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Connexins in The Skeleton

Joseph P. Stains[1] and **Roberto Civitelli**[2]

[1]Department of Orthopaedics, University of Maryland, School of Medicine, Baltimore, MD

[2]Division of Bone and Mineral Diseases, Department of Internal Medicine, Washington University in St. Louis, St. Louis, Missouri, United States of America

Abstract

Shaping of the skeleton (modeling) and its maintenance throughout life (remodeling) require coordinated activity among bone forming (osteoblasts) and resorbing cells (osteoclasts) and osteocytes (bone embedded cells). The gap junction protein connexin43 (Cx43) has emerged as a key modulator of skeletal growth and homeostasis. The skeletal developmental abnormalities present in oculodentodigital and craniometaphyseal dysplasias, both linked to Cx43 gene (*GJA1*) mutations, demonstrate that the skeleton is a major site of Cx43 action. Via direct action on osteolineage cells, including altering production of pro-osteoclastogenic factors, Cx43 contributes to peak bone mass acquisition, cortical modeling of long bones, and maintenance of bone quality. Cx43 also contributes in diverse ways to bone responsiveness to hormonal and mechanical signals. Skeletal biology research has revealed the complexity of Cx43 function; in addition to forming gap junctions and “hemichannels”, Cx43 provides a scaffold for signaling molecules. Hence, Cx43 actively participates in generation and modulation of cellular signals driving skeletal development and homeostasis. Pharmacological interference with Cx43 may in the future help remedy deterioration of bone quality occurring with aging, disuse and hormonal imbalances.

Keywords

Cx43; Cx37; gap junction; bone; signal transduction

1. Introduction

Bone is constantly formed and degraded in order to adapt to mechanical and metabolic demands. The ability to coordinate the function of bone forming and resorbing cells is essential for translating tissue level mechanical and metabolic inputs to single cell effectors for the maintenance of bone quality. Gap junctional intercellular communication is an

To Whom Correspondences should be addressed: Roberto Civitelli, MD, Division of Bone and Mineral Diseases, Washington University in St. Louis, Campus Box 8301, 425 South Euclid, St. Louis, MO 63110 USA, rcivitel@dom.wustl.edu, Phone: 314 454 8431, Fax: 314 454 5047.

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important mechanism of cell-to-cell communication in bone as in many other tissues. Gap junctions form by the pairing (docking) of two hexameric channels (connexons, or "hemichannels") on cell membranes of opposing cells to form an aqueous pore that permits the direct exchange of small molecules between the cytoplasm of the coupled cells. Connexons are composed by six subunits, connexins, which dictate the size and charge selective permeability of the gap junction channel [1, 2] (Fig. 1). There are 20 connexin genes in the mouse and 21 (plus one pseudogene) in humans [3] Although most commonly monomeric, connexons can form heteromeric connexons composed of different connexins. Heterotypic channels (docking of two connexons with different connexin composition) can also occur. This allows for large plasticity in the biophysical properties of gap junctions, dictating not only which molecules can be communicated, but also how the open/closed state of the channel is regulated, and what downstream signaling molecules can be recruited to the gap junction [4–6].

A metaphor of a computer network can describe the multicellular ensemble of gap junction-coupled cells. In this analogy, the cells represent the computers and the gap junctions loosely represent the modem/router. Connexin abundance determines the *bandwidth*, which can be fine-tuned through modulation of the open/closed state or abundance of the gap junction channels to affect *actual* bandwidth. Much like a computer network, the information exchanged across the intercellular network "administered" by gap junctions is diverse and dictates many functions. Hence, gap junctions can specifically restrict or permit certain types of information being exchanged among cells in the network. As discussed in this review, regulation of bone homeostasis by connexins exemplifies this concept (Fig. 2). Via different molecular mechanisms, connexin43 (Cx43) – the most abundant connexin present in bone cells (Figs. 3, 4), modulates bone modeling and remodeling, the response to hormonal and mechanical stimuli, and the expression of osteo-anabolic and osteo-catabolic genes. While much less is known about other bone connexins, Cx40, Cx45 and Cx46, very recent data reveals distinct roles for Cx37 in bone development and homeostasis.

2. Connexins in Human Skeletal Disease

In the last decade, mutations of Cx43 gene (*GJA1*) have been identified in patients with skeletal dysplastic syndromes. The increasing number of disease-causing Cx43 mutations and the breadth of the clinical spectrum related to such mutations provide a genetic demonstration that the skeleton is a major site of Cx43 action, a notion that had already emerged from animal studies. Advances in human genetics have also inspired the development of mouse models of human Cx43 disorders.

2.1 Oculodentodigital Dysplasia

A large number of *GJA1* mutations have been found in patients with oculodentodigital dysplasia (ODDD), a disease affecting multiple organs but primarily the skeleton, with characteristic craniofacial abnormalities (skull hyperostosis, pointed nose, enamel hypoplasia), aplastic or hypoplastic middle phalanges, syndactyly, and broad tubular long bones [7–9]. Underscoring a predominantly autosomal dominant inheritance, Cx43 mutants found in ODDD typically act as dominant negative; they are assembled in gap junctions but the intercellular channel is functionally defective [10–12]. However in some families, the

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ODDD phenotype has a recessive inheritance pattern. Further, the ODDD clinical spectrum can be variable, underscoring the complexity of Cx43 function in the skeleton (reviewed in [9]).

Mouse models of ODDD have been developed. A germline mutant generated through N-ethyl-N-nitrosourea mutagenesis, called *Gja1*$^{Jrt/+}$, harbors a heterozygous *Gja1* mutation (G60S). Although this mutation is not found in humans, the skeletal features of *Gja1*$^{Jrt/+}$ mice reproduce many of the features of ODDD patients, including syndactyly, enamel hypoplasia, and craniofacial anomalies [10]. Additionally, bone mineral density is abnormally low in *Gja1*$^{Jrt/+}$ mice, with decreased trabecular bone volume and reduced mechanical strength, features not yet described in the human disease. *Gja1*$^{Jrt/+}$ mice also exhibit thin cortical bone and enlarged marrow cavity in the femoral diaphysis. In another approach, the *Gja1* G138R point mutant – found in several ODDD families – was used to replace one wild type *Gja1* allele using the Cre/*loxP* method. Induction of a global *Gja1* to *Gja1*^{G138R} gene replacement using the ubiquitously expressed PGK-Cre in the mouse (cODDD^{PGK}) produced many of the multi-organ phenotypic features of human ODDD, including craniofacial abnormalities, but also decreased trabecular bone volume [12]. Conditional replacement of one wild type allele with the *Gja1*^{G138R} allele in cells of the chondro-osteogenic lineage by *Dermo1/Twist2-Cre*, which is expressed starting at E9.5 (cODDD^{TW2}) recapitulates all the skeletal defects seen in the global cODDD^{PGK} and *Gja1*$^{Jrt/+}$ mice, thus demonstrating that the osteogenic lineage is central for Cx43 modulation of skeletal development and homeostasis [13]. Consistent with the other two mouse ODDD models, whole body bone mineral density is reduced in cODDD^{TW2} mice, and this is associated with cortical thinning and a pronounced widening of diaphyseal cross sectional area; while trabecular bone is essentially unaffected [13]. As discussed more in depth later, expansion of the marrow cavity and cortical thinning are also seen in mice with conditional *Gja1* ablation in the osteogenic lineage. Thus, ODDD mutations in the mouse phenocopy most of the human disease, but they also reveal additional features not described in the human disease. The most prominent differences are in the cranial vault. While the skull is thickened in the human disease, mineralization of the skull is delayed and defective at birth in cODDD^{TW2} mice. Furthermore, low bone density has not been described in humans with ODDD, although the skeletal features of these patients have not been studied in detail. Notably, both the *Gja1*^{Jrt} and the *Gja1*^{G138R} mutants are dominant negative for channel function [10] and thus may also interfere with other connexins (*e.g.*, Cx37, Cx40, or Cx45) in forming functional channels in cells that express multiple connexins, such as osteolineage cells. In so doing, they may also interfere with gap junctional communication between osteoblasts (or osteocytes) and other cells present in bone that express a different repertoire of connexins (*e.g.*, vascular endothelial cells).

2.2 Craniometaphyseal Dysplasia

Craniometaphyseal dysplasia (CMD) is characterized by cranial hyperostosis and defective modeling of tubular bones, features shared with ODDD, but without syndactyly [14]. The dominant form of CMD is caused by mutations of the *ANKH* gene, encoding a pyrophosphate transporter [15]. Recently, 4 families with a recessive form of CMD have been reported carrying a *GJA1* mutation [16]. Remarkably, patients with recessive CMD

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have a characteristic flaring and undertrabeculation of the diaphysis of long bones, resembling the Erlenmeyer flask shape present in other metaphyseal dysplastic syndromes, and widening of the phalanges [16]. Intriguingly, while almost all the known ODDD mutations are upstream of the C-terminus of Cx43 and affect channel function [9], the mutant linked to recessive CMD, R239Q is located in the C-terminus, which is important for Cx43 interaction with microtubules and signaling molecules (see section 5.3). Lack of dental, ocular defects and syndactyly suggests that recessive CMD may not be part of the ODDD spectrum, possibly underlying different disease mechanisms linked to interference with specific functions of Cx43. The decreased trabecular bone mass without the cortical phenotype recently reported in mice overexpressing a Cx43 mutant lacking most of the C-terminus tail (see section 3) would be consistent with this hypothesis. Development of mouse models carrying the CMD-causing *Gja1* mutations might help clarify these open questions.

2.3 Other Skeletal Disorders Linked to Connexin Mutations

A *GJA1* mutation (R76H) has been found in one patient with Hellermann-Streiff syndrome (HSS), whose skeletal features partially overlap those of ODDD, in particular craniofacial dysmorphism, enamel hypoplasia, and clynodactyly [17]. Intriguingly, the R76H and R33X mutations have also been found in patients with ODDD [18]. Since these mutations are in the 3rd transmembrane domain of Cx43, it is likely that that both mutants disrupt Cx43 channel activity; hence, HSS may be part of the ODDD spectrum. Dysmorphic features of the upper extremities with fusion of skeletal elements in the wrist and phalanges are present in mice lacking Cx40 (*Gja5*$^{-/-}$) and to a lesser extent in heterozygous *Gja5*$^{-/+}$ mice [19]. Notably, similar dysmorphic features are present in mice with haploinsufficiency of T-box transcription factor, Tbx5 [19], a model of Holt-Oram syndrome, a cardiovascular disorder also characterized by dysmorphisms, sternal malformations and fusions of the upper limb bones [20]. Since Cx40 is a downstream target of Tbx5, these observations indicate that Cx40 is involved in upper limb patterning. Whether Cx40 is functionally active in adult bone is unknown. To date, there are no reports of human skeletal disorders that can be linked to Cx37 or Cx45, the other two connexins present in bone cells, although *GJA4* (Cx37 gene) polymorphisms are associated with low bone mass in Japanese men [21].

3. Cx43 in Skeletal Development and Homeostasis

Craniofacial abnormalities and developmental defects of the axial skeletal had been observed by *Gja1* “knockdown” in chick embryos in the late 1990s using antisense oligonucleotides [22]. However, it was the development of *Gja1*$^{-/-}$ mice by Drs. Janet Rossant and Gerald Kidder in 1995 [23] that provided the first genetic tool to understand Cx43’s biologic role in the skeleton. Although no major dysmorphisms are evident in *Gja1*$^{-/-}$ mice at birth, detailed skeletal analysis revealed reduced mineralization of the axial and appendicular skeleton and the cranial vault during embryonic development through E18.5, associated with delayed osteoblast differentiation [24]. These early in vivo studies from our group propelled research on the role of Cx43 in the post-natal skeleton.

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3.1 Cx43 Drives Cortical Bone Modeling

To overcome the post-natal lethality in *Gja1*$^{-/-}$ mice [23], several conditional gene deletion models (cKO) have been generated using promoter-Cre drivers that target the osteogenic lineage. Details on the different conditional *Gja1* ablation models have been discussed elsewhere in greater detail [25–27]. While the skeletal phenotype is more severe with broader *Gja1* ablation, there is a common long bone phenotype among all the *Gja1* cKO models, consisting of widening of the diaphyses, expanded marrow cavity, and decreased bone strength. This cortical modeling defect is secondary to increased endocortical bone resorption and increased periosteal bone formation. The important "take homes" emerging from these mouse genetic studies are as follows: (1) Cx43 in the osteoblast lineage plays an important role for osteoblast function, matrix production, and for modulation of secretory products that regulate osteoclastogenesis [13, 28, 29] and hematopoietic cells [30]. (2) Cortical bone is the primary site of Cx43 action, where it functions to restrain endocortical osteoclast development. It is not yet clear whether the accentuated periosteal bone formation represents an adaptive response to the endocortical expansion or a cell autonomous effect brought about by lack of Cx43. Nonetheless, increased bone resorption is key, as the bone resorption inhibitors, bisphosphonates, can rescue the cortical thinning and improve bone strength in adult mice [31]. (3) The increased endocortical resorption leading to an expanded marrow cavity in Cx43-deficient mice is caused by decreased osteoblast/osteocyte secretion of the osteoclast inhibitor, osteoprotegerin (OPG, *Tnfrsf11b*), or by increased production of the pro-osteoclastogenic factor, Receptor Activator of Nuclear factor κ-B Ligand (RANKL, *Tnfsf11*) or both [13, 29, 32]. (4) The mechanism of increased periosteal bone formation in *Gja1* cKO mice remains to be determined, even though decreased sclerostin (*Sost)*, a canonical Wnt antagonist and important bone remodeling regulator, may play a role [28, 33]. (5) Differentiated osteoblasts and osteocytes play a predominant role in mediating the effects of Cx43 deficiency on long bone geometry; whereas *Gja1* ablation in osteogenic precursors also decreases whole body bone mass, the consequence of increased cortical porosity and production of an under-mineralized matrix with abnormal collagen organization. (5) In a healthy, growing animal, the overall contribution of Cx43 is to supports osteoblast function and bone matrix production, osteocyte survival, and restricting osteoclastic bone resorption.

3.2 Cx43 in Trabecular Bone Remodeling

While Cx43 predominantly acts in cortical bone, in certain conditions an action on the trabecular compartment emerges. Earlier studies using the 2.3kb-Col1A1 promoter to ablate *Gja1* (cKO^{Col1A1}) reported reduced mass in skeletal segments high in trabecular bone, such as the spine using dual energy X-ray absorptiometry [34]. However, a trabecular phenotype was not confirmed in other *Gja1* cKO models [13, 35]. More to the point, germline mouse mutants mimicking ODDD (*Gja1*$^{Jrt/+}$ and *Gja1*$^{G138R/+}$) have reduced trabecular bone volume [10, 12, 36]; as have mice harboring a Cx43 truncation mutant that lacks the Cx43 C-terminus (*Gja1*^{K258Stop}) [37]. Interestingly, since the trabecular phenotype in *Gja1*^{K258Stop}-carrying mice occurs regardless of the presence or absence of Cx43 in differentiated osteoblasts and osteocytes, it is possible that the mutant acts as dominant negative on Cx43 at earlier stages of osteoblastogenesis. Reduced osteoblast precursor

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proliferation and/or differentiation would explain the reduced osteoblast number in the trabecular compartment reported in these mice [37], a scenario fully consistent with trabecular abnormalities observed in mice with a germline heterozygous ODDD mutation [10]. These mouse models have opened new questions about the role of Cx43 that requires further investigation.

3.3 Cx43 Favors Processes that Improve Bone Quality

There are numerous mechanisms by which Cx43 influences bone quality. In addition to the regulation of the RANKL/OPG ratio, as detailed above, several groups have shown that collagen processing, and specifically collagen cross-linking by lysyl oxidase (*Lox*), is abnormal in osteoblast *Gja1* cKO mice [13, 28], leading to disorganized fibrillar collagen, hypomineralized bone matrix and decreased bone strength; these abnormalities are particularly severe in cKO^{Tw2} mice, where Cx43 is absent in the entire chondro-osteogenic lineage [13]. As already noted, diminished sclerostin expression, a key regulator of bone homeostasis, may explain the enhanced osteo-progenitor proliferation and exuberant periosteal apposition observed Cx43 cKO knockouts [13, 32, 38, 39]. Therefore, Cx43 is a positive regulator of sclerostin, although this action is most likely indirect [32], as a consequence of either increased osteocyte viability [32], or stimulation of osteoblast differentiation [13]. Lastly, in the $Gja1^{Jrt/+}$ ODDD mice, deposition of a bone matrix markedly enriched in bone sialoprotein caused by osteoprogenitor expansion contributes to increase bone resorption by a direct effect of bone sialoprotein on osteoclastogenesis, an effect abrogated by crossing these mice into a $Bsp^{-/-}$ background [36].

4. Cx43 in Adaptive Responses to Skeletal Stimuli

While Cx43 plays a largely osteoanabolic role in healthy mice, remarkable complexities of Cx43 action in bone have emerged from adaptive responses to various stimuli, including hormonal challenges, mechanical loading and unloading, aging and fracture healing. This research has underscored the need to better identify and decode the unique molecular signals that are propagated by gap junction channels.

4.1 Hormonal Stimulation

The first evidence that Cx43 is involved in the elaboration of hormonallystimulated osteoanabolic signals was provided by the attenuated effect of parathyroid hormone (PTH) in cKO^{Col1A1} mice [34]. Daily administration of PTH-(1–34) at doses that activate bone formation and increase bone mass in normal mice induces severely attenuated responses in Cx43 deficient mice [34]. Earlier in vitro data demonstrated that interference with *Gja1* expression hinders PTH stimulation of cAMP production [40] and matrix mineralization by osteoblastic cell lines [41]. Although the mechanisms by which Cx43 modulates this hormonal responsiveness are not clear, these results underscore the importance of Cx43 in adaptive responses to a hormonal challenge at the tissue level. Intriguingly, Cx43 contributes to the diffusion of survival signals among osteoblasts following treatment with PTH-(1–34) via sequestration of βarrestin by Cx43 [35].

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4.2 Mechanical Stimuli

The widened and thinner cortices present in Cx43 deficient bones share striking similarities with changes in bone structure occurring after mechanical unloading (e.g. space flight, prolonged bed rest), as well as in aged bone [42]. There is no evidence that mice with a conditional *Gja1* deletion in bone forming cells age prematurely; however, it is quite clear that Cx43 is key for transduction of mechanical signal to skeletal cells. First, the enlarged marrow cavity and cortical thinning evident in *Gja1* cKO mice are not present at birth, but develop post-natally and become evident after the animals have been able to ambulate in normal gravity conditions [27], suggesting that the cortical modeling abnormalities reflect a mechano-sensing defect. Observations from different groups have corroborated this premise, leading to the conclusion that in the absence of Cx43 cortical bone perceives normal mechanical loading as a disuse scenario, resulting in abnormal activation of endocortical osteoclasts and eventual expansion of the marrow cavity [13, 29, 32, 33]. These studies have been recently reviewed in detail elsewhere [26, 27, 43]; and overall they provide compelling evidence that osteogenic cells at endocortical and periosteal surfaces respond in different and even opposite ways to application of mechanical load; absence of Cx43 amplifies such a difference. While in an early study from our group a 3-point bending protocol resulted in reduced bone endocortical bone formation in cKO^{Col1A1} mice [44], an opposite effect has been consistently reporter later at the periosteal surface. Using either cantilever bending of the tibia in cKO^{OG2} mice – in which *Gja1* is ablated by OG2-Cre in mature osteoblasts and osteocytes – [29], or axial tibial compression in cKO^{Tw2} mice [38], or ulna compression in cKO^{Dmp1} mice [45], to apply mechanical load, a consistent enhancement of periosteal bone formation response has been reported in Cx43-deficient mice relative to wild type mice, even though with some slight differences among the different experimental settings. Thus, lack of Cx43 increases the sensitivity of periosteal bone formation to mechanical load.

Cx43 also modulates response to skeletal unloading. Our group reported that cortical bone of *Gja1* cKO^{Col1A1} was insensitive to the effect of Botulinum Toxin Type A (BtxA)-induced muscle paralysis, whereas wild type mice experienced a significant expansion of the medullary cavity and cortical thinning associated with increased number of endocortical osteoclasts [33]. Such changes are essentially identical to those caused by loss of Cx43. Indeed, osteoclast number and cortical thickness after induction of muscle paralysis in wild type mice were very similar to control, non-BtxA injected cKO^{Col1A1} mice. In this study, there was no difference in the profound cancellous bone loss after BtxA injection between Cx43 deficient and wild type mice. These findings have been corroborated, in large part, by another study using the tail suspension method for mechanical unloading, which caused loss of both trabecular and cortical bone in wild type animals, while changes in cortical (and trabecular) bone were attenuated in *Gja1* cKO^{OG2} mice [46]. These findings reinforce the notion that the cortical geometry of Cx43-deficent bones resembles that of disuse bones.

4.3 Fracture Healing

Cx43 expression is upregulated in the fracture callus [47, 48]. Accordingly, fracture healing is impaired in *Gjai1* cKO^{Col1A1} mice [48], which is consistent with delayed osteoblast differentiation and bone formation [34]. This results in decreased bony fraction of the developing callus. There is a transient negative effect on osteoclastogenesis with an

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unexpected decrease in the RANKL/OPG ratio, the opposite of what is seen in the same and other models of osteolineage specific *Gja1* ablation in the context of normal bone physiology [13, 29, 32]. Intriguingly, β-catenin activity is decreased in the fracture callus of *Gjai1* cKO^{Col1A1} mice; and increasing β-catenin stability with LiCl restores normal bone formation in the callus [48]. These data exemplify the concept that Cx43 regulation of bone cell function is context dependent; specifically, Cx43 facilitates new bone formation in fracture healing, while it restrains periosteal bone formation in normal physiology. Likewise, Cx43 restrains bone resorption in normal conditions, while it increases osteoclastogenic signals in fracture healing. This context-dependent function of Cx43 may be related to specific extracellular cues that bone cells are exposed to during facture healing relative to normal physiology. Thus, the signals propagated by Cx43 gap junctions or hemichannels may be different, leading to distinct biologic outcomes.

4.4 Aging

An interesting observation made in *Gja1*$^{Jrt/+}$ mice has provided some insights on Cx43 role in the aging skeleton. Bone quality deteriorates with age at a faster rate in *Gja1*$^{Jrt/+}$ mice than in wild type mice [36]; trabecular bone mass and strength reach a minimum by 4 months of age, while they continue to decline up to 12 months of age in wild type mice. Somewhat similar results were seen in the cortical compartment. These data can be interpreted to suggest that: (a) loss of gap junctional communication abrogates the exchange of aging, or “catabolic” signals, thus preserving the bone from further deteriorating; or, (b) the Cx43 mutant causes bone quality to decline faster until reaches a minimum that is only reached in wild type mice during aging. In either case, these results strengthen the notion that Cx43 coordinates diffusion of signals that drive either bone anabolism or bone catabolism, depending on the physiologic context. Defining the molecular signals that mediate such changes can potentially uncover fundamental mechanisms by which bone quality can be optimized and maintained.

5. Mechanisms of Signal Modulation by Cx43 in Skeletal Cells

That adaptive skeletal responses to hormonal and mechanical stimuli are Cx43-dependent underscores the importance of the molecular signals that are propagated by Cx43 gap junctions. However, Cx43 connexons can also function as “hemichannels”, through which metabolites and signaling molecules can be released into the extracellular space. A third, emerging mechanism of Cx43 action is to provide a scaffold for signaling pathway component at the cell membrane, thus optimizing downstream signaling.

5.1 Gap Junction-Dependent Communication of Second Messengers

Gap junctions formed by Cx43 transmits numerous second messengers and signaling molecules from cell to cell. The first example is represented by mechanically stimulated intracellular Ca^{2+} waves, which propagate among osteoblasts and osteocytes in culture via gap junctions [49–51]. These observations have been corroborated by e*x vivo* imaging of Ca^{2+} signaling in intact bone, where gap junction-dependent propagation of Ca^{2+} oscillations were recorded in resting conditions and in response to fluid shear stress [52, 53]. These studies elegantly demonstrate that an elaborate interconnected communication

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network exists among osteocytes and osteoblasts. Indeed, establishing gap junctional communication between osteoblasts and osteocytes in co-culture is sufficient to increase expression of osteoblast genes [49, 54–56]. Furthermore, osteocytes can sense mechanical strain and communicate it to co-cultured osteoblasts to affect their function in a Cx43-dependent manner [49, 56, 57].

One key target of Cx43 action in osteoblasts and osteoprogenitors is Runx2, a master regulator of osteoblastogenesis. Going back to the computer network metaphor, increasing or decreasing the bandwidth of gap junctional intercellular communication by changes in Cx43 expression parallels changes of Runx2 transcriptional activity [58], and requires direct cell-to-cell contact [59]. Cx43 abundance also modulates the ERK and PKCδ pathways [58, 59]. One of the second messengers contributing to the activation of PKCδ, and in turn Runx2, is $InsP_7$, a product of inositol hexakisphosphate kinase [60], and of appropriate size (~740 Da) to pass through Cx43 gap junctions. However, while synthesis of $InsP_7$ is required for Cx43-dependent effects on PKCδ and Runx2, direct evidence of cell-to-cell transfer of $InsP_7$ or related metabolites has not been demonstrated. Nonetheless, involvement of Cx43 in Runx2 regulation is supported by downregulation of Runx2 downstream gene expression in cells isolated from Cx43-deficient mice [34, 61], and by a recent and still preliminary report demonstrating that compound heterozygous *Gja1*$^{+/-}$;*Runx2*$^{+/-}$ mice have a skeletal phenotype that largely phenocopies *Gja1* cKO mice [62].

Another mechanisms by which Cx43 regulates osteoblast gene expression is modulation of DNA binding affinity of the transcriptional activator Sp1 via the ERK signaling cascade [63, 64], which also affects transcriptional activity of another master regulator of osteogenesis, Osterix/Sp7 [65]. Among the many pathways that converge on ERK signaling is the cAMP/protein kinase A (PKA) system. Underscoring the importance of cell-cell diffusion of cAMP, interference with Cx43 using antisense oligonucleotides in osteoblastic cells reduces cAMP levels without affecting adenylate cyclase activity in response to PTH [66]. Notably, recent preliminary work suggests that Cx43 can amplify cAMP-dependent signaling and PKA-dependent gene transcription in osteoblasts in a cell-to-cell contact dependent manner [67]. As also noted, Cx43 can bind and sequester β-arrestin, thus preventing β-arrestin inhibition of cAMP signaling [60]. The resistance of *Gja1* cKO^{Col1A1} to the anabolic actions of intermittent PTH administration [34] provides further in vivo evidence of the importance of sharing cAMP among the network of connected osteoblasts and osteocytes.

5.2 Hemichannel-Mediated Paracrine Signaling

Exposure of osteocyte-like cells to fluid flow in culture increases cellular uptake of membrane impermeable fluorescent probes, a phenomenon interpreted as opening of Cx43 hemichannels [68]. This is associated with release of prostaglandins [68, 69] and ATP [70] into the extracellular fluid. Fluid flow-induced hemichannel opening requires physical interaction between Cx43 and α5β1 integrin, triggered by mechanical forces and activation of the Akt pathway [71, 72]. In an attempt to extend these observation in vivo, two transgenic mouse strains were generated using the 10kb-*Dmp1* promoter to drive expression of either a *Gja1* R76W point mutant, which can form hemichannels but not functional gap

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junctions, or a mutant lacking the coding sequence for amino acids 130–136 (Δ130–136), which is devoid of both gap junction and hemichannel function [73]. As noted earlier, mutations of R76 have been reported in ODDD patients [9]. The characteristic cortical phenotype of *Gja1* cKO mice – cross-sectional expansion and marrow cavity expansion, with increased endocortical bone resorption – was observed in the Δ130–136 expressing mice, but, quite surprisingly, not in those expressing the R76W mutant [73]. The latter result is in direct contrast with the cortical phenotype observed in the two existing ODDD mutants, $Gja1^{Jrt/+}$ and $Gja1^{G138R/+}$, both of which phenocopy the cortical abnormalities of *Gja1* cKO mice [10, 13]. Indeed, just like R76W, the G138R mutant forms non-functional gap junctions, while "hemichannel" activity (ATP release) is actually enhanced [12]. The use of different approaches (transgenic expression vs. targeted gene replacement) and different promoters to drive the genetic mutation might explain the discrepancy. Notably, the existence of Cx43 hemichannels has been challenged by a study showing that fluid flow-induced PGE_2 release and ATP-dependent dye uptake, typically attributed to Cx43 hemichannels, can occur in Cx43-deficient primary osteoblasts and are mediated by a $P2X_7$ receptor and pannexin-1 complex [74]. Thus, the in vivo evidence for a physiologic role of Cx43 hemichannels remains controversial.

5.3 Cx43 as a Docking Platform for Signaling Factors

There is increasing evidence that connexins actively participates in signaling by providing a scaffold for signaling complexes to the gap junction plaque. It is possible that each connexin recruits a unique profile of signaling effectors, thus resulting in specific downstream effects. This model would assign an additional biologic diversity to connexins, in addition to forming channels with specific biophysical properties. As mentioned, at least three signaling complexes interact with the Cx43 C-terminus, PKCδ, which upon FGF2 stimulation translocates from the Cx43 C-terminus to the nucleus [58–60], β-arrestin and α5β1 integrin [35, 71]. Although there is no direct evidence, it is likely that β-catenin and MAPK also interact with Cx43 C-terminus [75]. In osteoblastic cells, the Cx43 C-terminus is necessary but not sufficient to enhance cell signaling in vitro [71, 76]. In this context, the observation that female mice globally expressing the truncated allele $Gja1^{K258Stop}$ have low trabecular bone mass but normal cortical geometry, even in the absence of a wild type allele [37], suggests that the Cx43 C-terminus is dispensable for maintenance of normal cortical geometry, but it may be important for other actions of Cx43 in bone.

5.4 Cx43 and Apoptosis

Increased osteocyte apoptosis and empty osteocyte lacunae have been reported in both cKO^{hOC} and cKO^{DMP1} mice [32]. Since loss of osteocytes leads to increased osteoclast formation and bone resorption [77, 78], these observations would provide a mechanism for the increased endocortical resorption and the reduced abundance of sclerostin, an inhibitor of the Wnt/β-catenin cascade, observed in Cx43-deficient mice [32]. Consistently, *Gja1* knockdown by shRNA decreases viability of MLO-Y4 osteocytelike cells, while RANKL production increases and OPG expression is reduced [29, 32]. However, despite relatively conserved phenotypes, osteocyte apoptosis has not been consistently observed in all models of Cx43 loss of function [13, 36]. Accordingly, osteocyte apoptosis may contribute to, but it

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is not required for the development of the skeletal modeling abnormalities in Cx43 deficiency.

5 Other Connexins in the Skeleton

While significantly less studied than Cx43, recent data demonstrate that Cx37 (*Gja4*) does have a physiologic role in the skeleton. In contrast to Cx43, *Gja4*$^{-/-}$ mice have increased bone mass [79]. While both osteoblasts and osteoclasts express Cx37, the phenotype of these mice is secondary to reduced osteoclast differentiation and a concomitant reduction in bone resorption. No changes in osteoblast function were reported, suggesting that if Cx37 plays a role in osteoblasts it may be compensated for by other connexins. Notably, bone quality defects are more pronounced in males than females [79].

6 Translational Perspectives

Osteoblasts and osteocytes utilize connexins to communicate and coordinate signals in order to adapt to hormonal, mechanical and local signals. As we gain a better understanding of the information shared among this interconnected cellular network, the anatomic control of tissue modeling and remodeling will come to light. Gap junctions propagate both bone anabolic and catabolic signals depending upon the tissue context and physiological or adaptive conditions. Thus, there is value in designing modulators that could specifically disrupt or enhance the communication of these catabolic or anabolic signals, respectively. New compounds that modify gap junctions have been developed, although their mechanism of action is not completely understood [80]. However, the diversity of the downstream effects of Cx43 in bone and the advancing knowledge on specific signaling pathways regulated by Cx43 indicate that a more focused targeting would be more effective in achieving therapeutic potential, rather than inhibiting or enhancing Cx43 function as a whole. For example, it may be useful to modulate specific interactions between signal complexes and the C-terminus of Cx43 in order to inhibit signals that are catabolic to bone, while preserving the intercellular communication of signals that are anabolic to bone. To this end, defining the communicated *information* and how the intercellular network formed by gap junctions modulates the function of bone cells adds a new dimension to our understanding of bone modeling and remodeling at the tissue level. Such knowledge opens new avenues in the discovery of molecular targets using Cx43 as a platform for improving bone strength and optimizing response to therapy.

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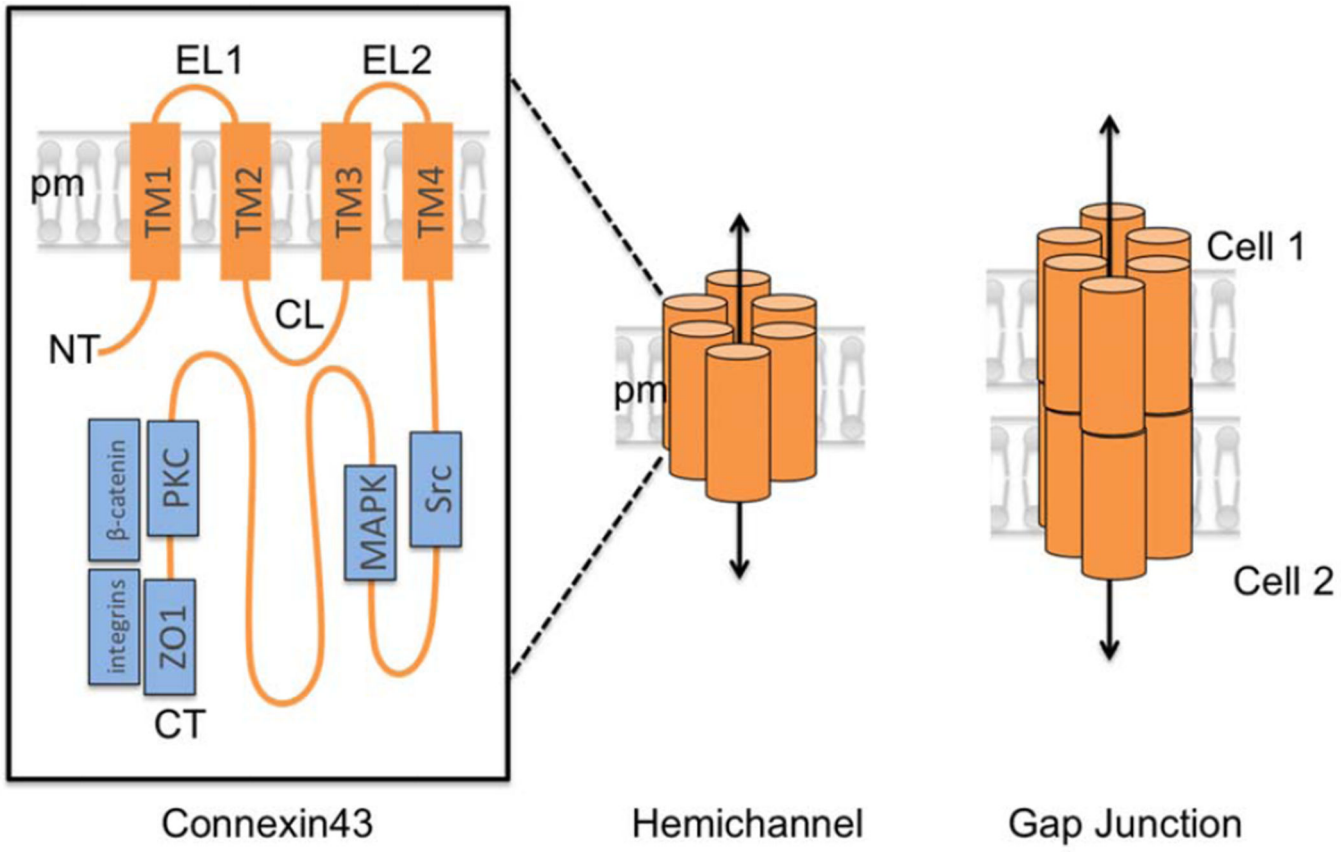

Fig. 1.

Structure and components of gap junctions. Gap junctions are composed of 6 connexin subunits. (Left) Connexins have 4 transmembrane domains (TM1–4), two extracellular loops (EL1–2), a cytoplasmic loop (CL) and cytoplasmic N (NT) and C-termini (CT). Protein complexes known to associate with the Cx43 CT are shown in blue. (Middle) Six connexin monomers assemble to form a connexon, which when inserted at the plasma membrane is called a “hemichannel”, which allows diffusion of small molecules between the cytoplasm and the extracellular space. (Right) When two connexons on the plasma membrane of adjacent cells dock, a continuous channel is formed between the two cells, permitting direct cell-to-cell communication.

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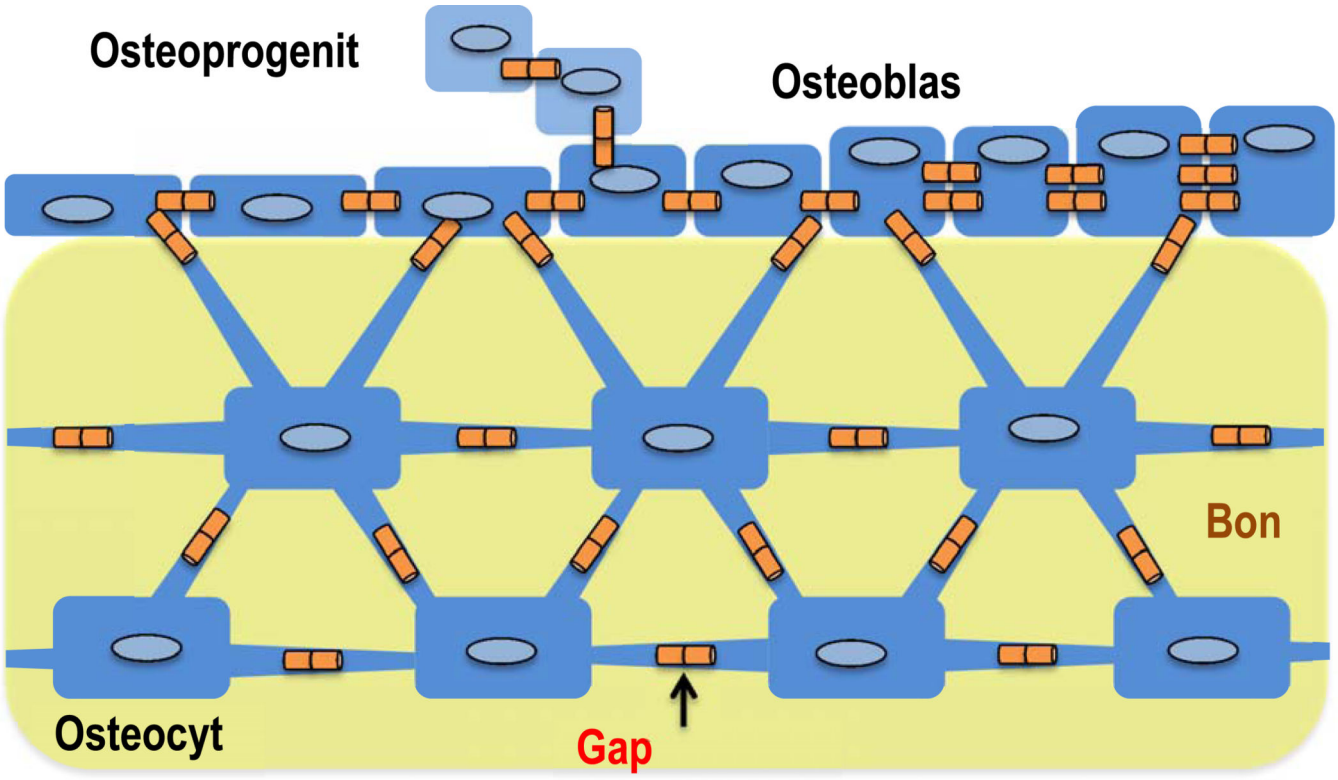

Fig. 2.

An elaborate network of gap junction coupled cells allows communication among bone embedded osteocytes, surface osteoblast and osteoprogenitors.

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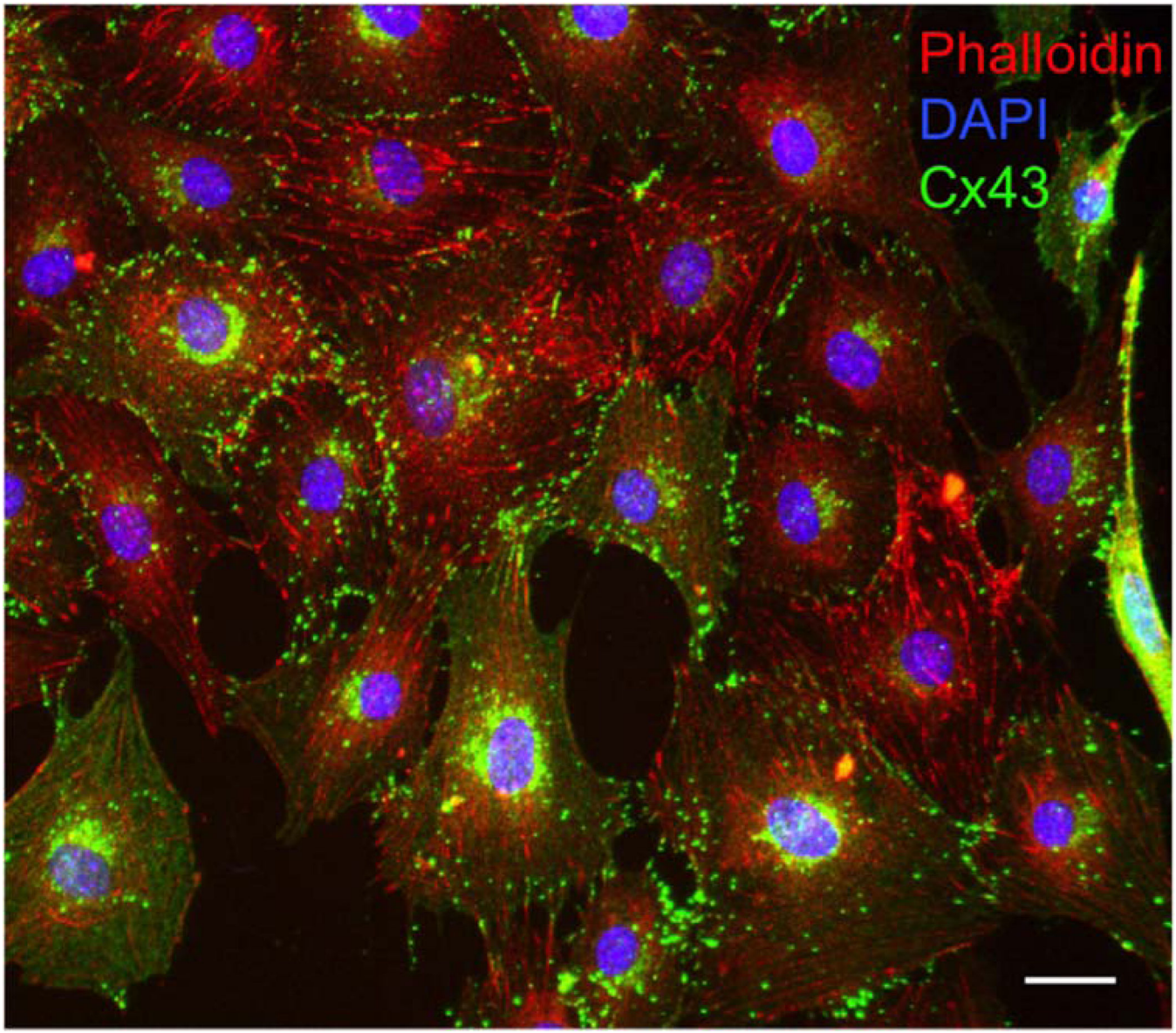

Fig. 3.

Cx43 in cultured MC3T3 osteoblastic cells. Immunofluorescence labeling of Cx43 (green), the actin cytoskeleton (red), and the nuclei (blue). Scale bar = 20 μm.

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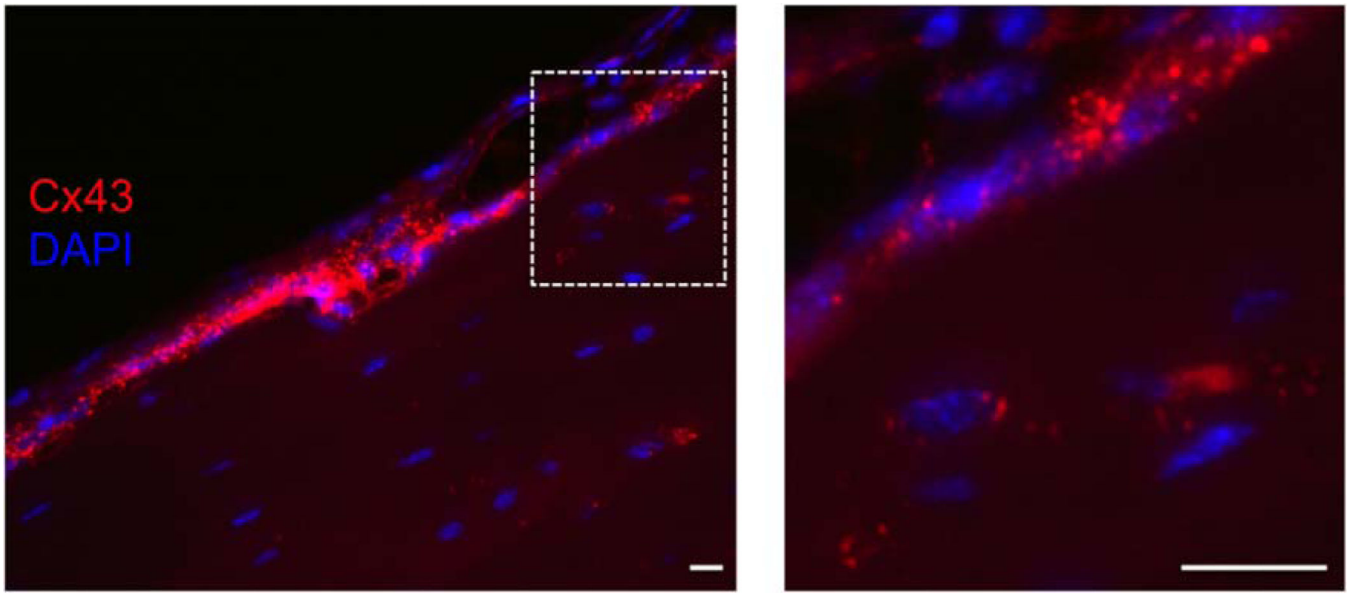

Fig. 4.

Cx43 in cortical bone. Immunofluorescence of Cx43 (red) with DAPI stained nuclei (blue). The boxed area enlarged on the right shows extensive Cx43 signal in surface cells and throughout the osteocytic network. Scale bar = 20 μm.