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Molecular Mechanisms of Osteoblast/Osteocyte Regulation by Connexin43

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Abstract

Osteoblasts, osteocytes and osteoprogenitor cells are interconnected into a functional network by gap junctions formed primarily by connexin43 (Cx43). Over the past two decades, it has become clear that Cx43 is important for the function of osteoblasts and osteocytes. This connexin contributes to the acquisition of peak bone mass and it is a major modulator of cortical modeling. We review key data from human and mouse genetics on the skeletal consequences of ablation or mutation of the Cx43 gene (*Gja1*), and the molecular mechanisms by which Cx43 regulates the differentiation, function and survival of osteogenic lineage cells. We also discuss putative second messengers that are communicated by Cx43 gap junctions, the role of hemichannels, and the function of Cx43 as a scaffold for signaling molecules. Current knowledge demonstrates that Cx43 is more than a passive channel; rather, it actively participates in generation and modulation of cellular signals driving skeletal development and homeostasis.

Keywords

Cx43; gap junction; bone; signal transduction; Runx2; Osterix

Introduction

Skeletal development (bone modeling) and skeletal maintenance in post-natal life (bone remodeling) requires the precise coordination of the activity of several cell types, including osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts. Indeed, extracellular and intracellular autocrine and paracrine signaling among these cells types is critical to both bone modeling and remodeling. One efficient means for the coordination of cellular functions is via the direct cell-to-cell communication of signals via gap junctions.

Gap junctions are intercellular channels formed by the pairing of hexameric array of connexin monomers (called a connexon or hemichannel) in the plasma membrane with a similar connexon in the plasma membrane of an adjacent cell, thus forming an aqueous pore between the two cells (Figure 1). Gap junction channels aggregate into gap junction plaques that can contain as many as thousands of channels and span from less than 100 nm to several μm in diameter. [1]. Gap junctions permit the direct intercellular exchange of ions, small molecules and second messengers. The result is a functional syncytium of shared signals,

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nutrients and small molecules among cells coupled by gap junctions. In addition, unopposed connexons can function as gap junction “hemichannels”, serving as direct conduits between the cytosol and extracellular fluid [2].

Connexin43 (Cx43; gene name, *Gjal1*) is the most abundant gap junction protein expressed in bone. Gap junctions composed of Cx43 allow the diffusion of molecules less than ~1200 Da. Other connexins, namely Cx45, Cx40 and Cx46 are expressed in cells of the osteoblast lineage (reviewed in [3, 4]). Cx37 has also been found in osteoblasts and is markedly enriched in osteocytes [5]. Substantially less is known about the role of these connexins in bone. Accordingly, this review will focus on Cx43, which over the past few years has attracted the attention of many researchers. New data from mouse genetics, human disease and zebrafish genetics have established the importance of Cx43 in bone modeling and remodeling. Substantial advances have also been made on molecular mechanisms of Cx43 biology in bone. As will be discussed in detail below, *in vivo* models of *Gjal1* deletion or mutation have converged towards strikingly consistent phenotypes, revealing novel, somewhat unpredictable and complex mechanisms by which gap junctions regulate bone cell function.

Cx43 in Skeletal Disease

In 2003, it was reported that point mutations in the human Cx43 gene (*GJA1*) causes oculodentodigital dysplasia (ODDD), an autosomal dominant disorder characterized by several skeletal abnormalities, including broad tubular long bones, craniofacial abnormalities, aplastic or hypoplastic middle phalanges, and syndactyly. [6]. At least 62 mutations spanning all the functional domains of Cx43 can cause ODDD [7]. Mechanistically, these mutant connexins act in a dominant negative fashion with respect to gap junction communication; they can form gap junctions but the intercellular channel is functionally defective [8-12]. Although cranial hyperostosis has been reported in some ODDD patients, their skeletal features and bone mass have not been systematically assessed. Nonetheless, the presence of a skeletal phenotype in these patients reinforces the notion emerged from early mouse genetic studies that gap junctions, and specifically Cx43, have a biologically relevant role in skeletal development and/or homeostasis.

To date, three distinct mouse models of ODDD have been described, and have served to characterize the molecular mechanisms of this disease and to further understand the biologic function of Cx43 in the skeleton. A mouse mutant with many phenotypic features of human ODDD was identified in an N-ethyl-N-nitrosourea mutagenesis screen, and called *Gjal1^{Jrt}*. This mouse mutant harbors a heterozygous *Gjal1* mutation not found in the human disease (*Gjal1* G60S), but the phenotype includes ODDD features, such as syndactyly, enamel hypoplasia, and craniofacial anomalies [8]. *Gjal1^{Jrt}* mice have low bone mineral density, decreased trabecular bone volume and significantly reduced mechanical strength relative to wild type littermates, features not described in the human disease. Additionally, in micro-computed tomography (μ CT) images *Gjal1^{Jrt}* mice appear to have thin cortical bone and enlarged marrow cavity in the femoral diaphysis, although this abnormality was not fully characterized.

A second ODDD mouse model was generated by conditional replacement of one wild type *Gjal1* allele with a *Gjal1* G138R mutant, driven by the ubiquitously expressed PGK-Cre (cODDD^{PGK}) [11]. The *Gjal1* G138R point mutation has been found in several cases of ODDD, and similar to *Gjal1^{Jrt}*, cODDD^{PGK} mice have many of the phenotypic characteristics of human ODDD, including craniofacial abnormalities and decreased trabecular bone volume relative to wild type controls. The cell autonomous nature of the skeletal abnormalities was established by conditional replacement of the *Gjal1* G138R allele exclusively in cells of the chondro-osteogenic lineage by using *Dermo1/Twist2-Cre*, which

is expressed starting at E9.5 (cODDD^{TW2}) [13]. At birth, these mice exhibit hypomineralized skulls, which by one month appear normally mineralized but remain smaller than the skulls of age-matched wild type littermates. Further, whole body bone mineral density is reduced in cODDD^{TW2} mice starting one month through at least 12 months of age. The reduced whole bone mineral density is caused by cortical thinning (20% relative to wild type) and a pronounced enlargement of diaphyseal cross sectional area, rather than by changes in trabecular bone. Notably, expansion of the marrow cavity and cortical thinning also occur in aging and disuse, raising the possibility that Cx43 may be involved in the pathophysiology of these processes. It is also important to note that these *Gjal* ODDD mutants are dominant negative, i.e. they can assemble in connexons but the gap junction channel is nonfunctional [12]. Therefore, some aspects of the skeletal phenotype could be caused by interference with other connexins expressed in bone (e.g., Cx37, Cx40, or Cx45) or by inhibition of gap junctional communication between osteoblasts (or osteocytes) and other cells present in bone (e.g., vascular endothelial cells).

Cx43 in Osteoblasts and Osteocytes Regulates Cortical Modeling

Before the discovery of *GJA1* mutations as a causative mechanism for ODDD, animal models of *Gjal* ablation had demonstrated that Cx43 has an important biologic function in bone. Using antisense oligonucleotides, significant defects in craniofacial and axial skeletal development were observed by *Gjal* “knockdown” in chick embryos [14]. A milestone step was achieved in 2000, with the report of a marked delay in ossification of the axial and appendicular skeleton and craniofacial abnormalities in *Gjal*^{-/-} mice [15]. Calvarial and long bone-derived osteoblasts isolated from these *Gjal*^{-/-} mice display delayed ossification and reduced expression of many osteoblast genes, suggesting a cell autonomous defect in osteoblastogenesis. Notably, these mice die shortly after birth due to severe cardiovascular malformations [16].

To assess the role of Cx43 in postnatal bone, conditional deletion models have been developed using different promoters to drive Cre recombination at different stages of the osteoblast differentiation program (Figure 2). As observed with the cODDD mouse models, conditional ablation of the *Gjal* gene in cells of the chondro-osteoblast lineage using *Derm1/ Twist2-Cre* (cKO^{TW2}) results in a small and hypomineralized skull at birth relative to wild type mice, a phenotype that, unlike in cODDD^{TW2}, persists as long as 1 month of age [13]. Also in contrast to cODDD^{TW2} mice, several skeletal dysmorphic features were detected in cKO^{TW2} mice, including hypoplastic skull with smaller parietal and interparietal bones, smaller mandible, reduced chest cavity from modestly shortened ribs, and mildly reduced length of the tibiae and femurs. Whole body bone mineral density is reduced as much as 40% in younger animals, recovering slightly after 4 months of age, yet remaining 8-12% lower even at 12 months relative to wild type littermates. Similar to the ODDD mutants, no trabecular bone phenotype was observed in cKO^{TW2} mice; however, cross-sectional area of the femoral diaphysis is increased (43% larger than wild type), while cortical thinning is pronounced (41% less than wild type), resulting in an expanded marrow cavity.

This cortical phenotype led to the key discovery that osteoblast/osteocyte Cx43 indirectly regulates osteoclastogenesis – lack of Cx43 results in a pronounced increase in osteoclasts on the endosteal surface of the cortex associated with cortical porosity, a hallmark of increased endocortical bone resorption. Indeed, the number of osteoclasts is abnormally increased on the endocortical surface of cKO^{TW2} bones, and Cx43 deficient bone marrow stromal cells exhibit a higher osteoclastogenic capacity relative to wild type cells, associated with failure to up-regulate osteoprotegerin [13]. The expanded cross-sectional cortical area implies periosteal expansion. Indeed, periosteal bone formation is significantly increased in

cKO^{TW2} mice, compared to wild type controls, whereas endocortical bone formation is decreased. Despite the consequent increased moment of inertia, which would predict increased resistance to bending by the wider cortical bone, Cx43-deficient bones are actually weaker and fracture at a lower load than wild type bones, implying abnormal material properties [13]. Accordingly, cKO^{TW2} bones exhibit several microstructural abnormalities, including disorganized collagen matrix with a woven bone appearance, disorganized fibrillar collagen network, and decreased cortical mineralization. These changes are associated with abnormal expression of several osteoblast and osteocyte genes, suggesting a defect in progression from the Runx2⁺ to Osterix⁺ stage. This is a key transition point in osteoblastogenesis, when cells become post-proliferative and begin producing bone matrix and other osteoblast and osteocyte gene products (Table I) [17]. Despite this osteoblast defect, the capacity of bone marrow stromal cells to produce osteogenic precursors is actually enhanced in cKO^{TW2} mice, perhaps owing to a markedly increased proliferative capacity. Hence, ablation of *Gjal* in the entire osteogenic lineage attenuates the capacity of pre-osteoblasts to fully mature, thus leading to production of an abnormal, mechanically impaired bone tissue. The increased osteogenic capacity upstream of the Runx2/Osx transition may reflect a compensatory mechanism to override the partial block of osteogenic differentiation, or an effect of Cx43 in regulating proliferative osteoblast precursors.

Osteoblast and Osteocyte Contributions to Cx43 Regulation of Cortical Modeling

Conditional ablation of *Gjal* at later stages of osteoblast differentiation leads to a similar but less pronounced phenotype. *Gjal* ablation using the 2.3kb *Colla1* (cKO^{Coll1}) promoter results in reduced whole body bone mineral density (~ 5% less than wild type controls), diminished cortical thickness (~20% less than wild type) and a pronounced widening of the cross sectional area (30% larger than wild type) at the femoral diaphysis [18, 19]. Similar to findings with *Gjal*^{-/-} and cKO^{TW2} mice, calvarial cells isolated from the cKO^{Coll1} mice exhibit delayed ability to form mineralized nodules and reduced expression of osteoblast genes in culture [18]. Intriguingly, studies in cKO^{Coll1} mice have also disclosed a novel function in Cx43 in regulating mobilization of hematopoietic stem cells from the bone marrow to the peripheral blood [2], an action that is most likely mediated by abnormal secretion of CXCL12, a cytokine abundantly produced by osteoblastic cells and directly regulated by Cx43 [20]. Conditional *Gjal* ablation using the human osteocalcin promoter (cKO^{hOC}), which is restricted to bone lining osteoblasts and osteocytes, has led to somewhat inconsistent results. While one group reported reduced cortical mineral density relative to wild type mice [21], another group using the same transgenic Cre model to ablate *Gjal* found no significant difference in cortical bone density [22]. Indeed, in the former study the abnormality is less severe than after broader *Gjal* deletion, and it is evident at 8 weeks of age persisting up to 6 months [21]. Importantly, expansion in periosteal and endocortical area and widening of the bone marrow cavity is consistently seen in cKO^{hOC} mice; but unlike the more broadly *Gjal* deleted models, there is no cortical thinning in cKO^{hOC} mice. As observed for cKO^{TW2} mice, the phenotype is largely restricted to cortical bone, where a significant increase in endocortical osteoclast number is observed relative to wild type mice. Notably at 8 weeks of age, parameters of osteoblast function (bone formation rate and serum PINP levels) are unchanged in cKO^{hOC} mice, suggesting that loss of Cx43 in late osteoblasts and osteocytes is less consequential than loss in the entire lineage, and the abnormalities are subtler. Nonetheless, a cortical phenotype clearly develops in cKO^{hOC} mice, even if less severe, implying that Cx43 in mature osteoblasts and/or osteocytes is involved in paracrine regulation of osteoclastogenesis and in periosteal expansion. Intriguingly, a reduction in circulating osteocalcin was observed, despite the increased periosteal bone formation, further suggesting that production of bone matrix products is abnormal in Cx43 deficient osteoblasts, a notion also supported by mild defects of collagen

crosslinking and down-regulation of several osteoblast and osteocyte genes (Table I) [21, 22].

An almost identical phenotype has also been reported by deleting *Gjal* using the *DMP1* promoter (8 kb, cKO^{DMP1}), resulting in 20% increase in femoral diaphysis cross sectional area attended by increased osteoclast number, but no difference in cortical thickness or whole body bone mineral density, and no trabecular abnormalities [21]. Although *DMP1-Cre* is believed to induce deletion primarily in osteocytes, recent data indicate that the *DMP1* promoter is widely expressed by bone lining cells in addition to osteocytes (less for the 8kb than the 10kb fragment), in a pattern not too dissimilar than the field of expression of *Bglap* [23, 24]. Therefore, it is not surprising that the phenotypes of cKO^{hOC} and cKO^{DMP1} mice are almost identical.

In summary, *Gjal* ablation early in the osteoblast lineage leads to lower than normal whole body bone mass, cortical widening and thinning, and a cell autonomous defect in osteoblast function. Deletion of *Gjal* at later stages does not affect whole body bone mass, whereas some aspects of the cortical phenotype, namely cross sectional widening, increased endocortical resorption and periosteal formation persist. Thus, Cx43 modulation of cortical modeling is in large part the result of its action on mature osteoblasts and/or osteocytes. However, actions of Cx43 in less differentiated osteoblast and osteogenic precursors contribute, since the cortical phenotype is patently more severe after broader *Gjal* ablation. At very early stages of osteogenic differentiation, Cx43 is also involved in regulating proliferation of osteoblast precursors. Thus, Cx43 is biologically important throughout the osteoblast differentiation program, serving primarily to modulate cortical modeling via regulation of both bone formation and endocortical bone resorption. While the effect on bone resorption is clearly indirect, most likely mediated by Cx43 modulation of osteoprotegerin production, the mechanisms by which Cx43 affects the function of bone forming cells remain to be elucidated. Both osteoblast cell autonomous (direct regulation of gene expression) and paracrine mechanisms via osteocytes (regulation of sclerostin secretion) have been proposed (reviewed in [4, 25]).

The role of Cx43 in cortical modeling has become increasingly complicated, as a series of recent publications have reported, rather unexpectedly, that Cx43 may negatively regulate periosteal bone formation response to mechanical load, so that in the absence of Cx43 cortical bone is more sensitive to mechanical load [19, 26-30]. More detailed discussion of this fundamental aspect of Cx43 biology is beyond the scope of this review, which focuses on topics relevant to the molecular mechanisms of Cx43 action in bone cells, and in particular the nature of the signal molecules that are propagated by gap junctions, the signaling pathways they affect, the identity of their molecular targets, and how Cx43 participates in “information sharing” among bone cells. Some open questions in this area include: Does Cx43 act simply as a passive channel for signal diffusion or is it an active participant in the signaling process that controls osteogenic differentiation and function? What is the role of gap junction “hemichannels”? Are there any other, non-gap junction related functions of connexins that contribute to bone homeostasis? Understanding the molecular mechanisms of Cx43 action in osteogenic cells will greatly aid in interpreting data obtained in animal models and the skeletal responses to hormonal, pharmacological and mechanical stimuli.

Gene Expression Regulation by Cx43 in Osteoblasts and Osteocytes

Most of the work aimed at understanding how Cx43 regulates cell function has focused on osteoblasts, as early studies showed defective osteoblast gene expression and osteoblast function in murine models of *Gjal* ablation [15, 18], observations corroborated by several *in vitro* studies [31-36] (Table I). More in depth analyses showed that Cx43 modulates genes

involved in matrix production at the transcriptional level, and that the master regulators of osteoblastogenesis, Runx2 and Osterix/Sp7, are downstream effectors of Cx43 action in cells of the osteoblast lineage. Overexpression of Cx43 in MC3T3-E1 osteoblasts increases the transcriptional activity of a Runx2 reporter in response to fibroblast growth factor-2 (FGF-2) treatment, while siRNA mediated knockdown of Cx43 suppresses Runx2-dependent transcription [37]. Interestingly, this effect is connexin-specific, as Cx45 overexpression does not mimic the effects of Cx43 on Runx2. Further, Cx43 overexpression increases Runx2 recruitment to the osteocalcin promoter, despite no effect on Runx2 expression levels, indicating that Runx2 transcriptional activity is regulated downstream of Cx43 function [37]. This is consistent with *in vivo* the observation that many downstream targets of Runx2 action are reduced by conditional *Gjal* deletion, while *Runx2* mRNA abundance is not affected [13]. The signal pathways mediating the action of Cx43 on Runx2 activity include the novel protein kinase C (PKC) family member PKC δ and the extracellular signal regulated kinases (ERK) [37, 38], two pathways previously reported to regulate Runx2 activity in osteoblasts [39-41]. Overall, the accumulated evidence favors the notion that activation of Runx2 by Cx43 is related to intercellular gap junction communication (IGJC) rather than to other functions of Cx43. First, the percentage of cells responding to FGF-2 stimulation of Runx2 activity is increased when Cx43 is overexpressed and reduced when Cx43 level or function is decreased [37, 38]. More to the point, the ability of Cx43 to enhance Runx2 activity is present when cells are cultured at a high density and cell-to-cell contacts are abundant, but it is abrogated by culturing cells at low density, thus limiting direct cell-to-cell communication [38]. Furthermore, under low density culture conditions the inhibition of Cx43 channel function with a pharmacologic channel blocker is ineffective at inhibiting Runx2 activity.

In addition to Runx2, Osterix (gene name, *Sp7*) is also a target of Cx43 action in osteoblasts. In cKO^{TW2} mice, there is a ~40% decrease in *Sp7* mRNA abundance in femurs of 3-month-old mice relative to control animals [13]. However, it is unclear whether this effect is secondary to reduced Runx2 transcriptional activity or whether Osterix is also regulated by Cx43. Previous *in vitro* experiments have shown that Cx43 stimulates binding of the transcriptional activator Sp1 to a CT-rich sequence of the rat *Bglap* and *Colla1* promoters downstream of ERK activation, a non-canonical Sp1-binding site also called Connexin Response Element (CxRE) [42, 43]. A similar mechanisms involving nuclear localization of Sp1 has been reported for Cx43 (and Cx45) regulation of CXCL12 transcription in bone marrow stromal cells [20]. Circumstantial evidence had suggested that Osterix might bind to the Sp1-binding CxRE in the rat osteocalcin proximal promoter (−70 to −57 relative to transcriptional start). First, Osterix had been shown to bind Sp1 cognates [44]. Second, Osterix regulates *Bglap* expression, though its precise binding cognate is unknown [44]. Third, the non-canonical CT-rich Sp1-binding CxRE in the rat and mouse proximal promoters is replaced by a canonical Sp1 cognate in human promoters. This striking conservation of function, despite divergence of the primary sequence, suggests that this sequence might be an important target of a master regulator of osteoblast function, such as Osterix. Indeed, in MC3T3 cells this Sp1-binding cognate is required for the transcriptional activity of Osterix on the rat *Bglap* promoter [45]. However, Osterix does not directly bind to this Sp1-binding site, rather Sp1 binds to this site in a Cx43-dependent manner, and this is a necessary step to allow co-recruitment of Osterix to a different upstream element (−92 to −87 relative to transcriptional start) in the rat *Bglap* promoter. Thus, Cx43 affects both Osterix transcriptional activity and expression.

A very similar model of Cx43 regulation of signaling pathways regulating factors important for skeletal homeostasis is observed in zebrafish. Loss of function mutations in the zebrafish *cx43* gene result in the “short of fin” phenotype, in which the length of the bony fin rays is markedly reduced [46, 47]. This phenotype can be recapitulated by knockdown of *cx43*

mRNA [46]. In contrast, a mutant with increased fin bone segment length (“another long fin”, *alfty86* mutant) has increased *cx43* expression, and fin length can be restored by knockdown of the overexpressed *cx43* [48]. In analogy to murine models, Cx43 regulates the expression of a key molecular mediator of bony fin growth, semaphorin3d, which signals through neuropilin2a and plexin a3 to regulate the proliferation and joint formation by osteogenic precursor cells, thus directing fin skeletal morphogenesis [49]. While there are considerable differences between bone growth in higher mammals and zebrafish, the concept that Cx43 is a conserved regulator of proliferation and differentiation of osteogenic cells is intriguing and biologically important. Semaphorin3d is only one example of modulators of skeletal homeostasis that are regulated by Cx43, and thus might be involved in the pathogenesis of the modeling abnormalities consequent to *Gjal* mutation or ablation, as detailed in a recent review [50].

Cx43 and Osteoblast/Osteocyte Apoptosis

In both cKO^{hOC} and cKO^{DMP1} mice, a 2- to 2.5-fold increase in TUNEL-positive osteocytes was observed in the cortical bone of the femoral midshaft relative to control mice at 5.5 months of age [21], with associated 6-fold and 30-fold increase in empty osteocyte lacunae, respectively. Since loss of osteocytes leads to increased osteoclast formation and bone resorption [51, 52], increased osteocyte apoptosis would explain the increased endocortical resorption and the reduced abundance of sclerostin, an inhibitor of the Wnt/ β -catenin cascade, observed in Cx43-deficient mice [21]. *In vitro*, *Gjal* knockdown by shRNA results in decreased viability of MLOY4 osteocyte-like cells, while increasing RANKL production and reducing OPG expression [21, 53]. Since the latter effect was not observed in primary osteoblasts it has been suggested that Cx43 regulation of osteoclastogenesis is primarily via the osteocyte. This conclusion would be consistent with *in vivo* data showing that expansion of the medullary cavity, which is attended by increased endocortical bone resorption, develops in mice in which *Gjal* deletion is restricted primarily to osteocytes (see above). Indeed, increased empty osteocytic lacunae are reported in cKO^{hOC} and cKO^{DMP1} mice. In contrast, no loss of osteocyte number is observed in cKO^{TW2} [13], despite the fact that *Gjal* is ablated in the osteocyte population in this model. Furthermore, bone marrow stromal cells from cKO^{TW2} mice, where *Gjal* ablation affects the entire osteogenic lineage, support a larger number of osteoclasts in stromal cell-macrophage co-cultures relative to wild type cells, and fail to up-regulate osteoprotegerin when cultured in osteoclastogenic medium [13], suggesting that at the very least osteoblasts also contribute to Cx43 regulation of osteoclastogenesis. This conclusion is consistent with the fact that the cortical modeling phenotype is more severe when *Gjal* is more broadly deleted than when *Gjal* ablation is more restricted to osteocytes (see above). Thus, the notion that Cx43 protects from osteoblast and osteocyte apoptosis remains controversial. It is possible that the discrepant results may be related to different ages at the time of examination, and different effectiveness of gene deletion, or mouse strain background differences. Nonetheless, the demonstration that Cx43 can contribute to the diffusion of survival signals among osteoblasts following treatment with parathyroid hormone, via sequestration of β arrestin by Cx43 [54], supports a role of Cx43 in regulating osteoblast and osteocyte viability.

Gap Junctions and Hemichannels in Osteoblasts and Osteocytes

Considerable attention has been directed to the biologic role of unopposed hemi-gap junction channels, or “hemichannels”, formed by Cx43 in osteocytes and osteoblasts. *In vitro*, mechanical perturbation of MLO-Y4 osteocytes with fluid flow induces uptake of low molecular weight, Cx43-permeable dye from the extracellular fluid. Since dye uptake is dependent on the presence of Cx43, such result is typically taken as evidence of opening of Cx43 hemichannels [55]. Based on this interpretation, opening of Cx43 hemichannels has been linked to fluid flow-induced release of prostaglandins [55, 56] and ATP [57] into the

extracellular fluid. Release of these factors would provide a paracrine mechanism for osteoblast regulation and ultimately for stimulation of bone formation near the site of the activated osteocytes. Studies by one group suggest that fluid flow-induced hemichannel opening is a result of a physical interaction between Cx43 and $\alpha 5\beta 1$ integrin, whereby the integrin heterodimer functions as the mechanical sensor of fluid flow-induced shear stress [58]. Accordingly, fluid flow-induced dye uptake can be abrogated by siRNA knockdown of $\alpha 5$, or exposure to a Cx43 channel blockers, or to a hemichannel-specific blocking antibody [58]. These intriguing data may in theory represent an important step forward to understanding mechanotransduction in the skeletal tissue. However, not only do these results still require corroboration *in vivo*, but the biologic model proposed is inconsistent with recent findings from different groups demonstrating that lack of Cx43 in osteoblasts/osteocytes actually favors periosteal bone formation response to mechanical load [26, 53]. Unfortunately, studying hemichannel function is hampered by the difficulty in distinguishing hemichannels from classic gap junctions, or from other, non-connexin transmembrane channels (see below). Although hemichannel-specific blocking antibodies or peptides are very helpful, more powerful genetic approaches are required.

An innovative co-culture model, in which MLO-Y4 osteocytes and hFOB osteoblasts were cultured on opposite sides of a permeable membrane, provided proof to the concept that osteocytes can modulate osteoblast activity in a Cx43-dependent manner in response to fluid flow [59]. Under these culture conditions, the two cells types are separated but can communicate via gap junctions; application of fluid flow to the osteocyte side induced a 40% increase in alkaline phosphatase activity in the osteoblast side. Importantly, this effect was blocked by the gap junction channel inhibitor 18 α -glycyrrhetic acid; whereas neither extracellular ATP nor prostaglandin E₂ could recapitulate the effects of fluid flow, suggesting that the effect is mediated by direct IGJC between osteoblasts and osteocytes. If hemichannels are involved it would likely be via alternate messengers.

Indeed, evidence is accumulating arguing against a biologic role of Cx43 hemichannels in bone. Foremost among this controversy are data from the Cx43 G138R murine models of ODDD dysplasia [11, 13]. As noted, the Cx43 G138R mutant can assemble in connexons but does not allow GJIC. In fact, Cx43 G138R increases release of intracellular ATP [11], possibly functioning as a membrane ATP channel. However, cODDD mice closely phenocopy osteoblast/osteocyte specific *Gjal* conditional deleted mice (*e.g.*, low bone mass, cortical thinning, increased femoral cross sectional area, increased endosteal osteoclasts) despite the fact that they have normal or even enhanced hemichannel activity [13]. Similarly, a preliminary and still unpublished report suggested that a *Gjal* mutant that removes gap junction function but not hemichannel activity results in a phenotype similar to *Gjal* deletion [60]. Even a conservative interpretation of such findings suggests that Cx43 hemichannels are not sufficient to compensate for lack of Cx43 gap junctions in regulating skeletal modeling and homeostasis. Such conclusions also underscore the limitations of *in vitro* data that frequently rely exclusively on pharmacologic agents to block Cx43 hemichannels, as many of the inhibitors used also block gap junction function. More recent data cast further doubts on the validity of evidence for hemichannel activity. Contrary to earlier findings [55], fluid flow-induced prostaglandin E₂ release and ATP-induced extracellular dye uptake occurred just as well in osteoblasts derived from Cx43-deficient mouse calvaria as in wild type cells [61]. Instead, these effects could be attributed to P2X₇-receptor and pannexin 1 activity, respectively. Pannexins are a class of proteins with high structural homology to connexins; they can form hexameric membrane channels, much like Cx43 hemichannels, but they function as conduits between the intracellular and extracellular space, and do not dock with pannexins in apposing cell membranes [62-64]. Indeed, pannexins are expressed in bone; in particular, pannexin 3 expression is directly regulated

by Runx2 [65] and promotes osteoblast differentiation [66]. Thus, some of the observations attributed to Cx43 hemichannels might instead be related to activation of pannexin channels.

In vitro evidence supports a model whereby Cx43 hemichannels are directly involved in mediating pharmacologic effect of bisphosphonates on the osteoblast lineage. Accordingly, hemichannel opening in response to bisphosphonate exposure activates Src/ERK signaling cascade, thus promoting osteoblast and osteocyte survival by inhibiting apoptosis [67, 68]. Bisphosphonates are potent inhibitors of bone resorption and their primary pharmacologic action is to inhibit osteoclast function and survival [69]. However, a positive effect on osteocytes could contribute to the anti-fracture efficacy of bisphosphonates independent of their effect on bone resorption [70]. Unfortunately, experiments using mouse models of conditional *Gjal* ablation have cast substantial doubts about the physiologic importance of Cx43 for the pharmacologic action of bisphosphonates. Although *Gjal* ablation in mature osteoblasts and osteocytes (cKO^{hOC}) removed the ability of alendronate to halt glucocorticoid-induced osteoblast and osteocyte apoptosis, it did not alter at all alendronate prevention of glucocorticoid-induced bone loss [71]. Furthermore, alendronate or risedronate prevented ovariectomy-induced bone loss equally well in wild type and osteoblast and osteocyte *Gjal* ablated (cKO^{Col1A}) mice, and actually rescued the main abnormalities of *Gjal* deficient bone, normalizing cortical thickness and bone strength [72]. These results confirm that osteoclasts are the primary target of bisphosphonates, and lack of any effects of bisphosphonate treatment on osteoblast number and bone formation rate on bone histomorphometry also argues against a significant pharmacologic effect on the osteogenic lineage [72]. Indeed, the anti-apoptotic effect, which is seen at low doses of bisphosphonates, is difficult to reconcile with their pro-apoptotic effect on osteoclasts and, at doses used in clinical settings, on osteoblasts [73]. Thus, this area remains highly controversial. It also remains to be determined how bisphosphonates may activate Cx43 hemichannels, since they do not bind Cx43 directly [74].

Second Messengers and Signals Shared via Cx43 Gap Junctions

A key question in gap junction biology, one that requires more attention than it is usually given, is what are the biologically relevant molecules that are being communicated through gap junctions? It is a complex question to address, as the communicated message(s) will likely depend upon the extracellular or intracellular cue that initiates signaling. However, some inroads have been made towards understanding the role of some signaling molecules as effectors of Cx43 function, and a lot of this progress has in fact been achieved in bone cells. One second messenger that can pass through Cx43 gap junctions is inositol 1,4,5 triphosphate (InsP₃) [75]. Furthermore, InsP₇, produced by inositol hexakisphosphate kinase, has been recently found to be required for Cx43 enhancement of Runx2 activity via the calcium independent PKC δ [76]. These water soluble second messengers are small enough (~740 Da) to pass through Cx43 gap junctions, though direct evidence of their cell-to-cell communication has not been demonstrated, yet. InsP₃ (or other inositol polyphosphates) is a likely candidate mediator of Cx43-dependent calcium wave propagation elicited by mechanical perturbation of osteoblasts and osteocytes [77-79]. Rapid calcium waves can be also propagated by ATP release and activation of purinergic receptors followed by opening of membrane calcium channels [77, 78, 80]. However, while chemical inhibition of gap junctional communication has little impact on long-range calcium wave propagation in MC3T3 cells [81, 82], in an *ex vivo* imaging system of intact bone both spontaneous and fluid flow-activated calcium oscillations among osteocytes, and to a lesser extent osteoblasts, were sensitive to gap junction inhibition [83, 84]. Intriguingly, the spatiotemporal properties of calcium oscillations are substantially different between osteoblasts and osteocytes [85], and it is conceivable that such a discrepancy may reflect the different anatomical environments and constraints surrounding the two cell types. For

example, gap junctions may permit more localized calcium signal diffusion among the osteocytic network and between osteocytes and osteoblasts on the bone surface; whereas a paracrine mechanism, such as extracellular ATP release, may be better suited to reach a large number of cells in a high cell density environment such as the bone marrow. Evidence also indicates that cyclic adenosine monophosphate (cAMP) is likely to be diffused among bone cells via Cx43 gap junctions. Knockdown of *Gjal* *in vitro* by antisense oligonucleotides reduces cAMP levels in ROS17/2.8 osteosarcoma cells in response to parathyroid hormone (PTH), without affecting adenylate cyclase activity [86]. Further, the ability of PTH to stimulate matrix mineralization by osteoblasts in culture is markedly reduced when gap junctions are inhibited [36]; and, of even more significance, cKO^{Col1A} are resistant to the anabolic effects of intermittent PTH administration [18]. Such hormonal resistance might in part be the result of defective cAMP communication. In summary, some progress has been made but much remains to be discovered about the identity of the biologically relevant molecules that are diffused via gap junctions.

Connexin43 as a Docking Platform for Signal Transduction

While many have shown that protein kinases can bind to Cx43, and specifically to the C-terminal tail of Cx43 [87], only recently has attention shifted from a view of these complexes as signaling *towards* Cx43 to regulate the open-closed state of gap junction channels, to a view of Cx43 as an active participant in recruiting the complexes that signal *from* the gap junction channel. Accumulating data suggest that connexins can recruit a unique profile of signaling factors that may determine which signals are transmitted among cells. Thus, it may not only be the permeability of the gap junction, but also the repertoire of signal molecules at the gap junctional plaque that determines the functional consequence of signal diffusion via gap junctions. This mechanism could provide additional functional diversity for connexins, whose channels have largely overlapping permeability and electric conductance. Indeed, signaling complexes by and large interact with the C-terminal tail, the least conserved domain of connexins [88]. Perhaps the unique function of a connexin protein is dictated not solely by the size and charge of the shared message, but also by the assemblage of signal molecules that determine the functional consequences of a shared message.

At least three such interactions with the Cx43 C-terminal tail have been shown to have biologic significance in osteoblasts and osteocytes. In MC3T3 osteoblastic cells, PKC δ transiently binds to the C-terminal tail of Cx43 before translocating to the nucleus, where it binds to and regulates Runx2 transcriptional activity [76, 89]. Likewise in the OB-6 osteoblast cell line, interaction between the Cx43 C-terminal tail and β arrestin is involved in the anti-apoptotic action of PTH [54]. In this model, the C-terminal tail of Cx43 is responsible for sequestering β arrestin, hampering its ability to suppress cAMP-dependent signaling cascades [54]. In fact such protein-protein interaction may not require gap junction channel activity. Finally, as noted earlier, in MLO-Y4 osteocytic cells the C-terminal tail of Cx43 complexes with $\alpha 5\beta 1$ integrin; this interaction is enhanced by application of fluid flow shear stress via activation of the phosphoinositide 3-kinase cascade, resulting in a conformational change of the integrin extracellular domain and opening of Cx43 hemichannels [58].

Summary and Perspectives

In summary, Cx43 plays a critical role in osteoblast and osteocyte biology. The osteocyte, one of the “permanent” cells of bone, utilizes Cx43 to signal among its network to regulate anabolic and catabolic responses and to maintain cell survival. Osteoblasts require Cx43 throughout their differentiation program, particularly as they progress through the Runx2 to Osterix transition. Loss of Cx43 in any of these contexts can affect cell survival and/or the

efficiency of a cellular response to an extracellular cue. We propose a model whereby cells sense an extracellular cue (*e.g.*, a growth factor or mechanical strain) that is then translated into a signaling response resulting in production of second messengers (Figure 3). As a result of cell coupling via gap junctions, these second messengers are propagated to adjacent cells. Locally recruited signaling complexes at the gap junction plaque then initiate signaling in the receiving, coupled cell. This mechanism of signal sharing allows multiple cells to respond to the stimulus, regardless of their ability to sense it (perhaps for lack of receptors, or because of certain anatomical constraints), thereby amplifying the overall response. For example, an osteocyte sensing mechanical load can send its signal to a surface osteoblast that does not sense a load; or an osteoprogenitor activated by growth factors can signal its neighboring cells to coordinate osteogenic differentiation and bone formation. Thus, under conditions of robust Cx43 expression, there is an amplification of the cell population's ability to respond to the cue by direct sharing of information. In contrast, when Cx43 expression is reduced, or when *Gjal* is mutated, the response is attenuated, as information is not communicated to all cells that could be able to respond. In bone, the result is reduced osteoblast maturation, aberrant gene expression and abnormal bone formation. The second messenger signal being shared is very likely to be specific to the extracellular cue and to the cell context, *e.g.* matrix embedded osteocytes or bone surface osteoblasts. Although gap junctions are likely to propagate both anabolic and catabolic cues the possibility that specific molecules carry out specific anabolic or catabolic functions via a particular connexin would in theory allow designing modulators that could specifically disrupt or enhance the cell-to-cell exchange 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 [90-92]. Understanding the molecular mechanisms by which connexins modulate skeletal homeostasis and response to mechanical and hormonal cues will help define potential applications of gap junction modifiers to improve bone structure and strength. Indeed, any intervention intended to impact the entire bone forming unit to reverse or slow down skeletal diseases will require an understanding of the intricate methods of intercellular exchange of information, such as those afforded by Cx43 among osteoblasts and osteocytes, for optimal efficacy.

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Figure 1. Gap junctions in osteoblasts

(A) The structure of a gap junction channel. The connexin monomer is a transmembrane protein with both the N- and C-terminal tails located in the cytoplasm, two cysteine (Cys) containing extracellular loops, and an internal cytoplasmic loop. The connexin monomers assemble into a hexameric connexon or hemichannel in the plasma membrane of one cell. Two hemichannels on adjacent cells can dock via the extracellular loops to form a continuous aqueous channel between the two cells. These gap junction channels can pass ions, small metabolites, and second messengers up to ~1kDa. (B) Immunofluorescent labeling of Cx43 (green) in MC3T3 osteoblasts in culture. The actin cytoskeleton is labeled in red and the nuclei in blue (DAPI). Abundant staining is observed in the plasma membrane at the interface of adjacent cells.

Figure 2. Conditional deletion models of Cx43 in bone

(A) A simplified schematic of osteoblast differentiation and relative time of expression of the various promoter-Cre used to conditionally ablate *Gjal* in the mouse. (B) Comparison of the skeletal phenotype of different *Gjal* conditional knockout mice. Numbers in parentheses represent a negative value. ND, not determined. NR, not reported. For osteoblast dysfunction and increased osteoclast number only a yes/no response is indicated. The magnitude of the dysfunction or increase is not compared. (C) Representative cross-sections of tibiae of 2-month-old wild type and cKO^{TW2} male mice generated by μ CT (VivaCT, Scanco, Switzerland). Location is 5 mm proximal to the tibio-fibular junction.

Figure 3. A model of Cx43 control of osteoblast/osteocyte survival and function

Upon receiving an extracellular cue (e.g., mechanical load, hormone, growth factor), bone cells elicit a small molecule/second messenger response that can be communicated among interconnected cells by Cx43. These communicated messengers converge on a finite number of signal cascades, such as ERK and PKCδ, to regulate cell survival, as well as Runx2 and Osterix transcriptional activity. Recruitment of signaling complexes to the C-terminal tail of the Cx43 molecule facilitates the efficiency of the signaling response in the recipient cell. By sharing such signals bone is able to respond to biochemical and mechanical cues in a coordinated fashion.

Table I

Osteoblast and Osteocyte Associated Genes Decreased by Loss of Cx43

Gene	Protein	Reference
<i>Colla1</i>	Collagen, type I, $\alpha 1$	[12, 17]
<i>Sp7</i>	Osterix	[12]
<i>Runx2</i>	Runx2	[17]
<i>Bglap2</i>	Osteocalcin	[12, 17]
<i>Sost</i>	Sclerostin	[12, 21, 22]
<i>Tnfrsf11b</i>	Osteoprotegerin	[12, 20, 21]
<i>Lox</i>	Lysyl oxidase	[12, 22]