO mice appeared slightly smaller. However, microscopic sections revealed that

enamel deposition in the KO mice was reduced and irregular (Fig 1D) in keeping with observations of amelogenesis imperfecta in humans with Orai1 defects (11).

Quantitative effects of Orai1 deficiency on bone were determined by micro-computed tomography (μCT). Vertebrae were compared because they have the most uniform trabecular structure. Orai1$^{-/-}$ vertebrae were not only much smaller than WT, but the mineralized bone showed discontinuities suggesting persistence of cartilage (Fig 2A). Although mice normally retain some cortical cartilage at birth, by three weeks the cortex should be fully ossified, as was evident in the control mice. In addition, vertebrae are normally filled with trabecular bone at three weeks, but in the in Orai1$^{-/-}$ mice trabecular bone was reduced (Fig 2A), though some regional variation was seen in all mice. Quantitative analysis of Orai1$^{-/-}$ vertebrae showed a significant reduction in trabecular thickness ($p=0.03$) with a trend toward decreased bone volume as a fraction of total volume ($p=0.06$) relative to the WT. There was also a trend toward reduced trabecular number ($p=0.11$) in Orai1$^{-/-}$. That statistical significance was not reached for some parameters reflects high intragroup variability and the low numbers of KO mice that survived long enough for analysis.

Cartilage and ossification in Orai1$^{-/-}$ and WT skeletons were then compared using alcian blue and alizarin red staining. Animals at three weeks could not be studied in whole mount, so key bones were dissected (Fig 2B). Alcian blue showed retained cartilage in bones of the skull in the Orai1$^{-/-}$ mice. There was also increased cartilage and decreased bone in the vertebrae of the KO compared to controls, consistent with the μCT results, and the long bones of limbs were only partially mineralized in the Orai1$^{-/-}$ mice.

Bone Formation and Degradation in KO and WT Animals

Decreased bone and retained cartilage in the knock-out mice were confirmed in tissue sections (Fig 3A). Cartilage is normally present at the growth plates of the vertebral bodies (left and right sides of images, Fig 3A) but in the Orai1$^{-/-}$ mice cartilage persisted at surfaces of the vertebral body that, at three weeks, normally are replaced by bone. Within the vertebral bodies in the KO, trabecular bone was reduced, and trabeculae were thin. This apparent defect in bone formation was unexpected and complicated interpretation of resorptive activity. Evaluation of lamellar bone by polarized light showed a marked predominance of woven bone in the KO, in contrast to predominantly lamellar bone structure in WT (Fig 3B). Woven bone is characteristic of the initial bone formed, but normally it is promptly resorbed and replaced by lamellar bone. Remodeling to mature lamellar bone depends on osteoclastic resorption of the woven bone. Prominent woven bone is consistent with reduced bone resorption and impaired osteoclastogenesis. However, marrow space was present in the bones of the KO, in contrast to the osteopetrotic bone that results from complete absence of osteoclast function.

The predominance of woven bone in the Orai1 mice might be driven by reduced recruitment of osteoclasts, perhaps related to slow bone formation, or might indicate a primary osteoclast defect. Consistent with decreased bone resorption in the KO, TRAP activity, which was abundant at the bone surface in the WT mice, was markedly reduced in Orai1$^{-/-}$ mice (Fig 3C). Few TRAP-positive cells were present and those that were present were small (7-10 μm

in diameter) (Fig 3D, arrows). This suggested that some features of osteoclast differentiation, including TRAP expression, were preserved in the Orai1$^{-/-}$ mice, but fusion of pre-osteoclasts to form large multinucleated cells was markedly inhibited, consistent with results from our previous *in vitro* studies (5). The absence of adequate osteoclast function was also reflected in the presence of mineralized cartilage in the KO, since mineralized cartilage is normally rapidly degraded by osteoclasts and essentially absent from WT mice at 3 weeks (data not shown).

The lack of typical osteopetrosis, however, suggested some retention of osteoclastic function. Although efficient bone resorption requires multinucleated osteoclasts, it has been reported that less differentiated mononuclear cells can resorb bone (15,16). To test the hypothesis that the small TRAP-expressing cells formed without Orai1 can degrade mineral, osteoclast precursors were cultured in RANKL to induce differentiation, without or with the Orai1 channel blocker N-(3,4-Dichlorophenyl)-propanamide (DCPA). DCPA inhibits Orai1 activity by blocking its activation by Stim1 in response to depletion of stored calcium (5). For these studies, cells were grown on slides coated with mineralized matrix. As we previously reported, TRAP positive mononuclear cells but no multinucleated osteoclasts were found in DCPA treated cultures (5). Nevertheless, trails of small resorption areas occurred in the DCPA treated cultures, and Lysotracker staining (Invitrogen) confirmed production of acid by cells in the DCPA treated cultures (not shown). Overall the total area resorbed was greatly decreased compared to control cultures without Orai1 inhibition (Fig 3F-G). These studies support the hypothesis that the TRAP positive mononuclear cells seen in the KO have reduced but not absent capacity for degradation of mineralized matrix.

Effects of Orai1 on Osteoblasts and Bone Formation

The reduction in mineralized bone evident by μCT and apparent with alcian blue/alizarin red staining suggested that orai1 deficiency might also impair the formation or function of osteoblasts. Indeed it is possible that the relative prominence of woven bone in KO mice reflects impairment in bone formation as well as resorption. To evaluate the effects of Orai1 deficiency on bone formation *in vivo*, we used a single calcein injection to label mineral as it was deposited in the WT and KO mice. Dual calcein labeling was not practical due to the poor condition of the KO mice. WT mice showed strong calcein labeling on cortical and trabecular surfaces as expected, but labeling was markedly reduced in the Orai1$^{-/-}$ mice (Fig 4A). Where bone did form in the KO, calcein labeled the surfaces, as expected, but with thin labels consistent with a paucity of mineral deposition relative to WT bone. Decreased calcein labeling did not appear to reflect a specific defect in the mineralization of the bone: increased osteoid (non-mineralized bone) was not seen in the KO. To determine whether reduced bone formation might reflect decreased osteoblast numbers, osteoblasts were labeled in vertebral sections using antibody to alkaline phosphatase. The area labeled by alkaline phosphatase in the Orai1$^{-/-}$ mice was significantly decreased compared to results from controls (Fig 4B).

The effect of Orai1 deficiency on osteoblasts could be indirect, since osteoclasts and lymphocytes (17,18) regulate osteoblasts and both appear compromised in the Orai1 KO. Alternatively, the bone formation defect in the Orai1 KO might indicate a novel role for

Orai1 in the regulation of osteoblast differentiation. Orai1 expression has not previously been studied in osteoblastic cells. To investigate the possibility that Orai1 deficiency resulted in an intrinsic osteoblast developmental defect, we examined osteoblasts for Orai1 expression. To exclude the possibility that the deficiency was species specific, we tested human osteoprogenitor cells grown in medium with or without ascorbate, glycerol-2-phosphate and hydrocortisone (factors that promote osteoblastic maturation and bone deposition). The presence of Orai1 protein in human osteoblasts was confirmed by Western blotting (Fig 4C), which raised the possibility of an increase in Orai1 expression with osteoblastic maturation. Orai1 mRNA was detected by RT-PCR and measured relative to GAPDH message using quantitative PCR. We found Orai1 expression was present in cells from both culture conditions; again there appeared to be relatively greater expression in the osteogenic medium (Fig 4D).

To evaluate the potential functional significance of Orai1 in osteoblasts, we examined the effects of the Orai1 inhibitor DCPA on human osteoblast differentiation *in vitro* (Fig 5A-D). As expected, in osteogenic medium, alkaline phosphatase activity was markedly increased, but this increase was significantly blunted by DCPA treatment (Fig 5C), consistent with impairment of osteoblast maturation when Orai1 function is blocked. Mineralization was evaluated using the calcium chelating dye alizarin red. After three weeks culture in osteogenic medium without inhibitor, mineralization was readily apparent; however, when Orai1 inhibitor was added, alizarin red staining revealed a marked reduction in mineralization (Fig 5D).

Osteoblastic differentiation was further evaluated using quantitative real time PCR to compare expression of osteoblast markers. Results were evaluated by analysis of variance to identify statistically significant differences; p values for comparison of cultures in differentiation medium without or with DCPA are shown on the figure with statistical results for additional comparisons reported in the figure legend. RNA was collected from cultures at the start of the treatment period (Ctl) and after 14 and 21 days, in growth or osteogenic differentiation medium, each with or without 50 μM DCPA (Fig 6A-C). In growth medium, osteoblast expression of Runx2, alkaline phosphatase, and type I collagen was relatively low and showed little or no increase with time. In contrast, Runx2 expression was markedly increased in cells cultured in differentiation medium, particularly at the earlier time point (Fig 6A); an increase in Runx2 expression was still detected when DCPA was added to differentiation medium but was significantly decreased compared to cultures with differentiation medium plus vehicle control. Similarly, alkaline phosphatase expression was increased at 14 and 21 days in osteogenic medium without inhibitor, but was significantly lower in DCPA treated cells (Fig 6B). The increase in expression of type 1 collagen in differentiation medium, was also significantly inhibited by addition of DCPA (Fig 6C). These results suggest an important role for Orai1 activity in osteoblast differentiation, and support the hypothesis that decreased bone in Orai1 KO mice may reflect an intrinsic osteoblast defect. This raised the further possibility that the osteoclast defects in Orai1 deficiency might reflect impaired osteoblast regulation of osteoclastogenesis. However, expression of RANKL and osteoprotegerin in osteoblast cultures was not significantly altered by Orai1 inhibition (Fig 6D).

Discussion

Our results identify Orai-mediated store-operated calcium influx, as a critical regulatory signal for both bone formation and degradation. Defects in Orai1 function were previously shown to cause T-cell abnormalities with immunodeficiency (19), mast cell abnormalities (20) and altered platelet function (21). Decreased size was also consistently noted for the Orai1 deficient mice but the presence of underlying skeletal changes was not examined.

The Orai1$^{-/-}$ skeleton has several peculiar properties likely reflecting the combined effects of both osteoclast and osteoblast abnormalities. A defect in osteoclast maturation was expected (5) and, indeed, mature multinucleated osteoclasts were essentially absent from the KO. Impaired osteoclast function was also suggested by the predominance of woven bone in the KO (Fig 3C), since osteoclast function is necessary for replacement of woven bone by lamellar bone. Yet, there appeared to be some calcified cartilage removal in the absence of Orai1, and TRAP expression, though much decreased, was not absent from Orai1$^{-/-}$ bone. We speculated that the mononuclear TRAP positive cells in the Orai KO mice, which comprise almost all of the TRAP-expressing cells in Orai1$^{-/-}$ bone, are capable of removing mineralized cartilage, albeit inefficiently. We therefore tested whether the TRAP positive mononuclear cells resulting from RANKL stimulation with concurrent Orai1 inhibition (5) could resorb mineralized matrix. These studies revealed markedly reduced but not absent matrix degradation (Fig 3E-F). Mononuclear cells with limited capacity for bone resorption have been reported previously: for example, macrophages from arthroplasty sites, though mononuclear, show a limited capacity for bone resorption (22).

However, bone formation was also profoundly impaired overall in Orai1$^{-/-}$ mice (Fig 4) and this likely contributes to the lack of a dense skeleton in the KO despite impaired bone resorption. The skeleton of the KO animal is not only much smaller than controls but shows reduced or delayed ossification of bone. Dental enamel is also affected (Fig 1D), a finding consistent with amelogenesis imperfecta, which was reported for humans with Orai1 defects (11). These findings suggest that there might be a general defect in mineral depositing cells in the absence of Orai1.

The Orai1$^{-/-}$ bone phenotype resembles in some respects those resulting from FGFR3 defects. FGFR3 deficiency causes thanatophoric dysplasia with greatly reduced growth of long bones. However, cartilaginous defects, which were not identified in the Orai1$^{-/-}$ animal, are prominent with FGFR3 deficiency (23). To define fully the cellular defects underlying the skeletal changes in Orai1 deficiency, further detailed in vitro studies will be needed.

A major signaling pathway stimulating osteoclast differentiation proceeds from the TNF-family receptor RANK through Traf6 to NF-κB activation (24) but calcium signals also appear to be critical (5, 25-27). Recent work points toward primary regulation of ER calcium release by IP3R family channels in osteoclast precursors (10,24,28). A second phase of calcium signaling, also critical to differentiation (5,29), appears to be mediated by plasma cell membrane calcium channels: our results suggest that Orai1 is such a channel.

The working model for calcium signaling in developing osteoclasts involves activation of phospholipase C gamma by RANKL and costimulatory signals, with a resulting rise in IP3 stimulating release of stored calcium (2). When Ca^{2+} is reduced in the endoplasmic reticulum, STIM1 undergoes a conformational change that causes it to aggregate with Orai1 in complexes at the plasma membrane called puncta, where Orai1 calcium channel activity is induced (7,9,30). The mechanism is known in some detail: a segment of STIM1 within the ER includes a Ca^{2+} binding EF hand domain with low Ca^{2+} affinity (31). Additional regulation is less well understood but appears to involve intermediate signaling proteins such as CRACR2A (32). STIM1 mutations in humans have been related to immune defects (33), but, like defects in Orai1 (19), have consequences in other tissues that are less well described. Interestingly, Masuyama et al. (34) identified a TRPV4 Ca^{2+} channel in osteoclasts and postulated a role for it in the terminal stages of osteoclast differentiation. Its function probably does not replace that of Orai1, in the main (given the severe phenotype of the Orai$^{-/-}$ animal). But it might be responsible for the limited resorptive activity retained by the mononuclear TRAP positive cells in Orai1$^{-/-}$ animals (Fig 3D).

In our previous in vitro studies, the most prominent osteoclast abnormality observed with Orai1 inhibition was a failure of precursor fusion; other components of osteoclast phenotype (e.g. expression of TRAP) appeared, at least partially, preserved (5). Consistent with in vitro findings, multinucleated osteoclasts were essentially absent from Orai1 knock-out mice, though some TRAP positive mononuclear cells were detected. The mechanisms underlying impaired cell fusion due to Orai1 deficiency remain to be defined. In T-cells, major targets of Orai-mediated calcium are the members of the NFAT family of transcriptional regulators, including NFATc1, that are activated by the calcium dependent phosphatase, calcineurin (35). Osteoclastogenesis also involves NFATc1 activation through dephosphorylation by calcineurin (2, 36), and the expression of genes such as DC-STAMP, implicated in pre-osteoclast fusion (37), can be directly regulated by NFATc1 (38). These observations suggest that the impaired osteoclast precursor fusion resulting from Orai1 deficiency might simply reflect inhibition of calcium-dependent NFATc1 activation.

Links between Orai1, NFATc1 and osteoclast differentiation are also suggested by recent in vitro studies using a transformed murine cell line that can be induced by RANKL to form osteoclast-like cells (39). As in our studies of primary human osteoclast precursors, Orai1 inhibition in the cell line markedly reduced RANKL-stimulated cell fusion. Moreover, after Orai1 knock-down in the cell line, it was shown that NFATc1 activation, as detected by measurement of nuclear NFATc1 protein, was not significantly increased by RANKL treatment. However, the cell line studies showed preserved RANKL-induction of some NFATc1 target genes (e.g. DC-STAMP) despite Orai1 inhibition. The cell line also showed significant RANKL-induction of TRAP after Orai1 knock-down, though stimulation was less marked than in controls (39); this result is consistent with the presence of residual TRAP positive (albeit mononuclear) cells in the Orai1 KO animals (Fig 3).

It appears that the effects of Orai1 deficiency on osteoclastogenesis do not correlate exactly with the changes in the NFATc1 activation even for genes known to be targets of NFATc1. Thus, while NFATc1 activation is likely impaired by Orai1 deficiency in osteoclast precursors, as in T-cells, mechanisms other than NFATc1 inhibition may contribute to the

failure of Orai1 deficient pre-osteoclast fusion. Normal osteoclastogenesis also requires calcium activation of calmodulin dependent kinase (CamK) to stimulate phosphorylation of CREB (1); CamK may represent an additional target of Orai1 mediated calcium signals in the osteoclast precursor. Further in vitro studies of Orai1 negative cells will be needed to clarify mechanism(s).

Calcium is also an important osteoblast regulator, for example through activation of calmodulin binding proteins such as CamK II (6). NFAT proteins are also activated in osteoblasts by calcium-calcineurin dependent dephosphorylation. Interestingly, increased extracellular calcium itself activates calcium signaling pathways through the calcium sensing receptor. The calcium signaling pathways in osteoblasts appear to involve both release of calcium stores and influx of extracellular calcium, but the latter has been attributed to calcium entry through voltage-gated channels (6).

We know of no precedents for calcium-release activated calcium entry as a requirement for normal osteoblast differentiation, but the results of our in vitro studies suggest an intrinsic defect in osteoblast maturation when Orai1 calcium signaling is inhibited. It is also possible that osteoblastic effects of Orai1 deficiency are, at least in part, indirect. The defects in osteoclast formation may affect osteoblasts by interfering with osteoclast-derived regulatory signals. However, most mutations that inhibit osteoclast formation or function have not shown reduced bone formation (40). In addition, there are precedents for immune cells, chiefly lymphocytes, regulating skeletal activity, so the developmental delay in bone formation might in part reflect impaired immune regulation of mesenchymal stem cells or osteoblast maturation (41,42). As yet these pathways are poorly defined and this should be regarded as speculative, particularly since the severe combined immunodeficiency mouse has no changes fetal skeletal development, although mild osteopenia develops as the animals age (43,44). The immune hypothesis is nonetheless attractive since it would tie the known immune defects of the Orai1$^{-/-}$ animal to the observed reduced osteoblast activity. The concept of immune regulation of bone cell differentiation has a strong following and is proposed as a new discipline, osteoimmunology (17,18). If we are successful in producing Orai1$^{-/-}$ MSC, the point should be easy to settle, although the mild skeletal phenotype of mice with severe combined immunodeficiency implies that lymphocyte impairment cannot be the sole cause of defective bone formation in the Orai1$^{-/-}$ mouse.

In conclusion, in Orai1$^{-/-}$ animals, multinucleated osteoclasts were greatly decreased, although mononuclear cells expressing TRAP were present. Calcified cartilage was degraded but less rapidly, and bone turnover was absent or greatly reduced. There was also a profound reduction in synthesis of bone in Orai1$^{-/-}$ animals: cortical bone appeared thinned and trabeculae were reduced. The observed retention of fetal cartilage potentially reflects both impaired removal of mineralized cartilage and decreased or delayed bone formation. Overall, the results from the Orai1$^{-/-}$ mice indicate an essential role for the Orai1 calcium channel in differentiation and function of osteoclasts and osteoblasts in vivo.

Acknowledgments

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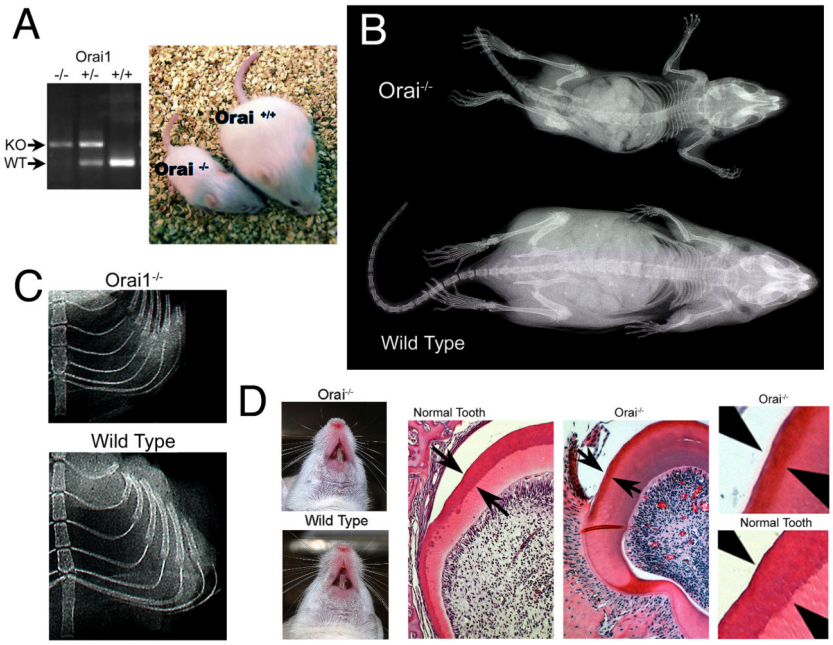

Figure 1. Skeletal phenotype and dentition of Orai1$^{-/-}$ mice

A. Genotyping and gross appearance The KO mice were smaller than WT littermates.

B. Radiographs of Orai1$^{-/-}$ and WT mice showed no gross defects. Bones in the KO were decreased proportionately relative to overall mouse size.

C. Radiographs of thoracic vertebrae and ribs. There is reduced bone thickness in the Orai1$^{-/-}$ animal.

D. Teeth in the Orai1$^{-/-}$ mouse were slightly smaller than in WT mice, but sections showed thinning and irregularity of the enamel in Orai1$^{-/-}$. Similar sections through the gingival sulcus are shown to minimize effect of plane of section. Left two frames, 450 μm, right frames 110 μm across.

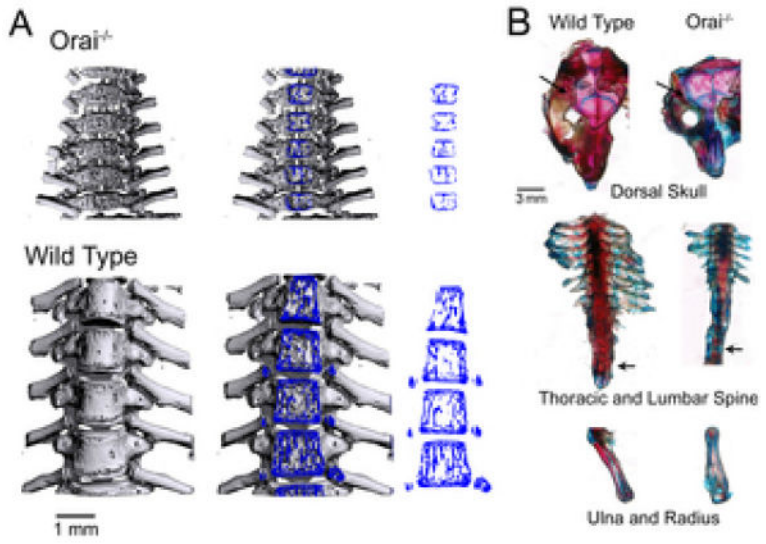

Figure 2. Analysis of Orai1$^{-/-}$ skeletons by μCT and alcian blue/alizarin red staining

A. Thoracic vertebrae from WT and Orai1$^{-/-}$ mice by μCT. Images (left and middle) are three-dimensional projections of the vertebrae (lowest is T12). Intact vertebrae (left), frontal sections with the virtual cut surfaces in blue (middle), and the cut surfaces separately (right). The vertebrae of Orai1$^{-/-}$ mice are smaller, show cortical discontinuities and reduced trabecular bone.

B. Skeletons stained with Alcian blue to identify cartilage and alizarin red to stain mineral. Comparison of skulls (upper frames) revealed retained cartilage in the KO (right) in areas mineralized in the WT (left). More cartilage remains in the skull around the nares in the Orai1$^{-/-}$, and cranial sutures are wider in the KO (arrows). The spines (middle frames) showed large regions of vertebrae remained cartilage in the KO (arrow) in contrast to WT, although there is regional variation. Replacement of cartilage by bone in the ulna and radius (lower frames) was incomplete in the KO. In controls, the shafts of the long bones were mineralized, with cartilage only at the joints.

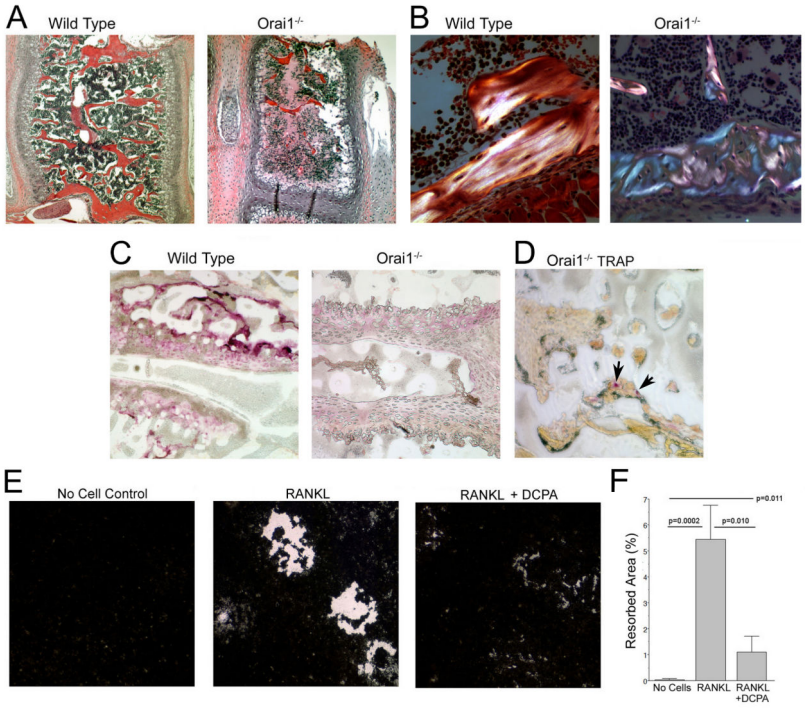

Figure 3. Bone, cartilage, and osteoclasts in sections of WT and KO bone, and osteoclast activity *in vitro* when Orai1 activity is suppressed

A. Increased cartilage and decreased bone in KO mice. Decalcified sections from KO (right) and WT (left) third lumbar vertebrae stained with hematoxylin and eosin. In the Orai1$^{-/-}$, the vertebral body retains cartilage on all sides, and has sparse, thin trabeculae. The control retains cartilage only at growth plates (gray, laterally) and has robust trabeculae. The growth plates are at the left and right sides of each frame. Images are 1.8 mm across.

B. Retention of woven bone in Orai1$^{-/-}$ mice. Collagen bundles are visualized by birefringence in polarized light. The cortex of a WT mouse vertebra (left) is mainly mature lamellar bone (parallel layers of collagen, bright). The KO retains woven bone (criss-cross pattern). Normally, the first bone synthesized when cartilage is replaced is woven bone, but woven bone is resorbed and replaced by lamellar bone. Images are 300 μm across.

C. Decreased tartrate resistant acid phosphatase (TRAP, magenta) in cartilage growth plates of Orai1$^{-/-}$ mice. Histochemical staining was performed on frozen sections of vertebrae from WT (left) and KO (right). The sections are centered on inter-vertebral discs and show the cartilaginous growth plates, which normally have high osteoclastic activity. Prominent TRAP is seen in WT but much less in the KO. Images are 750 μm across.

D. TRAP expressing cells in Orai1$^{-/-}$ mice. Higher power showed that the TRAP positive cells (arrows) in Orai1$^{-/-}$ are single small cells, generally not multinucleated. Image width is 750 μm.

E. Reduced mineral resorption by osteoclasts differentiated *in vitro* when Orai1 is inhibited. Human osteoclast precursors were treated with RANKL to induce osteoclast differentiation, in the presence (right) or absence (middle) of the Orai1 inhibitor DCPA, on slides coated with calcified matrix. Substrate resorption was evaluated by silver nitrate staining. Areas where substrate was resorbed are clear. Resorption was inhibited by addition of 50 μM

DCPA (right), compared to control (center). A no-cell control (left) has no resorption tracks. Note also that the resorption tracks in the Orai1 inhibited cultures are thin and threadlike, consistent with resorption by small, mainly mononuclear, cells. This reflects the paucity of multinucleated osteoclasts when Orai1 is inhibited.

F. The area of degraded mineral substrate. Cleared matrix area in cultures without cells (left), RANKL treated control cells (center), and cells treated with RANKL and the Orai1 inhibitor (right), are shown. DCPA resulted in a significant decrease ($p=0.01$), in resorption. Activity in DCPA was clearly greater than the no cell control ($p<0.01$). In each case, resorption activity was measured in three cell cultures for each condition, and a second experiment showed equivalent results (not shown).

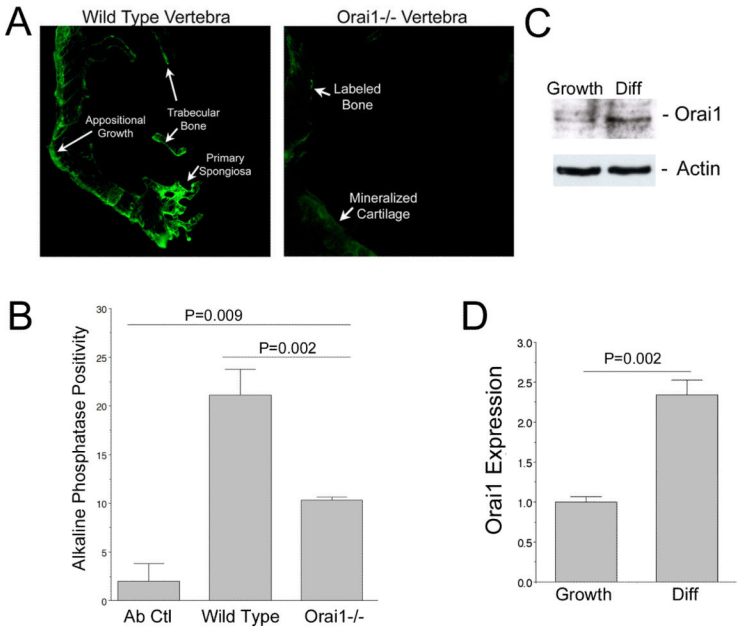

Figure 4. Osteoblast activity in Orai1$^{-/-}$ mice

A. Reduced mineralization in Orai1$^{-/-}$ mice. Fluorescent labeling of newly formed mineral in sections from WT (left) and KO (right) mice 2 days after calcein injection. Sections are of vertebrae with growth plate at the bottom; each image is 250 μm across. In the WT mice (left) there was calcein labeling along the bone surface (appositional growth), on trabeculae, and in the primary spongiosa, where bone replacing cartilage is remodeled. Labeled bone was markedly decreased in the Orai1$^{-/-}$ mice (right). Calcifying cartilage also labels with calcein, though these labels are diffuse. Some calcein labeling of mineralized cartilage is seen in the KO. Images are 250 μm across.

B. Alkaline phosphatase in vertebral bone of WT and Orai1 deficient mice. Decreased bone formation suggested that osteoblast numbers might be decreased. This was evaluated using an antibody for alkaline phosphatase that is highly expressed by active osteoblasts in bone. The total area labeling for alkaline phosphatase, measured in three sections of vertebrae, was decreased in Orai1$^{-/-}$ compared to the labeling measured in three sections from WT mice ($p=0.002$).

C. Osteoblast expression of Orai1 protein. Lysates from osteoblasts in basal (growth) or mineralizing (diff) medium were evaluated by Western blotting with anti-Orai1, and blots re-probed with anti-Actin to demonstrate equivalent sample loading. The results confirm expression of Orai1 by osteoblasts. Orai1 protein appeared increased in mineralizing osteoblasts in keeping with PCR results.

D. Osteoblast expression of Orai1 mRNA. Human osteoblasts in basal medium (Growth) or in differentiation medium containing ascorbate, glycerol-2-phosphate and hydrocortisone (Diff) were evaluated for Orai1 mRNA by quantitative PCR, relative to GAPDH, at 2 weeks. Orai1 message was present in the osteoblasts, with increased expression found in mineralizing cells ($n=5$, $p=0.002$). Results at three weeks and in a repeat experiment were similar (not shown).

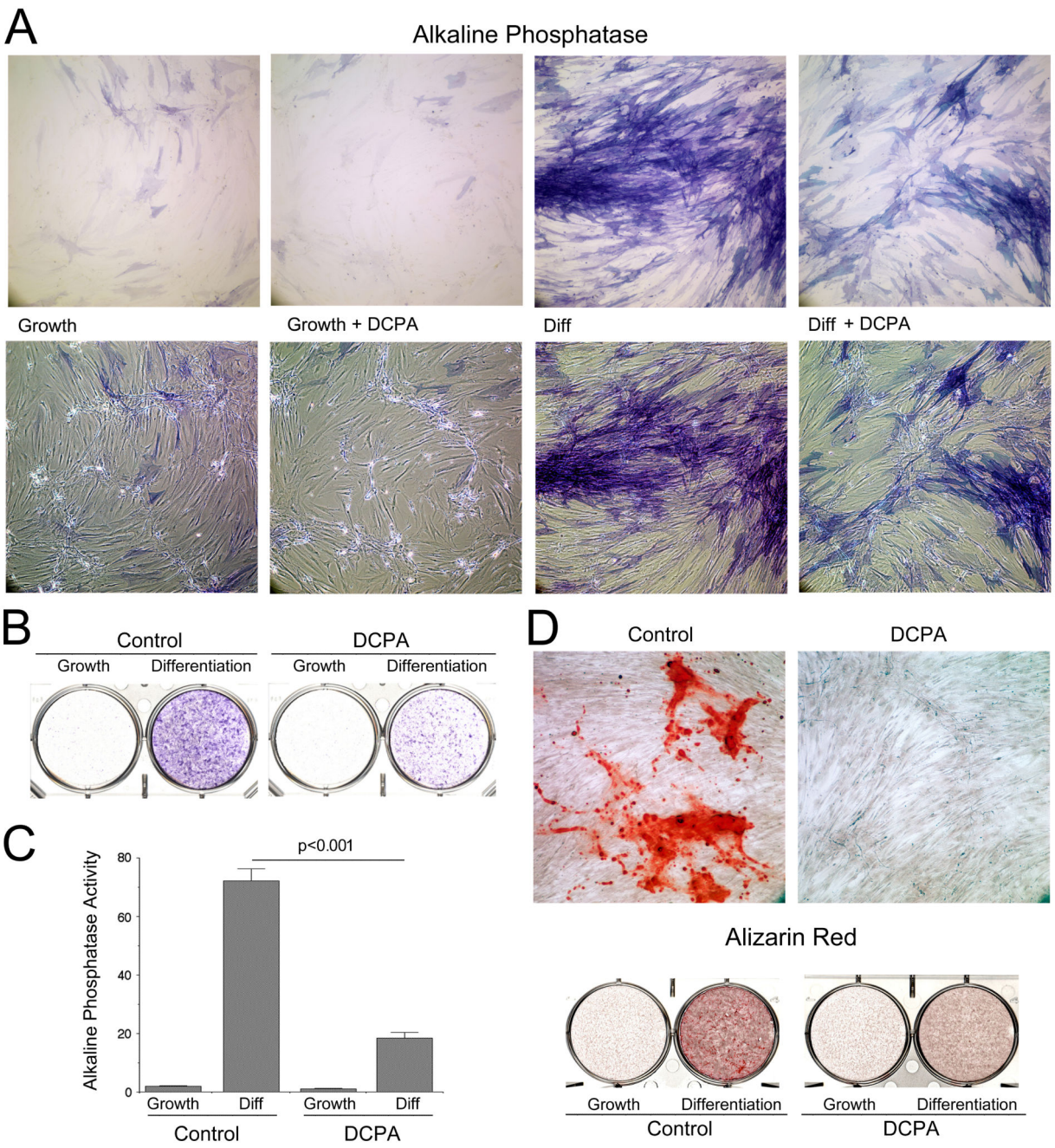

Figure 5. Effect of Orai1 inhibition on osteoblast differentiation and activity

A. Effect of the Orai1 inhibitor DCPA on alkaline phosphatase activity. Human osteoblasts were grown in basal medium (growth) or in medium with ascorbate, glycerol-2-phosphate and hydrocortisone (diff) to promote mineralization, with or without DCPA (or equivalent vehicle). Alkaline phosphatase activity (blue staining) was evaluated by light microscopy (upper row); a phase image of the same field is shown below. Little alkaline phosphatase activity was present in cells in growth medium. Alkaline phosphatase was substantially increased in cultures treated with ascorbate, 2-glycerol phosphate and hydrocortisone (diff)

but this effect was blunted by DCPA. The phase images, showing cell monolayers with and without DCPA, indicate that the reduction in alkaline phosphatase did not simply reflect a difference in cell numbers in the presence of the inhibitor. Results were similar in repeat experiments (not shown).

B. Alkaline phosphatase in cultures of human osteoblasts. In situ enzyme activity is shown for cultures (left to right) in growth medium, growth medium plus DCPA, differentiation medium, or differentiation medium plus DCPA. In differentiation medium, addition of DCPA reduced alkaline phosphatase activity. Wells are 3.5 cm across.

C. Alkaline phosphatase activity. For each condition in (A), alkaline phosphatase is measured as signal at 450-490 nm in assays of replicate cultures. DCPA significantly reduced alkaline phosphatase in mineralizing osteoblasts ($n=4$, $p<0.001$).

D. Mineral deposition by human osteoblasts is blunted by DCPA. Cells were cultured 3 weeks in basal medium (growth), or in mineralizing conditions (differentiation), each without or with DCPA. Alizarin red was used to stain calcium (bright red); representative cultures for each condition are shown (lower panel). As expected, little mineral was produced by osteoblasts in growth medium, with or without DCPA, but the photomicrographs show strong matrix labeling by alizarin red in cultures grown in differentiation medium without DCPA (control, upper left). However, when DCPA was added, alizarin red staining was mainly nonspecific (light red, upper right), without the focal strongly mineralized nodules that occur in differentiation medium alone. Wells are 3.5 cm in diameter; micrographs are 220 μm across.

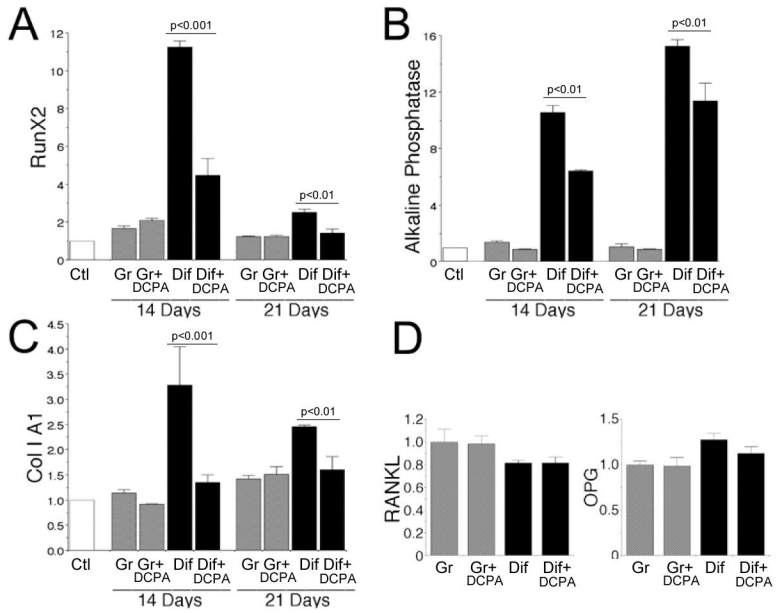

Figure 6. Effect of Orai1 inhibition osteoblast differentiation

Each assay shows expression relative to GAPDH mRNA in the same sample, mean ±SD, n=3. Differences were examined by analysis of variance. Repeat experiments gave similar results (not shown).

A. Expression of a transcriptional regulator of osteoblast differentiation, RUNX2. Human osteoblasts in growth medium (Gr), or in medium supplemented with ascorbate, glycerol-2-phosphate and hydrocortisone (Dif) are compared, without or with the Orai1 inhibitor DCPA (50 μM). Cells in mineralizing medium for 2 weeks showed significant up-regulation of RUNX2 compared to cultures in growth medium ($p<0.001$ without DCPA, $p<0.01$ with DCPA). The increase in RUNX2 with mineralizing medium was reduced by DCPA ($p<0.001$). While RUNX2 expression appeared lower in mineralizing medium at 21 days compared to 14 days, osteoblast RUNX2 expression was still significantly elevated ($p<0.01$) in the absence of DCPA. RUNX2 expression by osteoblasts in mineralizing medium plus DCPA for 21 days was not significantly different from pre-treatment or growth medium control cells.

B. Alkaline phosphatase mRNA. Alkaline phosphatase was significantly increased after 14 ($p<0.001$) or 21 days ($p<0.001$) in mineralizing medium compared to growth controls. Alkaline phosphatase expression by osteoblasts in mineralizing medium plus DCPA was reduced relative to mineralizing medium alone ($p<0.01$).

C. Expression of type 1 collagen (Col1A1). Expression was increased for osteoblasts in mineralizing medium ($p<0.001$) at 14 days. This increase was strongly attenuated by DCPA ($p<0.001$), though DCPA-treated cells in mineralizing medium expressed 30% more Col1A1 than cells in growth medium with DCPA ($p<0.05$). At 21 days, a reduced but still significant increase in Col1A1 expression was apparent in mineralizing medium without DCPA ($p<0.01$) but collagen expression in cultures with DCPA was no longer significantly elevated compared to cells in growth medium.

D. Expression of osteoclast regulatory proteins. RANKL and osteoprotegerin mRNA was assayed in human osteoblasts in growth (Gr) or mineralizing medium (Dif), with or without DCPA, for 14 days. Unlike markers of osteoblastic differentiation, expression of RANKL and osteoprotegerin did not vary significantly with DCPA.