BMP Signaling Mediated by BMPR1A in Osteoclasts Negatively Regulates Osteoblast Mineralization through Suppression of Cx43[†]

Running head: BMP signaling in OC regulates OC-OB crosstalk

Keywords: BMPR1A, osteoclast, osteoblast, connexin 43/GJA1, gap junction

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[†] This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jcb.25746]

Additional Supporting Information may be found in the online version of this article.

Received 05 August 2016; Revised 18 September 2016; Accepted 19 September 2016
Journal of Cellular Biochemistry

DOI: 10.1002/jcb.25746

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Number of figures: 5 figures and 1 supplemental figure

Grant information:

Contract grant sponsor: National Institutes of Health; Contract grant number: R01DE020843;

Contract grant sponsor: National Natural Science Foundation of China; Contract grant number: 81271111, 81320108011, 81500820, 81600843;

Contract grant sponsor: Ministry of Education of China; Contract grant number: 20120061130010;

Abstract

Osteoblasts and osteoclasts are well orchestrated through different mechanisms of communication during bone remodeling. Previously we found that osteoclast-specific disruption of one of the BMP receptors, *Bmpr1a*, results in increased osteoblastic bone formation in mice. We hypothesized that BMPR1A signaling in osteoclasts regulates production of either membrane bound proteins or secreted molecules that regulated osteoblast differentiation. In our current study, we co-cultured wild type osteoblasts with either control osteoclasts or osteoclasts lacking BMPR1A signaling activity. We found that loss of *Bmpr1a* in osteoclasts promoted osteoblast mineralization *in vitro*. Further, we found that the expression of *Cx43*/*Gja1* in the mutant osteoclasts was increased, which encoded for one of the gap junction proteins connexin 43/gap junction alpha 1. Knockdown of *Gja1* in the mutant osteoclasts for *Bmpr1a* reduced osteoblastic mineralization when co-cultured. Our findings suggest that GJA1 may be one of the downstream targets of BMPR1A signaling in osteoclasts that mediates osteoclast-osteoblast communication during bone remodeling.

Keywords: BMPR1A, osteoclast, osteoblast, connexin 43/GJA1, gap junction

Introduction

Bone remodeling is a dynamic process, which relies on the precise coordination of two principal cells within bone tissues: bone-formation activity of osteoblasts and bone-resorption activity of osteoclasts. It is well known that osteoblasts control osteoclast differentiation through cell-cell contact or paracrine, by expressing a number of factors, such as RANKL [Boyle et al., 2003], interleukin (IL) -1 [Lee et al., 2010] and -6 [Yoshitake et al., 2008], and tumor necrosis factor (TNF)-α [Atanga et al., 2011]. However, in the past few years, it is gradually recognized that osteoclasts do not just perform under the control of osteoblasts, they in turn influence osteoblasts as well. There are at least three modes of osteoclast-osteoblast communication. They can make direct contact, allowing membrane-bound ligands and receptors to interact and initiate intracellular signaling, i.e. family of ephrin ligands on osteoclasts and

Eph receptors on osteoblasts [Edwards and Mundy, 2008], and family of semaphorins on osteoclasts and the corresponding receptors on osteoblasts [Kang and Kumanogoh, 2013]. Osteoclasts can form gap junctions allowing passage of small water-soluble molecules between the two cell types [Batra et al., 2012]. Second, osteoclasts secrete diffusible factors and act on osteoblasts via paracrine, such as sphingosine 1-phosphate (S1P) [Ryu et al., 2006], platelet-derived growth factor BB (PDGF BB) [Kubota et al., 2002; O'Sullivan et al., 2007], and hepatocyte growth factor (HGF) [Grano et al., 1996]. Third, during bone resorption, osteoclasts release growth factors and other molecules from bone matrix, such as transforming growth factor-β (TGF-β) [Ota et al., 2013] and insulin-like growth factor-I (IGF-I) [Hayden et al., 1995].

Recently, we disrupted *Bmpr1a*, encoding BMP receptor type IA (BMPR1A), in an osteoclast-specific manner using a knock-in Cre mouse line to the *cathepsin K* locus (*Ctsk* $^{Cre/+}$; *Bmpr1a* $^{flox/flox}$) [Okamoto et al., 2011]. We found that deletion of *Bmpr1a* in differentiated osteoclasts increased osteoblastic bone formation, suggesting that BMP signaling through BMPR1A in osteoclasts negatively regulates osteoblast differentiation. However, it is largely unknown how BMP signaling in osteoclasts regulates osteoclast-osteoblast communication. We hypothesized that BMP signaling mediated by BMPR1A in osteoclasts regulates production of either membrane bound proteins or secreted molecules that affects osteoblast differentiation. In addition, since *Ctsk*-Cre-expressing cells were recently found to display markers and functional properties consistent with mesenchymal progenitors [Yang et al., 2013], another possibility is that the phenotype we observed in *Bmpr1a* conditional knockout mice is due to disruption of *Bmpr1a* in Ctsk-Cre-expressing mesenchymal progenitors. To test the hypothesis that BMPR1A signaling in osteoclasts regulate osteoblast function through communication between two types of cells, and to explore the molecular mechanisms, it is necessarily and important to set up an *in vitro* co-culture system.

Gap junctions are clusters of intercellular channels formed from hexamers of connexins (Cx) that connect adjacent cells and allow for the diffusion of ions, small molecules and second messengers between cells

[Kumar and Gilula, 1996]. In bone, gap junctional intercellular communication (GJIC) mediated by connexins plays important roles in regulating signal transduction among different bone cells, thus influences development, differentiation, modeling and remodeling of the bone. Connexin 43 (Cx43), also known as gap junction alpha-1 protein (GJA1) (encoded by *Gja1* gene), is a component of gap junctions. It is by far the most broadly and ubiquitously expressed member of the connexin family, being expressed in nearly all vital organs that include the brain, heart, bone, lungs, stomach and intestine [Laird, 2006]. Among the members of the connexin family, Cx43/GJA1 is the most abundantly expressed in bone cells, including pre-osteoblasts, osteoblasts, osteocytes and osteoclasts [Ilvesaro et al., 2000; Yellowley et al., 2000]. Physical contact of two adjacent cells facilitates the communication between the cells through Cx43/GJA1 [Lecanda et al., 2000; Meme et al., 2006]. It is known that Cx43/GJA1 positively regulates osteoblast differentiation and bone formation [Minkoff et al., 1999]. Besides, Cx43/GJA1 positively regulates osteoclastogenesis and bone resorption [Ransjo et al., 2003; Sternlieb et al., 2012]. Osteoclast-osteoblast interaction contributes to coupling of bone resorption and formation. However, GJIC between osteoclast and osteoblast is largely unknown.

In this study, to understand how BMP signaling in osteoclasts influences osteoblast differentiation, we co-cultured osteoclasts with osteoblasts *in vitro* and determined the extent of osteoblast mineralization which osteoclasts contributed to. We found that osteoclasts deficient for BMPR1A promoted osteoblast mineralization through up-regulation of a gap junction protein Cx43/GJA1 in osteoclasts. Our findings reveal a novel communication between osteoclasts and osteoblasts, which will further deepen understanding of bone biology.**Materials & Methods**

Mice

Generation of *Bmpr1a* null allele and conditional allele were previously reported [Mishina et al., 2002; Mishina et al., 1995]. Mice heterozygous null for *Bmpr1a* carrying tamoxifen (TM)-inducible Cre fusion protein Cre-ER^{TM} under the ubiquitin promoter (No. 008085, The Jackson Laboratory) (*Bmpr1a* +/-: Ubi-CreER^{TM}) were bred with mice homozygous for the conditional allele for *Bmpr1a* (*Bmpr1a* fx/fx)

[Mishina et al., 2002] (Fig. 1A) on a 12-hour light/dark cycle. Mice genotyped *Bmpr1a* fx/+: Ubi-CreER^{TM} (+)/(-) were designated as control (cont), and *Bmpr1a* fx/-: Ubi-CreER^{TM} (+)/(-) within the same littermate as mutant (mut). All animal experiments were performed in accordance with the policy and federal law of judicious use of vertebrate animals as approved by the Institutional Animal Care and Use Committee (IACUC) at University of Michigan.

Isolation and culture of osteoclasts

Control and mutant bone marrow cells were collected from the long bones (i.e. femora and humeri) of 1-month-old mice within littermates. After culturing overnight in α-MEM containing 10% fetal bovine serum (Hyclone) (full medium), non-adherent cells were harvested as bone marrow mononuclear cells (BMMCs). Proliferation of BMMCs was stimulated with 20 ng/ml Recombinant Murine Macrophage Colony Stimulating Factor (rmM-CSF) (PEPROTECH) for 4 days, followed by another 4 days of differentiation culture into osteoclasts using 20 ng/ml M-CSF and 50 ng/ml Recombinant Murine soluble Receptor Activator of NF-κB Ligand (rmRANKL) (PEPROTECH) [Yang et al., 2004]. Medium was changed every other day.

To determine efficacy of osteoclast formation, cells were differentiated using 20 ng/ml M-CSF and 50 ng/ml RANKL for 6 days, followed by tartrate-resistant acid phosphatase (TRAP) staining. The number of osteoclasts as well as the number of nuclei per osteoclast were counted. To determine bone resorption of osteoclasts, cells were seeded onto OsteoAssay Surface (Corning) and differentiated as stated above for 6 days. The resorption area was measured and was normalized by osteoclast number.

TM treatment and evaluation of gene recombination

Ubi Cre-ER^{TM} activity was induced by administering 100 ng/ml of (Z)-4-hydroxytamoxifen (TM) (Sigma-Aldrich) in culture for 4 days [Kamiya et al., 2008]. After TM treatment, cells isolated from control (*Bmpr1a* fx/+: Ubi-CreER^{TM} (+)/(-)) and mutant (*Bmpr1a* fx/-: Ubi-CreER^{TM} (+)/(-)) mice were

used as control and mutant cells, respectively. To detect Cre-dependent DNA recombination in *Bmpr1a*, genomic PCR was performed using primers fx1: 5'-GGTTTGGATCTTAACCTTAGG-3' and fx4: 5'-TGGCTACAATTTGTCTCATGC-3 [Mishina et al., 2002]. This primer set generates a 180-bp amplicon after Cre-dependent recombination, while over 1.5-kb amplicon is generated if no recombination is occurred (Fig. 1B) [Mishina et al., 2002]. To quantify deletion of *Bmpr1a*, genomic real-time quantitative PCR was performed using TaqMan Gene Expression Assays (*Gapdh*: Cat. # 4352932E and *Bmpr1a*: custom designed to detect exon 4, SKU # 4331348) (Fig. 1B). Protein level of P-SMAD1/5/9, representing BMP-SMAD signaling pathway, in control and mutant osteoclasts after gene recombination was assessed by western blot. Differentiated osteoclasts were stimulated with or without 100 ng/ml recombinant human bone morphogenetic protein 2 (rhBMP-2) for 30 min before cell lysis.

Osteoblast mono-culture and co-culture

Primary calvarial pre-osteoblasts were isolated from 1- to 3-day-old wild-type mice as reported [Liu et al., 2013]. 5 x 10^4 cells/well of osteoblasts were seeded in 24-well plate and the medium was changed to osteogenic differentiation medium (DM) (full medium containing 10 mM β-glycerophosphate and 50 μg/ml L-ascorbic acid) the next day. For osteoblast-osteoclast co-culture, 1 x 10^5 mature osteoclasts per well were added into osteoblast culture every 3 days and cultured in DM without M-CSF and RANKL (Fig. 1F). For each experiment, osteoclasts isolated from five control or mutant mice were pooled after confirmation of efficient Cre-dependent DNA recombination. Each experiment was repeated three times using different pooled samples.

Alizarin red staining

After co-culture for 14 days, cells were stained with 40 mM alizarin red S (Sigma-Aldrich) for 10 min at room temperature. The number of alizarin red stained nodules was counted. The percentage of nodule area was measured using Image J (RRID: SCR_003070). To minimize the effects of subjective bias when assessing the staining results, pictures were taken by one researcher (HZ), and measurements were

performed by another researcher in a blinded manner (CS). Then 10% w/v cetylpyridinium chloride (CPC) (Sigma-Aldrich) was used to dissolve bound alizarin red S and optical density was measured at 562 nm.

RNA isolation and quantitative real-time RT-PCR

After BMMCs were differentiated for 4 days (total 8 days after isolation), osteoclasts were stimulated with or without 100 ng/ml recombinant human bone morphogenetic protein 2 (rhBMP-2, R&D Systems) for 6 hours in full medium (10% serum). Total mRNA was isolated using TRIzol reagent (Life Technologies). For quantitative real-time RT-PCR analyses, equal amounts of RNA were reverse-transcribed using Superscript III first-strand synthesis System (Invitrogen) with oligo (dT) as a primer. The resulting cDNA templates were subjected to quantitative PCR using TaqMan probes (*Gapdh*: Mm99999915_g1; *Sema4d*: Mm00443147_m1; *Sema7a*: Mm00441361_m1; *Efnb2*: Mm00438670_m1; *Efna2*: Mm00433011_m1; *Acp5*: Mm00475698_m1; *Pdgfbb*: Mm00440677_m1; *Hgf*: Mm01135184_m1; *Cthrc1*: Mm01163611_m1; *Sphk1*: Mm00448841_g1; *Sphk2*: Mm00445021_m1; *Gja1*: Mm01179639_s1) by ABI PRISM 7500 (Applied Biosystems). Data were normalized to *Gapdh* expression using the $2^{-\Delta\Delta Ct}$ method.

Gja1 knockdown assay by adenovirus treatment

BMMCs were expanded using 20 ng/ml M-CSF for 4 days, and differentiated using 20 ng/ml M-CSF and 50 ng/ml RANKL for 2 days. The differentiated osteoclasts were transfected with vehicle control (Scrambled shRNA with GFP adenovirus, Ad-GFP-U6-shRNA, Vector Biolabs, Cat. No. 1122N) or mouse *Gja1* shRNA (Ad-GFP-U6-m-GJA1-shRNA, Vector Biolabs, No. shADV-280777) in serum free medium containing M-CSF, RANKL and 200 PFU/cell (multiplicity of infection, MOI) of adenoviral vector stock for 3 h. Then the medium was replaced to full medium containing M-CSF and RANKL. Forty-eight hours after transfection, osteoclasts were trypsinized and added to osteoblast culture as mentioned above.

Western blot analysis

Cells were lysed in RIPA buffer (20 mM Tris-HCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) containing protease inhibitor (Roche). The resulting lysates were run on 10% Mini-PROTEAN® TGX™ Gel (Bio-Rad) and transferred to Amersham Hybond-P membrane (GE Healthcare). The following antibodies were used (all are from Cell Signaling Technology): rabbit anti-connexin 43 (1:500, Cat. No. 3512, RRID: AB_2294590), rabbit anti-pSMAD1/5/9 (1:1000, Cat. No. 13820, RRID: AB_2493181), and rabbit anti-GAPDH (1:2000, Cat. No. 2118, RRID: AB_1031003). SuperSignal West Femto Chemiluminescent substrate was used to detect anti-connexin 43 and anti-pSMAD1/5/9, and Pico Chemiluminescent substrate (Thermo Scientific) was used to detect anti-GAPDH.

Statistical analysis

All results were expressed as means ± standard deviation of triplicate measurements with all experiments independently repeated three times. Unpaired Student's t-tests were used to evaluate statistical differences. Values of $p < 0.05$ were considered significant.

Results

Bmpr1a was efficiently disrupted in osteoclasts

In differentiated osteoclasts, genomic PCR results showed the appearance of a 180-bp band as well as a significant reduction in the intensity of the non-recombination bands (>1500 bp) occurred as early as 2 days after TM treatment (Fig. 1C). Quantitative genomic real-time PCR results showed a significant reduction of exon 4 compared to the same cells cultured without TM (Fig 1D). In control cells (*Bmpr1a* fx/+: Ubi-CreER™ (+)/(-), one floxed allele with one wild type allele, thus the maximum reduction of exon 4 is 50% after Cre-induced DNA recombination), we observed 38% and 27% reduction of the levels of exon 4 of *Bmpr1a* in undifferentiated and differentiated osteoclasts, while in mutant cells (*Bmpr1a* fx/-: Ubi-CreER™ (+)/(-), one floxed allele with one null allele), we observed 79% and 89% reduction in undifferentiated and differentiated osteoclasts. In control osteoclasts, BMP stimulation resulted in a strong

signal of P-SMAD1/5/9, whereas in mutant osteoclasts, BMP stimulation did not increase P-SMAD1/5/9 level as observed in control osteoclasts (Fig. 1E, 70% reduction in mutant osteoclasts compared with control). Taken together, these results suggested efficient deletion of *Bmpr1a* in both undifferentiated and differentiated osteoclasts by 4 days of TM treatment as evidenced by the significant reduction of BMP-SMAD signaling activity.

BMPR1A negatively regulated osteoclast formation

First, we investigated the impact of the loss of BMPR1A signaling during osteoclastogenesis and resorption activity using *in vitro* system. After 6 days of culture in differentiation media, the *Bmpr1a* deficient cells showed higher number of osteoclasts compared with control (2.7-folds, Supplemental Fig. S1A). However, mutant osteoclasts were smaller in size with fewer nuclei (Supplemental Fig. S1B). In terms of bone resorption function per osteoclast, there was no significant difference between control and mutant osteoclasts (Supplemental Fig. S1C). These results suggested that disruption of *Bmpr1a* in osteoclasts increased osteoclast formation, but attenuated further differentiation to fuse together. BMPR1A deletion did not influence osteoclast resorption activity.

Loss of *Bmpr1a* in osteoclasts promoted osteoblast mineralization

Alizarin red staining showed that presence of osteoclasts promoted mineralization of osteoblasts (Fig. 1F, and Fig. 2), i.e. co-culture showed higher number of mineralized nodules (1.6 folds and 2.9 folds in osteoblasts co-cultured with control and mutant osteoclasts respectively, compared with osteoblast mono-culture), and larger area of mineralized nodules (3.1 folds and 8.3 folds in osteoblasts co-cultured with control and mutant osteoclasts respectively, compared with osteoblast mono-culture), as well as more calcium deposit (16% and 32% increase in osteoblasts co-cultured with control and mutant osteoclasts respectively, compared with osteoblast mono-culture), as quantified in Fig. 2B. More importantly, osteoclasts with disrupted *Bmpr1a* further promoted osteoblast mineralization (77% increase in nodule number, 2.6 folds in nodule area, and 14% increase in calcium deposit, in osteoblasts co-cultured with

mutant osteoclasts compared with osteoblasts co-cultured with control osteoclasts) (Fig. 2). These results indicate that co-culture of osteoblasts with osteoclasts positively influences osteoblast mineralization, and suggest that BMP signaling through BMPR1A in osteoclasts indirectly, but negatively regulates osteoblast mineralization.

Expression of genes mediating osteoclast-osteoblast communication was changed

Since mutant osteoclasts promoted osteoblast mineralization in the co-culture system, we hypothesized that there were changes in mutant osteoclasts in secreted molecules or membrane bound proteins, which were involved in osteoclast-osteoblast communication. Based on published information, we selected 11 genes known to function in osteoclast-osteoblast communication and examined their expression [Batra et al., 2012; Edwards and Mundy, 2008; Grano et al., 1996; Kang and Kumanogoh, 2013; Kubota et al., 2002; O'Sullivan et al., 2007; Ryu et al., 2006; Takeshita et al., 2013]. We found that the expression of *Cx43*/*Gja1* was increased in mutant osteoclasts (~4 folds) compared with control osteoclasts in the absence or presence of BMP2 (Fig. 3A). The expression of *Efna2* was decreased (~30%) in mutant osteoclasts compared with control osteoclasts, in addition, 6 h of BMP2 treatment further lowered the expression of *Efna2* in both control and mutant osteoclasts (23% and 39% decrease in control and mutant osteoclasts, respectively, compared with no BMP2 treatment) (Fig. 3B). No overt changes were observed in other candidate genes (Fig. 3). EphrinA2 has been shown to mediate crosstalk between osteoclasts and osteoblasts [Irie et al., 2009], however, communication through Cx43/GJA1 between those two types of cells is largely unknown. Thus, we decided to further focus on *Cx43*/*Gja1* in this study.

Knockdown of *Cx43*/*Gja1* in osteoclasts decreased osteoblast mineralization

To explore a possibility that 6 h treatment was not enough or too long to observe the impact of BMP-2, we next examined the expression of *Cx43*/*Gja1* stimulated with BMP2 for different time periods. The expression of *Cx43*/*Gja1* decreased in both control and mutant osteoclasts after 6 h of stimulation, i.e., at 12 h and 24 h (Fig. 4A), but the expression of *Cx43*/*Gja1* was significantly higher in the mutant

osteoclasts at all time points examine. These results indicate that expression of *Cx43*/*Gja1* in osteoclasts was negatively regulated by BMP signaling, at least in part, through type 1A receptor.

To understand the role of Cx43/GJA1 in terms of osteoclast-osteoblast communication by knocking down *Cx43*/*Gja1* in mutant osteoclasts using adenovirus expressing shRNA. Strong GFP signal was detected 48 h after transduction that was also encoded by the adenovirus vector used for shRNA expression (Fig 4B). The transduction efficiency based on the percentage of GFP positive cells was approximately 80%. Quantitative RT-PCR results showed that the expression of *Cx43*/*Gja1* did not change 24 h after transduction. At 48 h, 72 h, and 96 h after transduction, the expression of *Cx43*/*Gja1* decreased by ~50%, compared with no transduction and scrambled shRNA (Fig. 4C). Cx43/GJA1 protein level revealed a 33% decrease at 48 h after transduction, an approximate 70% decrease at 72 h and 96 h after transduction (Fig. 4D). These results suggested that *Cx43*/*Gja1* was efficiently knocked down, and protein level was significantly reduced in the condition we employed. According to the co-culture scheme (Fig. 1F), we treated osteoclasts with the adenoviruses 48 h before adding to the co-culture (Fig. 4F).

Consistent with Fig. 2, alizarin red staining in Fig. 5 confirmed that co-cultured osteoblasts with osteoclasts enhanced osteoblast mineralization, compared with osteoblast mono-culture (OB only); mutant osteoclasts further promoted osteoblast mineralization (OB + mut OC), compared with control osteoclast (OB + cont OC) (Fig. 5). There was no difference between osteoblasts co-cultured with mutant osteoclasts (OB + mut OC) and mutant osteoclasts transduced scrambled shRNA (OB + mut OC sh-Scrb). However, after knocking down of *Cx43*/*Gja1* in mutant osteoclasts, the mineralization was significantly decreased (OB + mut OC sh-Gja1) compared with osteoclasts transduced scrambled shRNA (OB + mut OC sh-Scrb), but still higher than osteoblast mono-culture (OB only) (Fig. 5A and 5B). These results suggested that BMP signaling mediated by BMPR1A in osteoclasts, at least in part, negatively regulated osteoblast mineralization through suppression of Cx43/GJA1.

Discussion

Our present results demonstrated that osteoclasts deficient for BMPR1A promoted osteoblast mineralization *in vitro*. The expression of *Cx43/Gja1* was up-regulated in mutant osteoclasts compared with control osteoclasts. Knockdown of *Cx43/Gja1* in osteoclasts using shRNA decreased mineralization of osteoblasts when co-cultured *in vitro*. Our findings suggested that connexin 43 (Cx43)/gap junction alpha-1 protein (GJA1) encoded by *Gja1* gene is one of the downstream targets of BMPR1A signaling in osteoclasts which mediates osteoclast-osteoblast communication.

Gap junctions and Cx43/GJA1 play important roles in osteoclasts, including osteoclast formation, resorption and communication in the bone multicellular unit in bone remodeling [Ilvesaro and Tuukkanen, 2003; Ilvesaro et al., 2000; Ransjo et al., 2003]. Cx43/GJA1 positively regulates osteoclastogenesis *in vivo* [Sternlieb et al., 2012]. Blocking of Cx43/GJA1 affects the fusion of larger osteoclasts with four or more nuclei [Hobolt-Pedersen et al., 2014]. The connexin mimetic peptide GAP 27, that inhibits GJIC, decreases the numbers of TRAP positive osteoclasts and increases the number of apoptotic osteoclasts [Ilvesaro et al., 2001]. Gap junction inhibitors AGA or oleamide leads to a significant inhibition of resorption pits formation on bone slice [Ransjo et al., 2003]. These experimental evidences suggest the crucial role of GJIC in conveying stimulating signaling on osteoclast formation, survival and resorption. We previously reported that BMPR1A in osteoclasts negatively regulates osteoclastogenesis and bone resorption *in vivo* [Okamoto et al., 2011]. *In vitro* results using spleen cells indicates BMPR1A negatively regulates osteoclast formation and expression of osteoclast marker genes [Okamoto et al., 2011]. Together with the data shown in our current study, these results could be explained by increased *Cx43*/*Gja1* expression in the *Bmpr1a* mutant osteoclasts. In our current study, we found consistent results using bone marrow mononucleated cells, i.e. disruption of *Bmpr1a* increased osteoclastogenesis *in vitro*, reduced formation of multinucleated cells, and normal levels of resorption. Further investigation should shed a light on a molecular mechanism of how BMPR1A regulates osteoclastogenesis through Cx43/GJA1.

There are evidences showing that gap junctional intercellular communication (GJIC) occurs between osteoblast-osteoblast, osteocyte-osteocyte, osteocyte-osteoblast, and osteoblast/osteocyte-osteoclast. Transmission of soluble molecules via connexin gap junctions between osteoblasts allows propagation of specific signals and response to anabolic agents such as PTH [Chung et al., 2006; Lecanda et al., 1998; Stains and Civitelli, 2005]. Gap junctions at the tips of osteocyte cell processes respond to changes in the mechanical environment through stimuli such as mechanical loading, and transmit these signals through the osteocyte network to osteoblasts via gap junctions [Bonewald, 2011; Grimston et al., 2006; Yellowley et al., 2000]. In addition, GJIC plays an important role in osteoblast-osteocyte communication. Osteoblast/osteocyte specific *Cx43*/*Gja1* deficient mice have increased bone resorption and TRAP positive osteoclasts [Watkins et al., 2011; Zhang et al., 2011]. *In vitro* studies using *Cx43*/*Gja1* deficient MLO-Y4 cells, a cell line mimicking osteocyte characters, reveal an increase in the RANKL/OPG ratio, indicating that loss of *Cx43*/*Gja1* in osteocytes promotes osteoclastogenesis [Bivi et al., 2012; Zhang et al., 2011]. Bone marrow stromal cells (BMSCs) are shown to communicate with each other and the bone lining cells via gap junctions [Dorshkind et al., 1993; Doty, 1981].

However, the knowledge of the functions of GJIC between osteoclasts and other cell types is still naive. In our current study, we demonstrated for the first time that disruption of *Bmpr1a* in osteoclasts promoted osteoblast mineralization through up-regulation of *Cx43*/*Gja1* in osteoclasts. It is possible to speculate that increased Cx43/GJA1 results in increased trafficking of small molecules from osteoclasts to osteoblasts through GJIC. Another possibility is that the up-regulation of *Cx43*/*Gja1* alters osteoclast functions that secondarily influence osteoblast functions. Besides its participation in gap junctions and hemichannels, Cx43/GJA1 might affect cellular function by interacting with structural molecules (such as caveolin-1 and 3 [Giepmans, 2004; Liu et al., 2010], tight junction protein ZO-1 [Giepmans, 2004], N-cadherin [Dbouk et al., 2009], alpha-catenin [Dbouk et al., 2009], tubuin [Giepmans, 2004]), kinase/phosphatase (such as Src, PKA, PKC [Giepmans, 2004]), growth suppression molecule CCN3/NOV [Giepmans, 2004], cell motility molecule ephrin [Davy et al., 2006], and other intracellular

molecules (such as Cox-2, Hsp90 and beta-catenin [Dbouk et al., 2009]). The majority of these interactions are localized in areas of the cytoplasmic C-terminal tail of Cx43/GJA1, which does not participate in channel formation [Dbouk et al., 2009; Giepmans, 2004]. Thus increased expression of *Cx43*/*Gja1* may also increase interactions with intracellular molecules in our mutant osteoclasts, which secondarily promotes osteoblast mineralization. The system we employed here does not exclude a possibility that osteoclasts regulate osteoblast differentiation through secretion factors. It is an interesting future effort to understand the mechanisms of how Cx43/GJA1 involves in communication between osteoclasts and osteoblasts, especially necessity of direct cell-cell contact.

It is reported that BMP-2 induces expression of *Cx43*/*Gja1* in cardiomyocyte-like cells [Degeorge et al., 2008; Hou et al., 2013], in an *in vitro* mesenchymal cell condensation and chondrogenic differentiation model [Zhang et al., 2002], and in an embryonal mouse P19 cell line [Bani-Yaghoub et al., 2000]. While, in immortalized human granulosa cells, theca cell-derived BMP4 and BMP7 or oocyte-derived BMP15 down-regulates *Cx43*/*Gja1* expression and decreases GJIC activity [Chang et al., 2014; Chang et al., 2013]. It is also reported that BMP2, BMP4 and GDF5 can modulate *Cx43*/*Gja1* promoter activity in the osteosarcoma cell line ROS17/2.8, including positive and negative regulatory elements within *Cx43*/*Gja1* locus. Comparison of *Cx43*/*Gja1* promoter sequences from the human vs. mouse showed five regions with significant sequence conservation, two of which contained SMAD binding elements in conjunction with a BMP response element [Chatterjee et al., 2003]. In our current study, we found that BMP signaling mediated by BMPR1A down-regulated *Cx43*/*Gja1* expression in osteoclasts. These evidences suggest that BMP signaling differentially regulates *Cx43*/*Gja1* expression in a cell type dependent manner. However, more detailed mechanisms need to be further addressed, including whether *Cx43*/*Gja1* is a direct downstream target of BMP signaling in osteoclasts, whether aforementioned putative BMP responsive elements in the promoter of *Cx43*/*Gja1* are involving in transcriptional regulation of *Cx43*/*Gja1* in osteoclasts.

In conclusion, our study identified that disruption of *Bmpr1a* in osteoclasts promoted osteoblast mineralization when co-cultured. Up-regulation of gap junction *Cx43*/*Gja1* in mutant osteoclasts is responsible for the enhanced osteoblast function. Our findings reveal a novel communication pathway between osteoclasts and osteoblasts, which will help in understanding of bone biology.

Acknowledgements

This work was supported by grants from the National Institutes of Health (grant number R01DE020843 to YM), the National Natural Science Foundation of China (grant numbers 81271111 and 81320108011 to HS; grant number 81600843 to CS; grant number 81500820), and the Ministry of Education of China (grant number 20120061130010 to HS).

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: Study design: CS, YM and HS. Study conduct: CS, HZ and KL. Data collection: CS, HZ and KL. Data analysis: CS. Data interpretation: CS, HZ, YM and HS. Drafting manuscript: CS. Revising manuscript content: CS, HZ, KL, YM and HS. Approving final version of manuscript: CS, HZ, KL, YM and HS. CS takes responsibility for the integrity of the data analysis.

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Figure legends

Figure 1. *Bmpr1a* was efficiently disrupted in undifferentiated and differentiated osteoclasts. (A) Breeding was set up between the mice indicated. Bone marrow cells were harvested and Cre activity was induced during culture. (B) Levels of recombination were evaluated by genomic PCR (fx1 and fx4) and

quantitative genomic PCR for exon 4. (C) BMMCs were differentiated using 20 ng/ml M-CSF and 50 ng/ml RANKL and treated with TM for different days, followed by DNA extraction. Recombination levels were assessed by genomic PCR using primers fx1 and fx4. A 180-bp amplicon indicated Cre-dependent recombination, whereas an over 1.5-kb amplicon indicated no recombination. (D) BMMCs were treated with or without TM for 4 days in the presence of M-CSF only (undifferentiated) or differentiation medium (M-CSF and RANKL) (differentiated), followed by DNA extraction. Deletion efficiency of *Bmpr1a* locus was quantified by genomic real-time quantitative PCR. Levels of the presence of exon 4 were compared with the same stock of cells that were not treated with TM. (E) Control and mutant BMMCs were differentiated using M-CSF and RANKL for 4 days, and Cre activity was induced using tamoxifen at the same time. Then cells were stimulated with or without 100 ng/ml BMP-2 for 30 min, followed by cell lysis. P-SMAD1/5/9 level was detected by Western blot, and GAPDH was used as a loading control. (F) The scheme for osteoclast-osteoblast co-culture. BMMCs were expanded using M-CSF for 4 days and differentiated using M-CSF and RANKL for another 4 days. 1 x 10^5 osteoclasts/well were added to osteoblast culture every 3 days in osteogenic differentiation medium. cont: control, genotyped as *Bmpr1a* fx/+: Ubi-CreER^{TM} (+)/(-); mut: mutant, genotyped as *Bmpr1a* fx/-: Ubi-CreER^{TM} (+)/(-); het: heterozygous; cKO: conditional knockout; bp: base pair; TM: (Z)-4-hydroxytamoxifen; ex: exon; BMMC: bone marrow mononuclear cell; OC: osteoclast; OB: osteoblast; *: $p<0.05$; **: $p<0.01$.

Figure 2. Mutant osteoclasts deficient for *Bmpr1a* promoted osteoblast mineralization. (A) After co-culture for 14 days, cells were fix and stained for alizarin red. Comparison was done in triplicate. (B) The number of mineralized nodules was counted. The percentage of nodule area was measured and calculated. The staining was dissolved and the optical density was measured. Bar=2mm. cont: control; mut: mutant; OC: osteoclast; OB: osteoblast; *: $p<0.05$; **: $p<0.01$.

Figure 3. Osteoclastic expression profiles of candidate genes mediating osteoclast-osteoblast communication were changed. BMMCs were differentiated for 4 days, then cells were stimulated with or

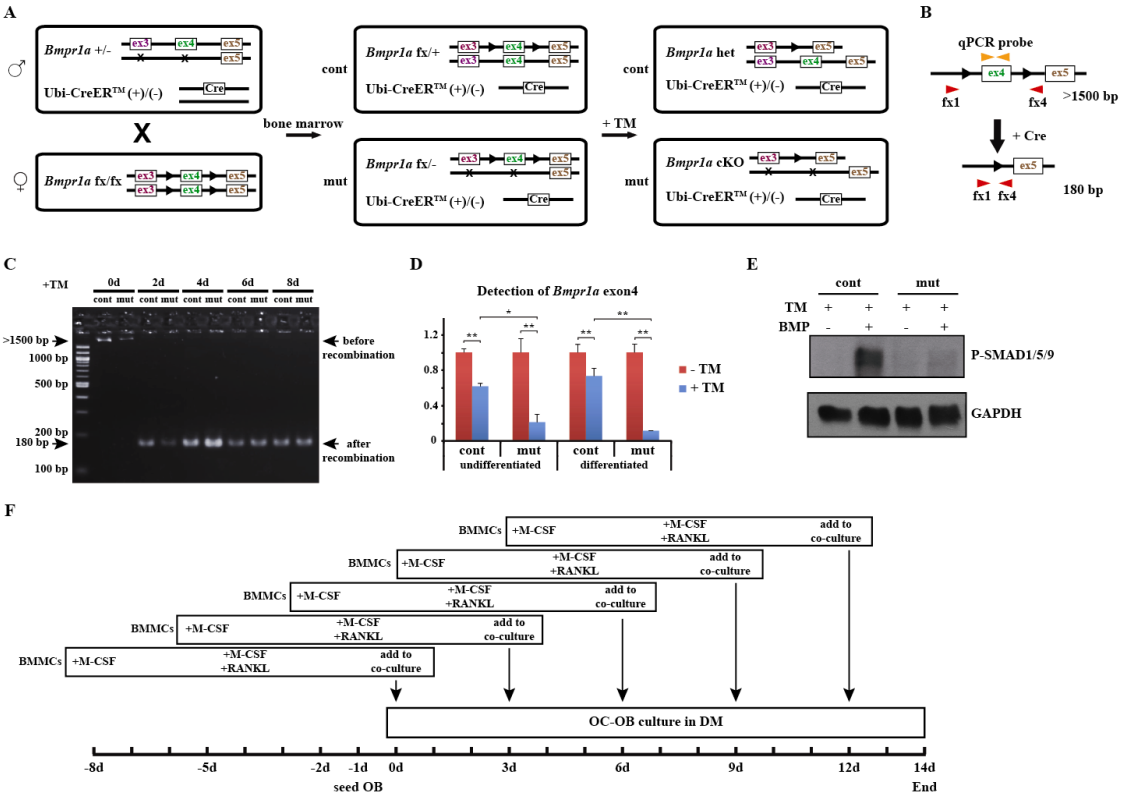
without BMP-2 for 6 hours followed by RNA extraction. Quantitative RT-PCR was performed. Gene expression levels in control osteoclasts without BMP treatment were calculated as 1. cont: control; mut: mutant; OC: osteoclast; *: $p<0.05$; **: $p<0.01$.

Figure 4. *Cx43*/*Gja1* was efficiently knocked down in osteoclasts by adenovirus treatment. (A) BMMCs were differentiated using M-CSF and RANKL for 4 days, then cells were stimulated with BMP-2 for different periods followed by RNA extraction. The expression of *Cx43*/*Gja1* was evaluated by quantitative RT-PCR. (B) Representative images of osteoclasts 48 h after transfection, showing GFP signals (lower panel) and the corresponding bright field (upper panel). (C) The expression of *Cx43*/*Gja1* after transfection. (D) Protein level of Cx43/GJA1 after transfection. (E) The scheme for co-culture when osteoclasts were treated with adenovirus. BMMCs were expanded using M-CSF for 4 days and differentiated using M-CSF and RANKL for another 4 days. Two days after differentiation, osteoclasts were transfected with adenovirus. 1 x 10^5 osteoclasts/well were added to osteoblast culture every 3 days in osteogenic differentiation medium. cont: control; mut: mutant; OC: osteoclast; BF: bright field; GFP: green fluorescent protein; Ad: adenovirus; Scrb: scrambled; *: $p<0.05$; **: $p<0.01$.

Figure 5. Mutant osteoclasts promoted osteoblast mineralization in the co-culture, and knockdown of *Cx43*/*Gja1* in mutant osteoclasts decreased osteoblast mineralization. (A) Cells were stained with alizarin red. Comparison was done in triplicate. (B) The number of mineralized nodules was counted. The percentage of nodule area was measured and calculated. The staining was dissolved and the optical density was measured. cont: control; mut: mutant; OC: osteoclast; OB: osteoblast; Ad: adenovirus; Scrb: scrambled; *: $p<0.05$; **: $p<0.01$.

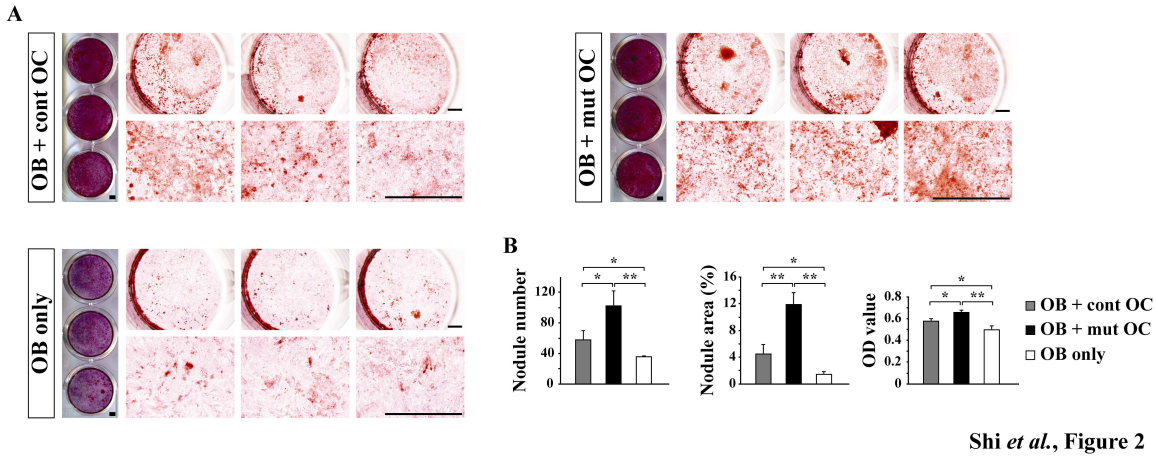
Supplemental Figure S1. *In vitro* osteoclastogenesis was increased with fewer number of nuclei, but resorption was not changed. Bone marrow mononucleated cells (BMMCs) were differentiated with M-CSF and RANKL for 6 days followed by TRAP staining. (A)The number of TRAP positive cells with no

less than 3 nuclei was counted as osteoclasts. (B) The number of nuclei per osteoclast was counted. (C) BMMCs were differentiated on a hydroxyapatite-coated well to examine resorption function of osteoclasts. The area of resorption as well as the number of TRAP-positive osteoclasts were measured and counted. Resorption area per osteoclasts was calculated. cont: control; mut: mutant; **: $p<0.01$.



Shi *et al.*, Figure 1

Fig 1-01 .



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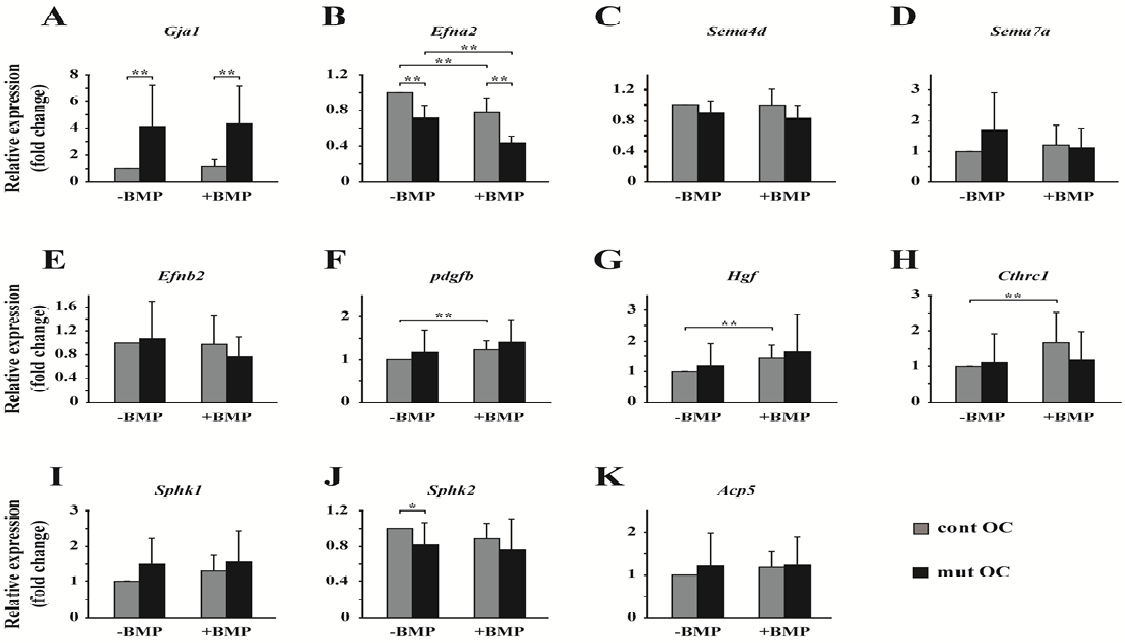
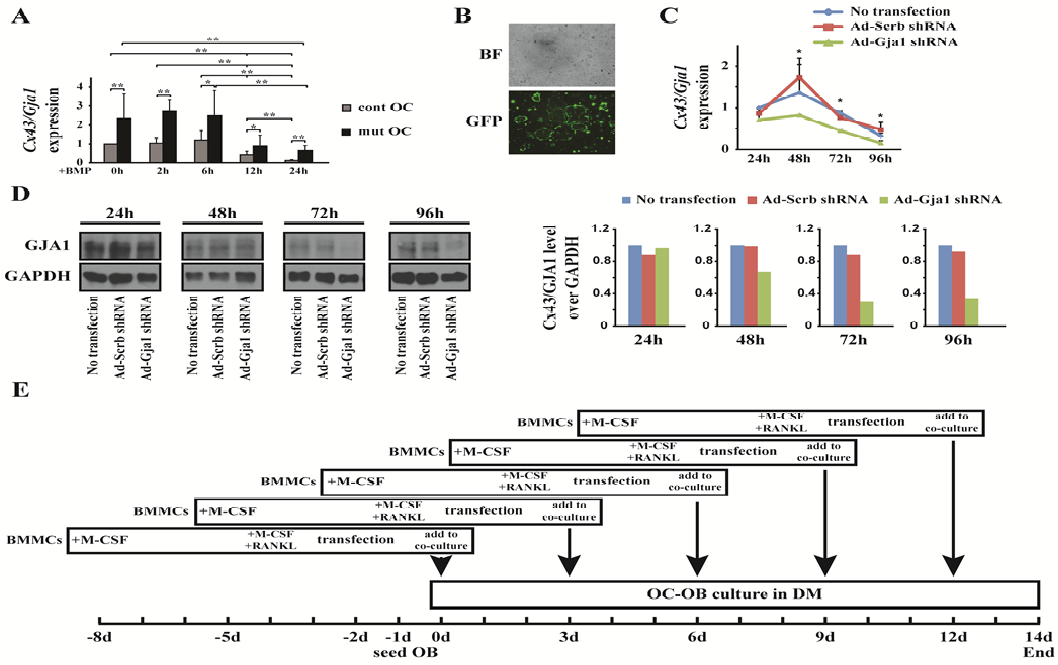


Shi *et al.*, Figure 3

Fig 3-01 .



Shi *et al.*, Figure 4

Fig 4-01 .

Fig 5-01 .

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