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Orai1 mediates osteogenic differentiation *via* BMP signaling pathway in bone marrow mesenchymal stem cells

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Abstract

Orai1 is a pore-subunit of store-operated Ca^{2+} release-activated Ca^{2+} (CRAC) channel that mediates Ca^{2+} influx in most non-excitable cells *via* store-operated Ca^{2+} entry (SOCE) mechanism. We previously demonstrated that Orai1 is involved in mediating osteogenic potential of mesenchymal stem cells (MSCs), but the underlying mechanism of this function remains unknown. Here, we report that Orai1 mediates osteogenic differentiation *via* bone morphogenic protein (BMP) signaling pathway in bone marrow MSCs (BMSCs). In osteogenic conditions, BMSCs derived from wild-type mice underwent osteoblastic differentiation and induced mineralization as demonstrated by increased alkaline phosphatase activity and alizarin red S staining, respectively. The expression of Runx2, a master regulator of osteoblast differentiation, and osteogenic differentiation markers were markedly increased in wild-type BMSCs under osteogenic conditions. In contrast, osteogenic conditions failed to induce such effects in BMSCs derived from Orai1-deficient (*Orai1*$^{-/-}$) mice, indicating that Orai1 is, in part, necessary for osteogenic differentiation of MSCs. We also found that BMP2 successfully induced phosphorylation of Smad1/5/8, the immediate effector molecules of BMP signaling, in wild-type BMSCs, but failed to do so in *Orai1*$^{-/-}$ BMSCs. Downstream target genes of BMP signaling pathway were consistently increased by osteogenic conditions in wild-type BMSCs, but not in *Orai1*$^{-/-}$ BMSCs, suggesting a novel molecular link between Orai1 and BMP signaling pathway in the osteogenic differentiation process. Further functional studies demonstrated that activation of BMP signaling rescues osteogenic differentiation capacity of *Orai1*$^{-/-}$ BMSCs. In conclusion,

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Orai1 regulates osteogenic differentiation through BMP signaling, and the Orai1-BMP signaling may be a possible therapeutic target for treating bone-related diseases.

Keywords

Orai1; BMP2; bone marrow stem cells; osteogenic differentiation

1. Introduction

Calcium ion (Ca^{2+}) is an indispensable element in living organisms that plays important roles in maintaining and regulating in normal biological processes [1, 2]. Although there are numbers of voltage-dependent calcium channels that regulates Ca^{2+} influx in excitable cells [3], it was shown that store-operated CRAC channels mediates Ca^{2+} influx in most non-excitable cells via SOCE [4].

Orai1 is an essential pore-subunit of CRAC channel that are extensively studied in immune cells such as T-cells [5]. When T-cells become stimulated, Ca^{2+} becomes released from the endoplasmic reticulum (ER) and quickly gets depleted. Ca^{2+} depletion from ER induces translocation of ER to the plasma membrane via physical interaction between ER-residing Stim1 and Orai1, leading to extracellular Ca^{2+} influx through Orai1. Intracellular Ca^{2+} plays an important role in mediating the downstream signaling pathways including the nuclear factor of activated T cells (NFAT) signaling pathway, which is required for T-cell activation [6].

Although Orai1 was originally identified from immune cells, increasing lines of evidence support a notion that Orai1 also plays an important role in other types of cells. Indeed, recent studies showed the importance of Orai1 in bone biology. In particular, Orai1 knockout mice showed osteoporotic phenotypes, and inhibition of Orai1 functions lead to impaired osteoclast and osteoblast differentiation [7, 8]. Similarly, we recently showed that Orai1 also mediates osteo/odontogenic differentiation and mineralization in dental pulp stem cells (DPSCs), indicating that Orai1 may play a critical role in mediating cell differentiation that leads to formation of mineralized matrixes.

Osteoblasts are bone-forming cells that are derived from mesenchymal precursors such as bone marrow mesenchymal stem cells (BMSCs). Differentiation of BMSCs into bone-forming osteoblasts requires orchestrated regulation of different signaling pathways such as BMP2 and Wnt [9]. BMP signaling elicits Smad1/5/8-dependent signaling transduction via BMP receptor type I and II (BMPRI and BMPRII, respectively), which ultimately lead to activation of gene expression such as *Runx2, Dlx5*, and *Osx* [10]. The activation of Wnt signaling pathway also lead to increased bone formation via β-catenin dependent (canonical) or β-catenin independent (non-canonical) signal transduction [11]. Although there are numbers of different signaling pathways during osteogenic differentiation, the involvement of Orai1 in this process in related to these signaling pathways remains to be elucidated.

Here, we used BMSCs isolated from *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ mice and demonstrated that Orai1 is indispensable in osteogenic differentiation. We further showed that Orai1 plays an

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important role in osteogenic differentiation by mediating the BMP signaling pathway, and reconstituting BMP signaling by overexpressing constitutively active BMPR1B rescued inhibited osteogenic differentiation in *Orai1*$^{-/-}$ BMSCs.

2. Materials and methods

2.1. Reagents and antibodies

The antibodies used for the western blot analysis were anti-p-Smad1/5/8 (#9511P, Cell Signaling), anti-Smad1 (#6944P, Cell Signaling), anti- β-catenin (#D59D7, Origene), and anti- Gapdh (sc-47724, Santa Cruz). Recombinant proteins, BMP2 (#355BM-010) and Wnt3a (#1234-WN), were all purchased from R&D Systems.

2.2. Cell cultures and animal study

Primary bone marrow stem cells (BMSCs) were isolated from the femurs obtained from 6-weeks-old mice. Briefly, inside of the mouse femur was flushed and cultured in the culture dish with α-MEM (Life Technologies, Carlsbad, CA), 20% FBS (Life Technologies), glutamine (200 mM; Life Technologies), and 2-mercaptoethanol (55 mM; Sigma-Aldrich, St. Louis, MO, USA). After culture for 16 hour, non-adherent cells were discarded, and the adherent cells were cultured. BMSCs that are under 5 passages were used. All experiments using mice in this study were performed according to the approved institutional guidelines from the Chancellor's Animal Research Committee (ARC # 2011-062).

2.3. Single-cell intracellular free Ca^{2+} imaging

Cells were plated on UV-sterilized coverslips one day prior to imaging. Next day, cells were loaded with 1 mM Fura 2-AM for 45 min at 25°C, and intracellular $[Ca^{2+}]_i$ measurements were performed. Briefly, cells were mounted in a RC-20 closed bath flow chamber (Warner Instrument Corp., Hamden, CT) and analyzed on an Olympus IX51epifluorescence microscope with Slidebook (Intelligent Imaging Innovations, Inc.) imaging software. Cells were perfused with Ca^{2+}-free Ringer's solution, and Ca^{2+} stores were passively depleted with 1 μM thapsigargin. Fura-2 emission was detected at 510 nm with excitation at 340 and 380 nm, and the Fura-2 emission ratio (340/380) was acquired at every 5-s interval after subtraction of background. For each experiment, 50–100 individual cells were analyzed using OriginPro (Originlab) analysis software. Acquisition and image analysis including measurement of Pearson's correlation co-efficient was performed using Slidebook (Intelligent Imaging Innovations, Inc.) software and graphs were plotted using OriginPro8.5 (Originlab).

2.4. Retrovirus production and cell transduction

BMSCs were transduced with retroviruses capable of overexpressing the constitutively active BMPR1B. The pLPCX empty vector was purchased from Clontech (Mountain View, CA, USA), and pLPCX-BMPR1B(Q203D) was purchased from the Addgene (#12642, Cambridge, MA, USA) [12]. These vectors were used to prepare retroviruses as described previously [13]. These vectors were transfected into GP2-293 universal packaging cells (Clonetech) along with pVSV-G envelope plasmid using lipofectamine 2000 (Life Technologies). Two days after transfection, the virus supernatant was collected and

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concentrated by ultracentrifugation. The virus pellet was suspended in a serum-free α-MEM and was used for immediate infection or stored in −80°C for the later use. BMSCs at the passage 2 or 3 were infected with these retroviruses in the presence of 6 μg/ml polybrene for three hours. All of these viruses consistently gave more than 90% of infection efficiency [13]. Drug selection of cells began at 48 hours after infection with 1 μg/ml puromycin. The drug resistant cells were maintained in subcultures as described above.

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated and cDNA were made as described previously [14]. qRT-PCR was performed in triplicates for each sample with LC480 SYBR Green I master (Roche, Indianapolis, IN, USA) using universal cycling conditions on LightCycler 480 (Roche, Indianapolis, IN, USA). A total of 55 cycles were executed, and the second derivative Cq value determination method was used to compare fold-differences. The primer sequences are available upon request.

2.6. Western blotting

Cells were washed twice with PBS before treatment with ice-cold lysis buffer [20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100]. The cells were then scraped and incubated on ice for 10 min. Cell debris was separated by centrifugation at 20,000 *g* at 4°C for 20 min and the supernatant was collected for Western blot analysis after 8 or 10 % SDS-PAGE. After electrophoresis, proteins were transferred to immobilized membrane (Millipore, Chicago, IL), which was subsequently blocked with 5% non-fat milk for 1 h at room temperature. Then, membrane was incubated with primary antibodies, and probed with the respective secondary antibodies conjugated with HRP. The signals were obtained using ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA).

2.7. Alkaline phosphatase (ALP) staining/activity and Alizarin Red S (ARS) staining

ALP staining/activity was performed using ALP staining kit (86R-1KT, Sigma-Aldrich, Inc.) according to the manufacturer's protocol. ALP activity was measured using pNNP substrate and alkaline buffer solution (Sigma-Aldrich, Inc.). For ARS staining, cells were fixed with 1% formalin/PBS for 10 min and stained with 2% ARS solution (pH 4.1 to 4.3 with 10% ammonium hydroxide) for 30 min at the room temperature. ARS solution was removed, and cells were washed with ddH_2O. The plates were photographed using both microscope and camera. ARS staining was further quantified by destaining in 10% acetylpryidinium chloride (Sigma-Aldrich, Inc.) and measuring at 562 nm using the microplate reader.

2.8. Cryostat sectioning and ALP staining

The freshly isolated femur was snap-frozen in hexane using liquid nitrogen and 2-methyl butane and was embedded in a 5% carboxymethyl cellulose (CMC) gel. Five μm thick sections were prepared using Kawamoto's film method (Cryofilm transfer kit; Finetec, Tokyo). The sections were fixed in ice-cold 5% acetic acid in ethanol and subjected to ALP staining using ALP staining kit (86R-1KT) according to the manufacturer's protocol.

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2.9. Statistical analysis

The outcome measurements from experiments were displayed as the mean ± standard deviation. To compare the means of outcome measurements, a student t test was conducted using SPSS 23 software (IBM Corp, Somers, NY). p values < 0.05 was considered significant.

3. Results

3.1. Orai1 is required in osteogenic differentiation and mineralization of BMSCs

Previously, we showed that Orai1 inhibits odontogenic differentiation of DPSCs [15]. To examine whether Orai1 also has the similar effects on BMSCs, we isolated BMSCs from wild-type (*Orai1*$^{+/+}$) and Orai1-deficient (*Orai1*$^{-/-}$) mice and examined their osteogenic potentials. We first confirmed that SOCE is impaired in *Orai1*$^{-/-}$ BMSCs, indicating that Orai1 deficiency functionally inhibited Ca^{2+} influx (Fig. 1A). When *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ BMSCs were induced to undergo osteogenic differentiation, they exhibited significant suppression of ALP and ARS staining as well as ALP activity (Fig. 1B–D). Also, femurs from *Orai1*$^{-/-}$ mice exhibited a diminished ALP staining pattern when compared to the femurs from *Orai1*$^{+/+}$ mice (Fig. 1E). Furthermore, while osteogenic markers such as *Alp, Bsp, Dspp*, and *Dmp1* were all significantly induced in *Orai1*$^{+/+}$ BMSCs, these genes were not induced in *Orai1*$^{-/-}$ BMSCs (Fig. 1F–I), suggesting Orai1 is required for osteogenic differentiation and mineralization.

3.2. Expression of Orai and Stim genes during Orai1$^{+/+}$ and Orai1$^{-/-}$ BMSC differentiation

To further examine the involvement of other genes associated with SOCE, we screened for the expression patterns of Orai and Stim family members. Similar to our previous study, the expression of *Orai1, Orai2, Orai3, Stim1* and *Stim2* was significantly induced as early as day 7 and then decreased afterward during osteogenic differentiation in *Orai1*$^{+/+}$ BMSCs (Fig 2, A–E). As expected, *Orai1*$^{-/-}$ BMSCs exhibited no expression of *Orai1* (Fig. 2A). On the other hands, expression of Orai2, Orai3, Stim1, and Stim2 was not induced initially but significantly induced toward the late stages of osteogenic differentiation (Fig. 2, A–E).

3.3. Involvement of Orai1 in the BMP2 and Wnt signaling pathways

Osteogenic differentiation is mediated by different signaling pathways including BMP2 and Wnt [9]. To delineate whether Orai1 is involved in these signaling pathways, we first screened for expression of the downstream target genes in each signaling pathway. During osteogenic differentiation, all tested downstream targets of BMP2, *i.e., Runx2, Dlx5*, and *Osx*, were consistently induced in *Orai1*$^{+/+}$ BMSCs, but not in *Orai1*$^{-/-}$ BMSCs (Fig. 3 A). Among the Wnt target genes, only *Axin2* were increased in *Orai1*$^{+/+}$ BMSCs (Fig. 3B), suggesting that Orai1 is associated with the BMP2 signaling pathway. To further delineate immediate effects of these signaling pathways, we treated *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ BMSCs with recombinant BMP2 and found an increased phosphorylation of Smad 1/5/8 in *Orai1*$^{+/+}$ but not in *Orai1*$^{-/-}$ BMSCs (Fig. 3C). We failed to observe activation of β-catenin by Wnt3a (Fig. 3D). Our findings indicate that Orai1 is involved in the BMP2 signaling pathway.

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3.4. Constitutively activated BMP receptor rescued inhibition of osteogenic differentiation in Orai1$^{-/-}$ BMSCs

Because the BMP2 signaling pathway was suppressed in *Orai1*$^{-/-}$ BMSCs, we examined whether reconstituting the BMP2 signaling pathway in *Orai1*$^{-/-}$ BMSCs can rescue osteogenic potential. To do so, we used BMRP1B(Q203D), a constitutively activated BMP receptor 1B with phosphor-mimic mutation at amino acid residue 203 [12]. When we overexpressed BMRP1B(Q203D) in *Orai1*$^{-/-}$ BMSCs and allowed them to undergo osteogenic differentiation, we found that osteogenic differentiation was significantly rescued in *Orai1*$^{-/-}$ BMSCs as determined by ALP staining (Fig. 4A), ALP activity (Fig. 4B), and ARS staining (Fig. 4C). In line with this finding, expression of the BMP2 downstream target genes, *Runx2, Dlx5*, and *Osx*, were all rescued in BMRP1B(Q203D) expressing *Orai1*$^{-/-}$ BMSCs (Fig. 4 D–F). Collectively, our data indicate that the BMP signaling pathway is required for the Orai1-mediated osteogenic differentiation.

4. Discussion

Previous findings including our study suggested that Orai1 is involved in osteogenic and odontogenic differentiation to form mineralized tissues [7, 8, 15]. Nonetheless, the molecular link between Orai1 and osteogenic differentiation has not been reported. Here, we provide compelling evidence that Orai1 plays a critical role in osteogenic differentiation by mediating the BMP signaling pathway. To the best of our knowledge, our study is the first report showing the molecular link between Orai1 and BMP/Smad signaling pathway in osteogenic differentiation.

Ca^{2+} plays a significant role in bone biology; highly mineralized bone matrix is formed by osteoblasts that mediate deposition of calcium and phosphate onto the Type I collagen matrixes [16]. High extracellular Ca^{2+} has been shown to enhance osteogenesis of human MSCs *in vitro* [17–19], and osteoblasts are known to propagate Ca^{2+} signals [20]. In line with these findings, vitamin D, the major regulator of calcium homeostasis and bone mineralization, is known to stimulate osteogenic differentiation *in vitro* [21, 22]. Similarly, estrogen also induces osteogenic differentiation [23]. Vitamin D and estrogen are known to induce a rapid Ca^{2+} influx in cells [24– 26]. As such, it is tempting to speculate that Orai1 may play a critical role in these processes by allowing Ca^{2+} influx into the cells in which Ca^{2+} participates in signal transduction to enhance bone formation.

Our study showed that reconstituting the BMP signaling pathway using constitutively active BMPR1B rescued suppression of osteogenic differentiation in *Orai1*$^{-/-}$ BMSCs (Fig. 4). Such observation implies that Orai1 may play in the upstream of Smad1/5/8 complex. The binding of BMP2 onto the BMP receptor complex dimerizes the type I and type II BMP receptors, leading to phosphorylation and activation of the type I BMP receptor which, in turns, phosphorylates Smad1/5/8 complex [27]. Therefore, it would be interesting to examine whether Ca^{2+} influx through Orai1 are required to make the BMP receptor complex fully functional to phosphorylates Smad complexes upon BMP2 binding.

Previous studies showed that extracellular Ca^{2+} induced gene expression of BMP2 [28, 29], and the BMP2 induction was associated with the ERK signaling pathway [30]. BMP2 can

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induce bone formation by autocrine fashion [31]. Therefore, our study, along with previous literature, suggests that BMP2 and Ca^{2+} signaling may form a positive loop in exacerbating bone-forming effects.

It is interesting to note that, although ALP activity was significantly suppressed in *Orai1*$^{-/-}$ BMSCs *in vitro* when compared to the *Orai1*$^{+/+}$ BMSCs, mineralizing capacity of these cells were not suppressed as significantly as seen in ALP activity (Fig. 1B–D). Similarly, ALP staining was significantly suppressed in femurs obtained from *Orai1*$^{-/-}$ mice (Fig. 1E), but these mice do develop bone, albeit to lesser degree [7, 8]. These data suggest that there may exist alternative mechanisms that compensate the role of Orai1. A closer examination reveals that there is significant induction of *Orai2, Orai3, Stim1*, and *Stim2* expression toward the late stages of osteogenic differentiation in *Orai1*$^{-/-}$ BMSCs (Fig. 2). We previously observed that when *Orai3* expression was knocked down, there was an increased expression of Orai1 [32]. As such, it is possible that, toward the late stages of osteogenic differentiation, there may exist overlapping functions of Orai1 with Orai2 and Orai3 such that Orai2 and Orai3 may have compensatory roles in osteogenic differentiation, especially during the mineralization process (Fig. 1B and D).

In conclusion, we showed that Orai1 plays a critical role in BMP2-mediated osteogenic differentiation in BMSCs. Further studies on mechanistic aspects of Orai1 involvement in osteogenic differentiation in the BMP2/Smad pathway may provide potential therapeutic targets to improve bone-related diseases such as osteoporosis.

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Research highlights

- Orai1 is required in osteogenic differentiation and mineralization of BMSCs.
- Orai1 regulates osteogenic differentiation through BMP signaling.
- BMP signaling rescues osteogenic differentiation capacity of *Orai1*$^{-/-}$ BMSCs.

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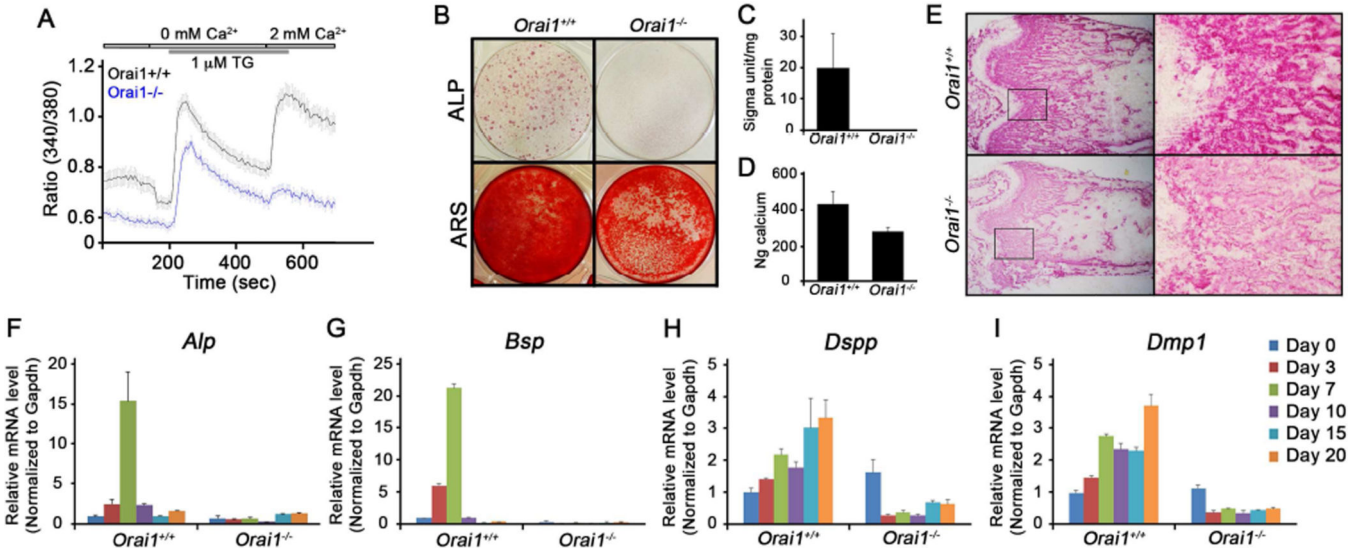

Figure 1. Orai1 is required in osteogenic differentiation and mineralization of BMSCs

(A) Intracellular Ca^{2+} imaging assay was performed to confirm ORAI1 function. (B) ALP and ARS staining of BMSCs isolated from *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ mice. (C) Quantification of ALP activity. (D) Quantification of ARS staining. (E) ALP staining of femur from *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ mice. (F–I) qRT-PCR for *Alp, Bsp, Dspp*, and *Dmp1* during osteogenic differentiation in *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ BMSCs.

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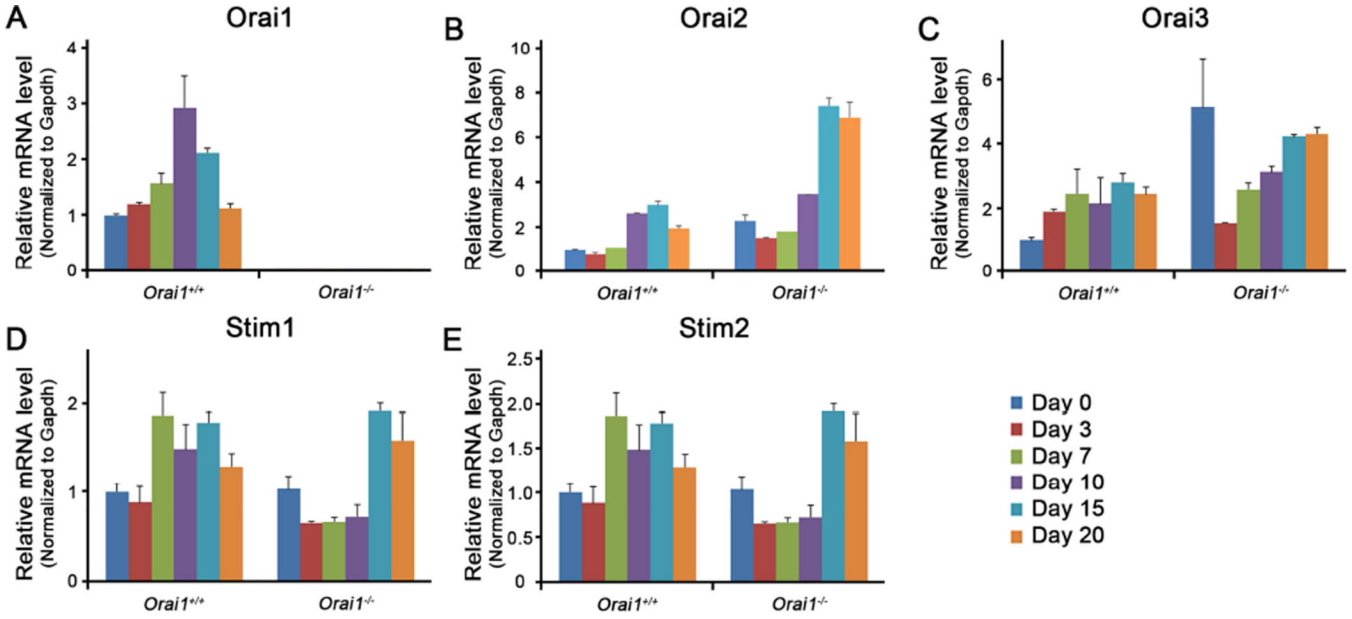

Figure 2. Expression of Orai and Stim genes during osteogenic differentiation in *Orai1*$^{+/+}$ or *Orai1*$^{-/-}$ BMSCs

(A–E) qRT-PCR for *Orai1, Orai2, Orai3*, *Stim1*, and *Stim2* during osteogenic differentiation in *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ BMSCs.

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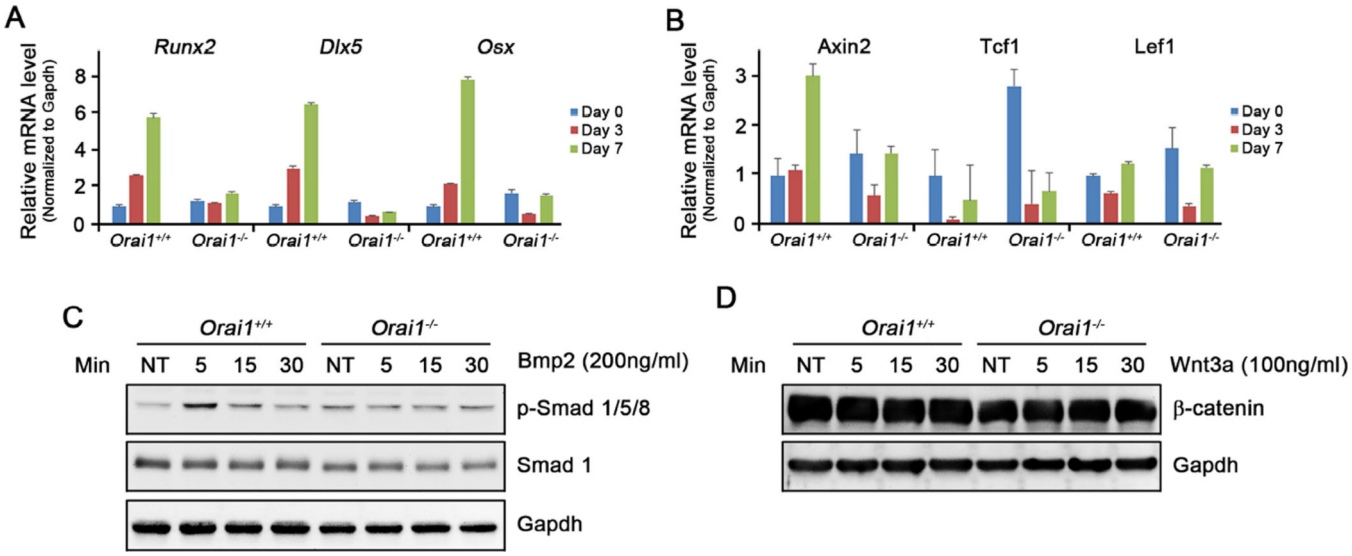

Figure 3. Involvement of Orai1 in the BMP2 or Wnt signaling pathway

(A) qRT-PCR for BMP2 downstream targets (*Runx2, Dlx5*, and *Osx*) during osteogenic differentiation in *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ BMSCs. (B) qRT-PCR for Wnt target genes (*Axin2, Tcf1*, and *Lef1*) during osteogenic differentiation in *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ BMSCs. (C) Western blotting for p-Smad 1/5/8 and Smad1 in *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ BMSCs following BMP2 treatment. (D) Western blotting for β-catenin in *Orai1*$^{+/+}$ and *Orai1*$^{-/-}$ BMSCs following Wnt3a treatment.

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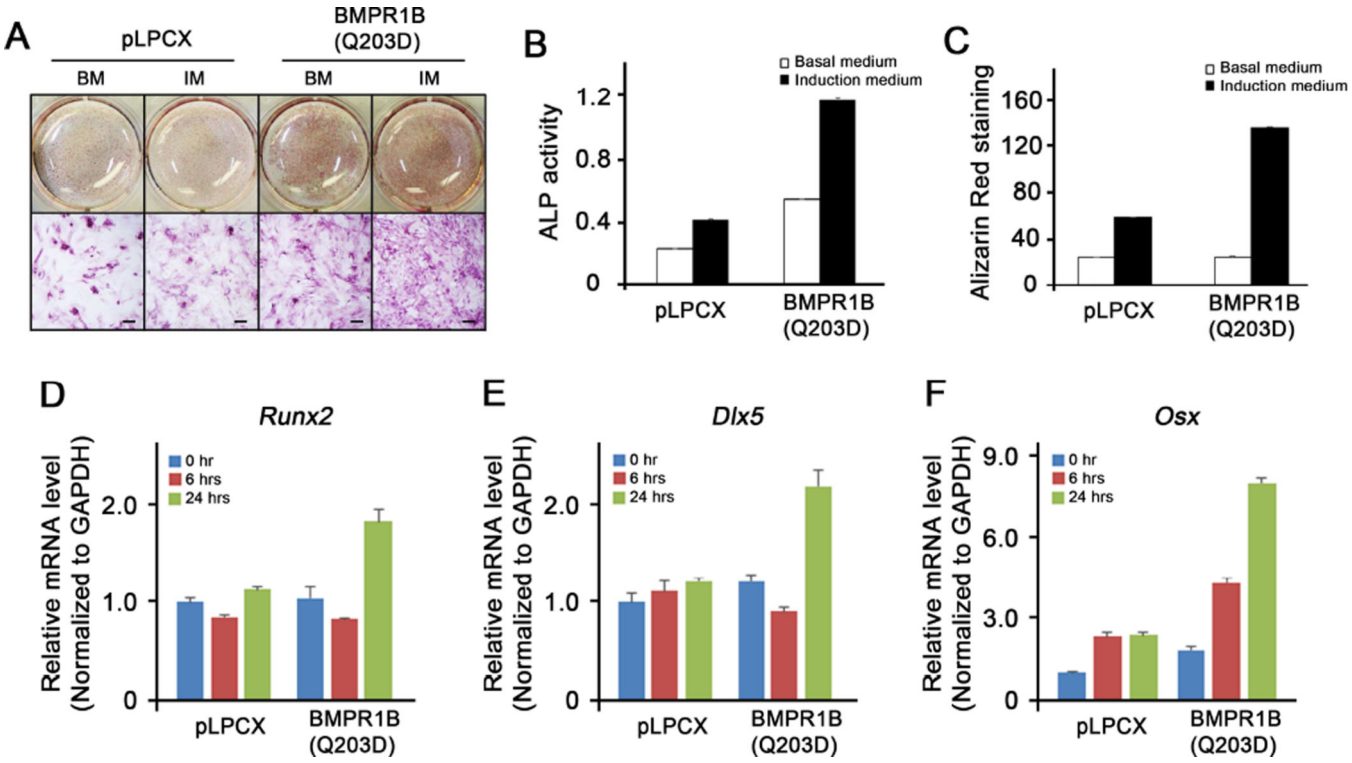

Figure 4. Constitutively activated BMP receptor rescued inhibition of osteogenic differentiation in *Orai1*$^{-/-}$ BMSCs

(A) ALP staining of *Orai1*$^{-/-}$ BMSCs transduced with retroviruses expressing pLPCX and BMRP1B (203D). (B) Quantification of ALP activity. (C) Quantification of ARS staining. (D–F) qRT-PCR for BMP2 downstream targets (*Runx2, Dlx5*, and *Osx*) during osteogenic differentiation in *Orai1*$^{-/-}$ BMSCs expressing pLPCX and BMRP1B (203D).