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Communication-dependent mineralization of osteoblasts via gap junctions

Yukihiko Hashida [a,b,c], Ken-ichi Nakahama [a,*], Kaori Shimizu [a,d], Masako Akiyama [a], Kiyoshi Harada [b], Ikuo Morita [a,c]

[a] Section of Cellular Physiological Chemistry, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Japan
[b] Section of Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Japan
[c] Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Japan
[d] Section of Oral Implantology and Regenerative Dental Medicine, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Japan

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ABSTRACT

Connexin43 (Cx43) is a major gap junction (GJ) protein in bone and plays a critical role in osteoblast differentiation. Several studies show that osteoblast differentiation is delayed by Cx43 ablation. However, the precise mechanism underlying the role of Cx43 in osteoblast differentiation is not fully understood. Firstly, we analyzed the phenotype of a conditional knockout mouse, which was generated by mating of an osterix promoter-driven Cre expressing mouse with a Cx43-floxed mouse. As expected, delayed ossification was observed. Secondly, we demonstrated that the cell communication via gap junctions played an important role in osteoblast differentiation using a tamoxifen-inducible knockout system in vitro. Genetic ablation of Cx43 resulted in both the disruption of cell-communications and the attenuation of osteoblast mineralization induced by BMP-2, but not by ascorbic acid. Moreover, restoring full-length Cx43 (382aa) expression rescued the impairment of osteoblast cell-communication and osteoblast mineralization; however, the expression of the Cx43 N-terminal mutant (382aaG2V) did not rescue either of them. Comparing the gene expression profiles, the genes directly regulated by BMP-2 were attenuated by Cx43 gene ablation. These results suggested that the cell-communication mediated by gap junctions was indispensable for normal differentiation of osteoblast induced by BMP-2.

Introduction

Gap junction (GJ) channels are made up of two apposing cells by docking via a half channel (called a hemichannel) and provide a direct cell–cell communication [1]. Gap junctional intercellular communication (GJIC) allows small molecules of less than 1 kDa, including ions, second messengers, and metabolites, to transfer from one cell to another. To date, 21 connexins (Cx), components of GJ, have been identified with distinct tissue-specific distributions in humans [2,3]. Cx43 is the most abundant gap junction protein expressed in bone cells and it plays important roles in bone remodeling [4,5]. Moreover, age-related decreases in Cx43 expression and GJIC function have been reported [6–8]. A large number of studies have indicated that Cx43 has a critical role in osteoblast differentiation and function. Fernando et al. reported that Cx43-deficient mouse embryos exhibited delayed ossification and osteoblast dysfunction [9]. Thereafter, some osteoblast lineage-specific Cx43-null mice were established using the Cre–loxP system and the bone phenotypes were analyzed. Conditional Cx43 gene (*Gja1*) ablation in osteoprogenitors or osteoblasts using a *Dermo1/Twist2* or α_1*(I)collagen* promoter resulted in a similar phenotype, characterized by expansion of the marrow cavity and cortical thinning [10,11]. Mice with conditional ablation of *Gja1* at a later stage of osteoblast differentiation using an *Osteocalcin* or *DMP-1* promoter exhibited a milder phenotype than the mice described above, although an expanded marrow cavity area was observed [12–14]. These results suggested that the differentiation stage-specific roles of Cx43 in osteoblasts may be involved in bone formation. Importantly, mutations in the human Cx43 gene caused oculodentodigital dysplasia (ODDD), an autosomal-dominant disorder characterized by skeletal abnormalities [11,15]. According to mutation analysis of *Gja1* in ODDD, these mutations are closely related to its GJIC function [16]. On the other hand, several GJIC-independent roles of Cx43 in bone homeostasis, especially as a mechanical sensor, were reported by Jiang et al. [17,18]. To explore the molecular mechanism by which Cx43 ablation caused inhibited osteoblast differentiation, analysis of osteoblast differentiation was performed in vitro. The expression of Cx43 also affected osteoblast differentiation induced by growth factors such as parathyroid hormone (PTH) or fibroblast growth factor 2 (FGF-2) [19,20]. However, the precise mechanism by which Cx43 regulates osteogenesis is not clearly understood.

In this study, we generated new conditional knockout mice (cKO) by crossing osterix (*Sp7*) promoter-driven Cre recombinase expressing

* Corresponding author at: Section of Cellular Physiological Chemistry, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan. Fax: +81 3 5803 0212.
E-mail address: nakacell@tmd.ac.jp (K. Nakahama).

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mice and mice homozygous for the floxed Cx43 gene. We found that these Osx1-Cre;Cx43$^{f/f}$ mice exhibited almost the same phenotype to previously described osteoblast-specific cKO mice. To clarify the role of Cx43 in osteogenesis, we established a tamoxifen inducible-Cx43 ablation system in osteoblasts in vitro using mice expressing the estrogen receptor (ERT2)-Cre recombinase fusion protein. Calvarial osteoblasts were isolated from ERT2-Cre;Cx43$^{f/f}$ mice and cultured with differentiation medium after cells were pretreated with/without 4-hydroxytamoxifen (4-OHT). We found that GJIC formed with Cx43 plays an important role in osteoblast differentiation induced by BMP-2, but not by ascorbic acid. This is direct evidence for the involvement of GJIC in osteoblast differentiation. Furthermore, microarray analysis revealed a change in the gene expressions related to BMP-2 signaling by ablation of Cx43 in osteoblasts.

Materials and methods

Conditional knockout mice

To ablate Cx43 in osteoblasts, Cx43$^{f/f}$ mice (stock No. 008039, Jackson Lab., USA) were mated with Osx1-Cre mice (stock No. 006361, Jackson Lab., USA). Osx1-Cre;Cx43$^{f/f}$ (Cx43cKO) and Cx43$^{f/f}$ (WT) mice were used in our experiment.

To study the effect of Cx43 expressed in osteoblasts, we established Cx43 KO mice induced by 4-OHT. Cx43$^{fl/fl}$ mice were mated with ubiquitin C promoter-driven ERT2-Cre mice (stock No. 008085, Jackson Lab., USA). ERT2-Cre;Cx43$^{f/f}$ mice were used in our experiment. Animal handling and experimental procedures were approved by the Animal Care and Use Committee of the Tokyo Medical and Dental University.

Alizarin red and alcian blue staining of calvaria from newborn mice

Skeletal preparations from newborn mice were stained with alizarin red and alcian blue. Briefly, specimens were fixed in ethanol overnight, and cartilage was stained with 0.015% alcian blue (Sigma, USA) for 24 h. After digesting soft tissue with 2% KOH (Sigma, USA), mineralized bones were stained with 0.0075% alizarin red (Sigma, USA) in 1% KOH. Samples were rinsed with water and kept in 1% KOH/20% glycerol (Sigma, USA) until complete clearing. The ossification centers and cartilage tissues were shown as red and blue, respectively.

μ-CT analysis

After fixation with EtOH, femurs were analyzed using micro-computed tomography (Micro-CT, inspeXio SMX-100CT, Shimadzu, Japan). Images were collected with the following parameters: X-ray tube voltage, 75 kV; tube current intensity, 140 μA; corn-CT scan mode, normal; slice thickness, 0.014 mm; number of views, 600; average number, 16. Structural analysis was performed in an area from 1.5 to 2.5 mm below the growth plate using the TRI/3D-BON software package (RATOC System Engineering, Tokyo, Japan).

Isolation and culture of primary mouse calvarial osteoblasts

Primary osteoblast cells were isolated from the calvaria of 0 to 1-day-old ERT2-Cre;Cx43$^{f/f}$ mice. Osteoblasts were cultured in alpha-modified Eagle's medium (αMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics with or without 1 μM 4-hydroxy tamoxifen (4-OHT) in 60 mm collagen-coated dishes at 37 °C, 95% humidity and 5% CO_2 for 3 days. 4-OHT was added once at the beginning of the three-day culture. Osteoblast cells were seeded on 48-well plate (IWAKI, Japan, 50,000 cells/well) with 10% FBS containing α-MEM. Osteoblast differentiation was induced by osteogenic medium (α-MEM containing 10% FBS, 10 mM β-glycerophosphate (β-GP), 50 μg/ml ascorbic acid (AA) or BMP-2). The medium was replaced every second day. After fixation of the cells with 75% ethanol at 4 °C for 60 min, the cells were stained with 4 mM alizarin red S solution, pH4.2, at room temperature for 10 min.

Western blot analysis

Membrane protein was collected using M-PER Eukaryotic Membrane Protein Extraction Reagent (Thermo Scientific, USA) containing a protease inhibitor. Total protein concentrations were determined by a Protein Assay kit (Bio-Rad, USA). The protein samples were separated on 12% SDS-polyacrylamide gels and electrotransferred onto PVDF membranes, then blocked with 5% skim milk (BD transduction, USA) in TBS-T (0.05 M Tris HCl, 0.150 M NaCl and 0.2% Tween-20, pH7.6). The membranes were incubated with anti-Cx43 (BD transduction) at 1:1000 or anti-BMPR1A (abcam) at 1:500 in 5% skim milk in TBS-T at 4 °C overnight. After rinsing with TBS-T, the membranes were incubated with an HRP-conjugated anti-IgG antibody (GE Healthcare, UK) at 1:5000 dilution at 4 °C for 2 h The blots were developed using a chemiluminescence method (ECL-Prime, GE Healthcare). The band images were obtained using LAS-1000 (Fujifilm, Japan). The blot was treated with stripping buffer (2% SDS, 100 mM 2-mercaptoethanol and 62.5 mM Tris–HCl (pH 6.7)) at 50 °C for 30 min, and then reprobed with anti-Pan cadherin polyclonal antibody (Sigma-Aldrich, 1:2000 with TBS-T) for normalization of protein levels.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) from primary osteoblasts cultured with α-MEM supplemented with 10% FBS and 50 μg/ml ascorbic acid for 10 days or 100 ng/ml BMP-2 for 1, 3, or 5 days. Five μg total RNA was subjected to reverse transcription. The cDNA samples were amplified with Platinum SYBR Green qPCR SuperMix-UDG with ROX. Quantitative analysis was performed using the comparative Ct Method (7500 Real-Time PCR System, Applied Biosystems, USA). The data were normalized to GAPDH. Primers were as follows:

5′-GGATCGCGTGAAGGGAAGAAGC-3′ (forward) and
5′-GAGTGGGGCCGTTGGTGAGGAG-3′ (reverse) for mouse Cx43;
5′-TGGCTGCGCTCTGTCTCT-3′ (forward) and
5′-GATGCGTTTGTAGGCGGTCTTCA-3′ (reverse) for mouse OCN;
5′-TACCGGCCACGCTACTTTCTTTAT-3′ (forward) and 5′-GACCGCCA GCTCGTTTTCATCC-3′ (reverse) for mouse BSP;
5′-CCAGTGGGTAGAGGGTTTGA-3′ (forward) and
5′-AGAAATCCGAGAAGCACGAA-3′ (reverse) for mouse Id1;
5′-CCCTTCTCAAGCACCAATGG-3′ (forward) and
5′-AAGGGTGGGTAGTCATTTGCATA-3′ (reverse) for mouse Sp7;
5′-CCACTGGATCTGTCCGATTCT-3′ (forward) and
5′-GGTCGTACACCGCATAGAGG-3′ (reverse) for mouse Smad6;
5′-GCATTCCTCGGAAGTCAAGAG-3′ (forward) and
5′-CCAGGGGCCAGATAATTCGT-3′ (reverse) for mouse Smad7;
5′-GGCCTGCTTGGCTTTTCT-3′ (forward) and
5′-CCAAGTGCAGGCAAGGTC-3′ (reverse) for mouse Hey1;
5′-ACTCAGCCGTCTGTGCCTCA-3′ (forward) and
5′-GGAGGCCTCGGTGGACATTA-3′ (reverse) for mouse Col1a;
5′-TACAGCAACAGGGTGGTGGAC-3′ (forward) and
5′-GTGGGTGCAGCGAACTTTATT-3′ (reverse) for mouse GAPDH.

Plasmid construction

Complementary DNA fragment coding full-length mouse $Cx43_{382aa}$ was amplified by a polymerase chain reaction (PCR), using Pyrobest DNA polymerase (Takara, Japan), 5′-AAAAGAATTCATGGGTGACTGGAG CGCCTT-3′ (forward), 5′-AAAAGGATCCTTAAATCTCCAGGTCATCAG-3′ (reverse) and mouse osteoblast cDNA as a template. The mutation of