Biochemical and Biophysical Research Communications xxx (2016) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Sung Hee Lee [a], Yongtae Park [a], Minju Song [a], Sonal Srikanth [b], Sol Kim [a], Mo K. Kang [a, c], Yousang Gwack [b], No-Hee Park [a, c, d], Reuben H. Kim [a, c, **], Ki-Hyuk Shin [a, c, *]

[a] The Shapiro Family Laboratory of Viral Oncology and Aging Research, UCLA School of Dentistry, Los Angeles, CA 90095, USA
[b] Department of Physiology, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
[c] UCLA Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095, USA
[d] Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA

ARTICLE INFO

Article history:
Received 7 April 2016
Accepted 13 April 2016
Available online xxx

Keywords:
Orai1
BMP2
Bone marrow stem cells
Osteogenic differentiation

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

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

* Corresponding author. UCLA School of Dentistry, Center for the Health Sciences, Room 43-003, 10833 Le Conte Ave, Los Angeles, CA 90095, USA.
** Corresponding author. UCLA School of Dentistry, Center for the Health Sciences, Room 43-009, 10833 Le Conte Ave, Los Angeles, CA 90095, USA.
E-mail addresses: rkim@dentistry.ucla.edu (R.H. Kim), kshin@dentistry.ucla.edu (K.-H. Shin).

http://dx.doi.org/10.1016/j.bbrc.2016.04.068
0006-291X/

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 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 h, 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 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 h 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

Please cite this article in press as: S.H. Lee, et al., Orai1 mediates osteogenic differentiation via BMP signaling pathway in bone marrow mesenchymal stem cells, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.04.068