Published in final edited form as:

Biochem Biophys Res Commun. 2019 February 12; 509(3): 728–733. doi:10.1016/j.bbrc.2018.12.173.

Connexin43 Regulates Osteoprotegerin Expression via ERK1/2 - dependent Recruitment of Sp1

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Abstract

In bone, connexin43 expression in cells of the osteoblast lineage plays an important role in restraining osteoclastogenesis and bone resorption. While there is a consensus around the notion that the anti-osteoclastogenic factor, osteoprotegerin, is a driver of this effect, how connexin43 regulates osteoprotegerin gene expression is unclear. Here, we showed that loss of connexin43 decreased osteoprotegerin gene expression and reduced ERK1/2 activation. Conversely, overexpression of connexin43 increased osteoprotegerin expression and enhanced ERK1/2 activation. This increase in phospho-ERK1/2 is required for connexin43 to induce activity from the osteoprotegerin proximal promoter. Connexin43 increased promoter activity via a specific 200 base pair region of the osteoprotegerin promoter located at –1486 to –1286 with respect to the transcriptional start site, a region which includes four Sp1 binding elements. Further, activation of this promoter region required an intact functional connexin43, as hypomorphic or dominant negative connexin43 mutant constructs, including one with increased hemichannel activity, were unable to stimulate osteoprotegerin expression as strongly as wild type connexin43. Using chromatin immunoprecipitations, we show that connexin43 expression enhanced the recruitment of Sp1, but not Runx2, to the osteoprotegerin proximal promoter. In total, these data show that connexin43-dependent gap junctional communication among osteoblast cells permits efficient ERK1/2 activation. ERK1/2 signaling promoted the recruitment of the potent transcriptional activator, Sp1, to the osteoprotegerin proximal promoter, resulting in robust transcription of anti-osteoclastogenic factor, osteoprotegerin.

Keywords

Gap Junctions; Connexin43; Osteoblast; Osteoprotegerin; Osteoclast

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Conflicts of Interest

The authors declare no conflicts of interest.

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1. Introduction

In the skeleton, osteoblasts, osteoprogenitors, and osteocytes share and amplify signals through connexin43 (Cx43, gene name *Gja1*)-containing gap junctions. This gap junctional intercellular communication between bone cells coordinates bone formation and bone remodeling to maintain cortical bone integrity [1, 2]. Loss or defects of Cx43 lead to skeletal dysplasia and alterations in cortical bone quality and geometry that closely approximate aging and disuse [2, 3]. During aging, osteocyte connectivity and connexin43 expression is remarkably diminished, consistent with a loss of gap junctional communication [4, 5]. Thus, understanding the molecular controls by which gap junctional networks affect bone represents an important therapeutic target. Decades of work on Cx43 biology in bone has demonstrated that it functions to communicate a diverse set of small molecules between bone cells to regulate an array of signal transduction pathways. These signaling pathways subsequently regulate osteoblast differentiation, restrain osteoclastogenesis, maintain osteocyte viability, and permit the proper organization and orientation of collagen in the bone extracellular matrix [1, 6–8].

Among the most striking and conserved phenotypes observed in mice selectively lacking Cx43 in the osteoblast lineage is an expansion of the marrow cavity as a result of hyperactive osteoclastic bone resorption on the endocortical, marrow facing surface of bone [9–12]. Enhanced osteoclastogenesis in a subset of Cx43 deletion or mutation models can also result in cortical thinning and increased cortical porosity [10, 12, 13]. The expansion of the marrow cavity caused by osteoblast-lineage deletion of Cx43 can be fully rescued by administration of the antiresorptive bisphosphonates, risedronate or alendronate, which inhibit osteoclast activity [14]. Given the osteoblast-lineage specificity of these Cx43 deletion models, the bone resorbing phenotype is due to a non-cell autonomous effect on the osteoclast lineage mediated by osteoblasts/osteocytes. The osteoblast lineage controls osteoclast formation and activity by regulating the ratio of the pro-osteoclastic factor, receptor activator of NF- κ B ligand (RANKL), and its anti-osteoclastogenic decoy receptor, osteoprotegerin (OPG) [15]. The RANKL to OPG ratio is elevated in the models of Cx43 deletion, most frequently attributed to a loss of OPG expression in Cx43 deficient osteoblasts [9–12, 16].

Despite the fundamental nature of Cx43 control on the expression of the OPG and the restraint of bone resorption, little is known about the molecular basis of its control. Here, we showed that Cx43 abundance regulated the transcription of the OPG encoding gene, *Tnfrsf11b*, from its proximal promoter. Specifically, decreasing Cx43 abundance reduced OPG expression and phospho-ERK1/2 levels. Conversely, increasing Cx43 abundance enhanced OPG expression by activating ERK1/2, increasing the recruitment of Sp1 to a 200 base pair region of the OPG proximal promoter containing four GC-rich Sp1 sites. In total, these data establish a molecular basis for the restraint of osteoclastogenesis by Cx43 coupled osteoblast lineage cells.

2. Materials and methods

2.1. Chemicals, antibodies and reagents

The following antibodies and reagents were from Cell Signaling Technologies: U0126, rabbit anti-phospho-ERK1/2 (#9101), total- ERK1/2 (#9102), and horseradish peroxidase-conjugated secondary antibodies. The mouse anti-GAPDH antibody (#MAB 374) was from Millipore. Rabbit anti-Cx43 (#C6219) was purchased from Sigma. The chromatin immunoprecipitation (ChIP) grade Sp1 antibody (#39058) was from Active Motif, and the anti-Runx2 antibody (#D130–3) from MBL International. All other chemicals were purchased from Sigma, unless indicated otherwise.

2.2. Cell culture

Primary murine long bone osteoblasts were isolated from *Gja1^{flox/flox}* mice (C57BL/6J background) mice and cultured as described [17, 18]. All animal procedures were performed with approval by the Animal Care and Use Committee at the University of Maryland School of Medicine. For *in vitro* deletion of the *Gja1*/Cx43 allele, primary osteoblasts from *Gja1^{flox/flox}* mice were transduced with adenovirus encoding GFP (control) or CRE recombinase in the presence of 0.5 µg/ml poly-L-lysine, as described [17]. UMR106 rat osteosarcoma cells were purchased from ATCC and were cultured, as described [19].

2.3. Western blotting

Western blotting of osteoblast whole cell extracts was performed, as described [20, 21]. Equal amounts of protein, as determined by BCA assay, were subjected to SDS-PAGE and transferred by semi-dry blotting to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were blocked in 5% non-fat dry milk, probed with the indicated primary and secondary antibodies, and detected with enhanced chemiluminescence detection reagents (Pierce). Blots were acquired and analyzed using an EpiChem gel documentation system (UVP Bio). All western blotting experiments were repeated a minimum of three times.

2.4. Plasmid constructs and transfections

The empty vector control (pSFFV-neo) or rat Cx43 expression vector (pSFFV-Cx43) have been described previously [22]. Cx43 constructs carrying loss of function point mutations identified in oculodentodigital dysplasia [23–26]. pSFFV-Cx43 G138R and pSFFV-Cx43 R76S were generated by Mutagenex from the pSFFV-Cx43 backbone vector, by a G412C and C266A mutations in the *Gja1* open reading frame, respectively (NCBI Reference Sequence: NM_012567.2). The –1486 to +133 OPG luciferase reporter (–1486 OPG Luc) and the –1286 to +133 OPG luciferase reporter (–1286 OPG Luc) have been described [27].

For transient transfections, cells were seeded at 2.8×10^4 cells/well in 48-well plates. After 24 hours, the cells were co-transfected with the appropriate plasmids with JetPRIME reagent (Polypus), as described [28]. The empty vector control (pSFFV-neo) or rat Cx43 expression vector (pSFFV-Cx43, pSFFV Cx43 G138R, or pSFFV Cx43 R76S) were co-expressed with the indicated OPG luciferase reporter. The MEK inhibitor, U0126 (10µM), was added to cells 16 hours prior to harvest. DMSO was used as a vehicle control. 48 hours post-transfection, cells were lysed and luciferase activity was monitored using a Berthold Centro

LB 960 luminometer, as described [19]. All plasmid DNA was prepared using an endotoxin free plasmid maxi prep kit (Qiagen).

2.5. Chromatin Immunoprecipitations and qPCR

Chromatin immunoprecipitations (ChIP) were performed using the ChIP-IT Express kit (Active Motif, #53008), according to manufacturer's suggestions. The rat OPG promoter primers were: Forward, AAC CCT GCC CCT GAT AAG AAA and Reverse, AGG TAG TCA CCC AGC TCT GCT T. Bead fractions were amplified by qPCR. For qPCR, input and beads fractions from the ChIP assays or reverse transcribed mRNA were prepared and examined, as described [29]. For ChIPs, data were normalized to input fractions. Controls were normalized to a value of 1. Primer sets for *Gja1*, *Tnfrsf11b*, *Hprt*, *Gapdh*, and *Rpl13* are available upon request.

2.6. Statistical analysis

Data presented are mean $\pm$ SD. Statistical significance was assessed by comparing mean values using a t-test, or an ANOVA with a Holm-Sidak's post-hoc multi-comparison test, as appropriate, using GraphPad Prism (v7).

3. Results and Discussions

A key function of Cx43 in osteoblast/osteocyte lineage is to restrain osteoclast activity and the subsequent loss of bone mass [10, 14]. This is accomplished through modulation of the expression of the ratio of the pro-osteoclastogenic factor, RANKL, and its negative regulator, OPG [9–12, 16]. However, the molecular basis of how this is regulated is unclear. Here, we show by quantitative RT-PCR that genetic deletion of the *floxed Gja1* alleles in primary osteoblasts was sufficient to markedly reduce osteoprotegerin (gene name: *Tnfrsf11b*) gene expression levels (Fig. 1A and B). One of the ways that Cx43 influences the expression of osteoblast genes is via the ERK1/2 pathway [20, 30–36]. Here, we observed that loss of Cx43 in primary osteoblasts decreased phospho-ERK1/2 levels (Fig. 1C).

In contrast to the loss of function, Cx43 overexpression can increase OPG/*Tnfrsf11b* gene expression in MC3T3 osteoblasts *in vitro* [20]. Consistent with the findings in MC3T3 cells, overexpression of Cx43 markedly increased transcription of an OPG proximal promoter luciferase promoter construct (–1486/+133 OPG-Luc reporter) in UMR106 cells (Fig. 2A). Cx43 expression also increased phospho-ERK1/2 levels relative to an empty vector control (Fig. 2B). In addition to ERK-dependent control, there are also ERK-independent pathways by which Cx43 influences osteoblast gene expression [5, 16, 19, 37–42]. Thus, to assess the contribution of the altered ERK1/2 signaling caused by Cx43 expression on OPG transcription, we examined the effects of treatment with the MEK inhibitor, U0126, on expression of the –1486/+133 OPG-Luc reporter. Treatment of the cells with U0126 dramatically decreased transcription from this promoter, preventing Cx43 overexpression from enhancing OPG promoter activity and reducing phospho-ERK1/2 levels in the cells (Fig. 2A and C).

Cx43-dependent regulation of ERK1/2 influences osteoblast gene expression via phosphorylation and activation of the transcription factors, Sp1 and Runx2 [20, 30–35]. Both

Sp1 and Runx2 sites are found in the OPG proximal promoter (Fig. 3A) [27, 43]. Specifically, the OPG proximal promoter has four Sp1 binding sites in the region from -1486 to -1286, relative to the transcriptional start site, and none from -1286 to +133. However, within the -1286 to +133 region of the OPG proximal promoter, there are two Runx2 binding sites [27]. Thus, we examined how changes in Cx43 expression may impact transcription of the OPG gene through these Runx2 and Sp1 binding elements. Expression of Cx43 increased the basal transcription of the -1486/+133 OPG-Luc reporter (Fig. 3B) containing both Sp1 and Runx2 binding elements, but Cx43 expression failed to stimulate transcription from the -1286/+133 luciferase OPG-Luc reporter, which contains the Runx2 binding elements, but not Sp1 binding sites (Fig. 3C). These data suggest that Cx43 regulates OPG transcription via the Sp1 but not the Runx2 transcription factor binding elements.

Cx43 can act not only as a source of direct intercellular communication via gap junction channels, but also as a hemichannel, serving as a direct conduit between the cytoplasm and the extracellular space [7, 44, 45]. Additionally, Cx43 has some structural functions independent of its channel [46–48]. In order to evaluate if the regulation of OPG expression required direct, intercellular communication via gap junction channels or one of these other functions, we overexpressed two mutant connexin constructs, Cx43 G138R and R76S, which are disease causing mutations contributing to oculodentodigital dysplasia and have hypomorphic or dominant negative function for gap junctional intercellular communication [24–26]. Notably, the Cx43 G138R mutant has been shown to have hyperactive hemichannel function, despite defects in intercellular communication [24]. Consistent with the requirement of intercellular communication through functional Cx43 channels as a driver of OPG expression, neither expression of Cx43 G138R or Cx43 R76S were able to stimulate OPG transcription from the -1486/+133 OPG-Luc reporter as robustly as wild type Cx43 (Fig. 3B). None of the connexin constructs had any effect on the -1286/+133 luciferase OPG-Luc reporter that lacks identified Sp1 binding elements. These data are supportive of the notion that intercellular communication through Cx43 containing channels increased OPG transcription from the Sp1 rich domain located between -1486 to -1286 of the OPG proximal promoter. Further, these effects on OPG likely require intercellular communication rather than by hemichannels or structural function of Cx43 independent gap junctional communication.

Finally, to confirm that ERK1/2 activation converged on the recruitment of Sp1 to the OPG proximal promoter, we performed chromatin immunoprecipitations (ChIPs) in transfected UMR106 cells. Wild type Cx43 expression enhanced the association of Sp1 with the OPG promoter compared to empty vector or mutant Cx43 constructs (Fig. 4A). In contrast, Runx2 association with the OPG proximal promoter occurred at the same rate independent of Cx43 overexpression (Fig. 4B). These data confirm that functional Cx43, capable of supporting direct intercellular communication, increases the association of Sp1 with the OPG promoter.

In total, these data strongly support that Cx43-dependent gap junctional communication among osteoblast cells permits efficient ERK1/2 activation. The gap junction-dependent communication activation of ERK1/2 leading to the recruitment of the potent transcriptional activator Sp1 to the OPG proximal promoter, leading to robust transcription of OPG and

suppression of osteoclastogenesis (Figure 4C). These data underscore the fundamentally important role for Cx43 in the control of ERK1/2 signaling, and its multifaceted influence on osteoblasts. These data also validate a Cx43-ERK1/2-Sp1 axis that appears to be conserved among different cell types [32, 35, 49–51]. Likewise, given the fundamental role of Cx43 in the restraint of cortical bone resorption and similarities between loss of Cx43 to bone changes observed during aging or disuse, these data add to molecular controls by which gap junctional networks affect bone and provide additional therapeutic targets for bone loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The –1486 to +133 OPG luciferase reporter (–1486 OPGLuc) and the –1286 to +133 OPG luciferase reporter (–1286 OPGLuc) were generously provided by Dr. Malayannan Subramaniam (Mayo Clinic, Rochester, MN).

Funding

This work was supported by a grant, R01-AR063631 (JPS), from the National Institutes of Health/National Institute for Arthritis, Musculoskeletal and Skin Diseases. NRG was supported by an institutional training grant (NIGMS, T32 GM008181).

Abbreviations:

Cx43	Connexin43
OPG	osteoprotegerin
RANKL	receptor activator of NF- κ B ligand
ERK1/2	extracellular signal regulated kinase 1/2
ChIP	chromatin immunoprecipitation
GFP	Green Fluorescent Protein

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Highlights

- Cx43 gain or loss of function regulates OPG expression from its proximal promoter
- Cx43 regulates ERK1/2 to enhance Sp1 binding to the –1486/–1286 OPG promoter
- Direct gap junctional intercellular communication is required to stimulate OPG

Figure 1. Loss of Cx43 in osteoblasts reduces OPG expression and ERK1/2 activation. qPCR of (A) OPG (*Tnfrsf11b*) and (B) Cx43 (*Gja1*) following Cre-mediated deletion of Cx43 by adenoviral transduction and expression of Cre recombinase in primary osteoblasts from *Gja1**lox/lox* mice (n=10 to 12). **, p-value <0.01, ***, p-value < 0.001 relative to control (GFP) by t-test. (C) Representative western blot of phospho-ERK1/2 and Cx43 protein abundance following deletion of *Gja1* from primary osteoblasts. GAPDH is used as a loading control. Bar graph shows the ratio of phospho-ERK 1/2/total ERK1/2 (n=3).

Figure 2. ERK1/2 is required for the Cx43-dependent effects on transcription from the OPG proximal promoter.

(A) Luciferase reporter assay performed in UMR106 osteoblasts co-transfected with a -1486/+133 OPG promoter driven luciferase reporter construct (-1486/+133 OPG Luc) and an empty vector (EV) control or Cx43 expression vector (Cx43) and treated with the ERK1/2 pathway inhibitor, U0126 (10 μ M, 16h) or DMSO vehicle control (n=4/group). ***, p-value < 0.001 relative to empty vector (EV) control sample by ANOVA. (B) Representative western blot of whole cell extracts of UMR106 treated as above and blotted for phospho-ERK1/2 and GAPDH, as a loading control. Bar graph shows the ratio of pERK1/2/GAPDH (n=3). **, p-value relative to empty vector (EV) control by t-test. (D) Representative western blot showing phospho-ERK1/2 and Cx43 expression in UMR106 osteoblasts following transfection with empty vector (EV) control or Cx43 expression vector (Cx43).

Figure 3. A functional Cx43 channel stimulates OPG promoter activity from a region between -1486 to -1286 relative to the transcriptional start site.

(A) Diagram of the OPG proximal promoter. Numbers represent the number of nucleotides relative to the transcriptional start site (+1). Four Sp1 sites located in the region from -1486 to -1286 are shown, as are the two Runx2 binding elements located at -994 to -990 and -309 to -303, respectively. (B) Luciferase reporter assay performed in UMR106 osteoblasts co-transfected with -1486/+133 OPG Luc or -1286/+133 OPG Luc plasmid and either an empty vector (EV) control, wild type Cx43 expression vector (Cx43), or mutant Cx43 plasmids bearing the G138R or R76S mutation. ***, p-value < 0.001 relative to empty vector (EV) control sample by ANOVA.

Figure 4. A functional Cx43 channel regulates Sp1 recruitment to the OPG proximal promoter. (A) ChIP performed in UMR106 osteoblasts transfected with an empty vector (EV) control, wild type Cx43 expression vector (Cx43), or mutant Cx43 plasmids bearing the hypomorphic G138R or R76S mutation and precipitated using Sp1 antibodies. Data show result of qPCR on bead fractions amplified by the primers to the OPG proximal promoter (n=4/group). Data are normalized to inputs. The empty vector (EV) control is set to a value of 1. ***, p-value < 0.001 relative to empty vector (EV) control sample by ANOVA. (B) (A) ChIP performed in UMR106 using Runx2 antibodies (n=4/group). (C) Model of Cx43-dependent regulation of *Tnfrsf11b*/OPG gene expression and inhibition of osteoclastogenesis.