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## Connexins and pannexins in the skeleton: gap junctions, hemichannels and more

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### Abstract

Regulation of bone homeostasis depends on the concerted actions of bone-forming osteoblasts and bone-resorbing osteoclasts, controlled by osteocytes, cells derived from osteoblasts surrounded by bone matrix. The control of differentiation, viability and function of bone cells relies on the presence of connexins. Connexin43 regulates the expression of genes required for osteoblast and osteoclast differentiation directly or by changing the levels of osteocytic genes, and connexin45 may oppose connexin43 actions in osteoblastic cells. Connexin37 is required for osteoclast differentiation and its deletion results in increased bone mass. Less is known on the role of connexins in cartilage, ligaments and tendons. Connexin43, connexin45, connexin32, connexin46 and connexin29 are expressed in chondrocytes, while connexin43 and connexin32 are expressed in ligaments and tendons. Similarly, although the expression of pannexin1, pannexin2 and pannexin3 has been demonstrated in bone and cartilage cells, their function in these tissues is not fully understood.

### Keywords

bone; cartilage; tendon; ligament; connexin; pannexin

## 1. Introduction

Musculoskeletal systems are faced with a plethora mechanical and systemic signals that require tightly organized cell responses to occur in order to maintain structural and functional integrity [1]. Coordinated cellular responses to these extracellular cues can occur directly or indirectly through communicative channels, including gap junctions, connexin hemichannels and/or pannexins channels. For example, in bone, osteoblasts and osteocytes form an extensive interconnected network, which express robust amounts of connexin43

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The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

(Cx43), as well as other connexins and pannexins [2,3]. This osteogenic network, interconnected by Cx43 in particular, is vital to how bone responds to mechanical load and mechanical unloading stimuli, as well as how bone responds to hormonal and growth factor cues to regulate bone quality [4,5]. In other musculoskeletal tissues like tendon, ligaments and cartilage, it is less clear how the cells that compose these systems use connexins and pannexins to regulate function. Yet, as it will be discussed below, growing evidence demonstrates a substantial contribution of these communicative channels to the optimal function of these cells. This review will focus on the presence and roles of connexins and pannexins in osteoblasts/osteocytes, osteoclasts, tenocytes, chondrocytes and ligamentous fibroblasts.

Bone homeostasis is controlled by the coordinated actions of osteoblasts, the bone-forming cells, and osteoclasts, the bone-resorbing cells [4]. Osteocytes, cells derived from osteoblasts that became enclosed by bone matrix, are thought to be the main regulators of the differentiation and function of osteoblasts and osteoclasts. Osteoblasts originate from osteochondroprogenitors, the same cells that give origin to chondrocytes, and their differentiation occurs through changes in gene expression that can be affected by changes in connexin levels. The function and viability of osteocytes are also affected by connexins. Osteoblast and osteocytes control osteoclast differentiation by producing the pro-osteoclastogenic cytokine receptor activator of nuclear factor kappa-B ligand (RANKL) and the anti-osteoclastogenic cytokine osteoprotegerin (OPG) [6]. The ratio between these 2 molecules dictates osteoclast differentiation, and, as will be detailed below, is highly regulated by Cx43 expression. Furthermore, connexins also affect osteoclast differentiation directly. In cartilage, tendon and ligament, the role of connexins and pannexins are only just beginning to come into focus. The data that are coming in suggest that there are some conserved pathways among cells of the skeletal systems by which connexins and pannexins may regulate cell signaling, differentiation, and function.

## **2. Expression of connexins and pannexins at tissue and cellular level**

### **2.1. Connexins: gap junctions and hemichannels**

Connexins permit the rapid dissemination of shared molecules and ions among cells of the musculoskeletal system via cell-to-cell communication. Connexins can link cells directly in the form of classic gap junction channels in which hexamers of connexins assemble a pore structure in the plasma membrane of one cell and then docks with a connexin pore on an adjacent cell, forming a continuous, aqueous channel between the 2 cells. Small molecules roughly 1kDa or less can diffuse through these channels, permitting cells to directly and efficiently share signal molecules, ions and other low molecular weight molecules [7]. Gap junctions facilitate both electrical and chemical (i.e. second messenger) coupling [8]. In addition, numerous factors, including posttranslational modifications, dynamically regulate the open/closed state of the gap junction channel and the abundance of connexins influence downstream signaling as well. Therefore, connexins and gap junctions are more than passive channels that link cells together. Recent data have suggested that connexins can pass larger biomolecules, including nucleic acids, suggesting another level of functional coupling of cells [9,10]. In addition to classic gap junction channels, unopposed connexin pores, also

known as hemichannels, can function to exchange small molecules between the intracellular space and the extracellular fluid. As will be discussed in more depth below, this function may explain the expression of connexins in tissues with limited cell-to-cell contact. There are 21 connexin genes [7]. Most tissues express 1 or more of the genes. Cx43 (gene name Gja1) is the most abundant and most heavily studied gap junction protein expressed by skeletal cells. Additionally, Cx45 (gene name Gjc1), Cx46 (gene name Gja3) and Cx37 (gene name Gja4) have also been described in skeletal system and, as will be discussed in greater detail, their contribution to tissue function is emerging.

## 2.2. Connexins in bone

Cx43 is the most highly expressed connexin in all bone cell types, including osteocytes, osteoblasts and osteoclasts [11–14]. Cx45 and Cx46 expression has been detected in osteoblastic cells [2,15,16] and in recent studies, the expression of Cx37 has been demonstrated in osteoblasts, osteocytes and osteoclasts [17,18]. The gap junctional coupling of bone cells results in the formation of an elaborately interconnected functional syncytium among the bone-embedded osteocytes and bone progenitors and osteoblasts on bone surfaces.

## 2.3. Connexins in other skeletal tissues

Cartilage is a specialized but flexible connective tissue made up of collagen fibers and proteoglycans that are secreted by chondrocytes. Donahue and colleagues reported the expression of Cx43 and the presence of functional gap junction channels in chondrocytes isolated from bovine articular cartilage [19]. Further studies demonstrated that, in addition to high levels of Cx43, human chondrocytes express Cx45, Cx32 and Cx46 [20]. In addition, using an animal model in which the Cx29 gene was replaced by LacZ, it was recently shown that this connexin is expressed in chondrocytes of the fibrous cartilage of the intercalated discs and in the epiphysis of the vertebrae [21].

Ligaments are composed of fibrous tissue connecting bones or cartilages, serving to support and strengthen joints. Expression of Cx43 [22,23] as well as of Cx32 [24], Cx40 and Cx45 [25] was found in cells from periodontal ligaments. Cx43 expression and the presence of gap junctions has also been detected in medial collateral ligaments [26]. Of note, the localization of Cx43 staining differs in isolated ligament cells in vitro from that of intact ligaments.

In tendons, a fibrous tissue synthesized by tenocytes that attaches muscle fibers to bone or cartilage, microscopy studies showed the presence of Cx43 in cell processes and of Cx32 in cell bodies of cells from rat flexor tendon [27]. The same 2 connexins are expressed in equine [28], avian [29] and sheep [30] tendon. The 2 connexins exhibit distinct localization, with Cx43 found in the tip of the cell processes as well as between cell bodies and Cx32 only between cell bodies [27].

## 2.4. Pannexins in bone, cartilage, ligament and tendon

There are 3 pannexins genes, namely Panx1, Panx2, and Panx3, which encode proteins with strikingly similar structural topology to connexins. Despite their structural similarities, pannexins have little sequence homology with connexins and function exclusively as an

unpaired channel, thereby communicating signals, primarily adenosine triphosphate (ATP), directly between the cytoplasm and the extracellular space [31,32]. Panx1 and Panx3 are broadly expressed, including in skeletal cells [33,34]. Panx1 is found in murine osteoblastic cells [35], whereas Panx3 is expressed in various osteoblastic cell lines and primary calvaria cells and in hypertrophic chondrocytes [36–39]. Panx2 was thought to exhibit a more restricted expression, being most abundant in the brain, spinal cord and neurons [40–42]. However, recent studies showed that Panx2 has a wider expression pattern [43] and can be detected in osteoblastic cells [36]. Panx1 expression was also found in periodontal ligaments [44], while there are no reports of pannexin expression in tendons.

### 3. Connexins in bone

#### 3.1. In vitro studies

In vitro gain-of-function and loss-of-function studies have made clear that Cx43 controls osteoblast and osteocyte function and influences cell survival. Early studies demonstrated that the relative abundance of Cx43 in cells of the osteoblast lineage impacted their ability to differentiate, with increased coupling via Cx43 promoting osteoblast differentiation and function and inhibition of Cx43 reducing osteoblast differentiation and function [45–48]. In Cx43 knockout models, there is a cell autonomous dysfunction in the osteoblast lineage, including reduced expression of markers of osteoblast differentiation [49–54]. Cx43 regulates osteoblastogenesis and cell survival, not only by the passive exchange of second messengers between cells, but also by actively modulating signal transduction cascades. Increasing Cx43 levels in osteoblasts or osteocytes increases extracellular signal-regulated kinase (ERK) signaling [55–57]. This activation of ERK by Cx43 is caused by cell-to-cell communication of signals, as direct cell-to-cell contact is required for this effect, and the percentage of cells that become ERK-positive in response to a specific cue is increased when Cx43 is overexpressed [58]. Similarly, Cx43 gain-of-function increases protein kinase C delta (PKC $\delta$ ) activation by fibroblast growth factor 2 (FGF2) in cultured osteoblasts [58,59]. Cx43 also alters protein kinase A-dependent signaling by sequestering  $\beta$ -arrestin in osteoblasts stimulated with parathyroid hormone (PTH) [60]. In fact, such interactions may represent a fundamental way that Cx43 influences signaling cascades.  $\beta$ -arrestin forms a complex with the Cx43 C-terminal domain in PTH-treated osteoblasts. This interaction with Cx43 prevents  $\beta$ -arrestin from blunting cyclic adenosine monophosphate (cAMP)-dependent signaling from the PTH receptor, leading to enhanced cell survival. Furthermore, deletion of Cx43 or removal of the C-terminal domain prevents the sequestration of  $\beta$ -arrestin by Cx43 and abolished the ability of PTH to promote osteoblast survival. Similarly, the Cx43 C-terminus is required for ERK activation resulting in cell survival [60]. In addition, PKC $\delta$  was shown to physically bind to the Cx43 C-terminal domain [61] and interactions with the C-terminus are required for Cx43 overexpression to enhance osteoblast signaling and gene expression following FGF2 administration [62]. Another example of this structure-function relationship between the C-terminal domain of Cx43 and signaling cascades in cells of the osteoblast lineage is the interaction of Cx43 with  $\alpha$ 5 $\beta$ 1 integrin in osteocytes [63,64]. In vitro studies show that the interaction between Cx43 and  $\alpha$ 5 integrin permits the opening of Cx43-based hemichannels in response to fluid flow shear stress. Once opened, these

osteocyte Cx43-based hemichannels have been implicated in the release of autocrine/paracrine effectors, including ATP and prostaglandin E2 [65–67].

Cx43 can modulate osteoblast/osteocyte function by regulating numerous effectors. Increasing or decreasing Cx43 expression or function in osteoblast cell lines alters specificity protein 1 (Sp1) recruitment to osteoblast promoters, leading to changes in gene expression [68,69]. This effect is mediated downstream of ERK activation with Cx43 supporting robust ERK activity and concomitant Sp1 recruitment, while disruption of Cx43 function attenuates ERK activation leading to diminished Sp1 recruitment [56]. Indeed, the modulation of an ERK/Sp1 axis by Cx43 has been shown in other cell systems, suggesting functional conservation [70–72]. In bone cells, this Cx43-dependent recruitment of Sp1 can also enhance the recruitment and transcriptional activity of specific protein 7 (Sp7)/Osterix, a master regulator of osteoblastogenesis, to osteoblast promoters [68]. Similarly, Cx43-dependent ERK activation promotes cell survival signals downstream of bisphosphonates [55,57]. Cx43 can also effect signaling through an inositol polyphosphate/PKC $\delta$  cascade to influence another master regulator of osteoblastogenesis, Runt-related transcription factor 2 (Runx2) [58,59,73]. In addition, loss of Cx43 reduces the bone morphometric protein 2 (BMP2)-responsiveness of osteoblasts [74].

Overall, these Cx43-influenced signals impact the expression of osteoblast genes involved in the control of differentiation (i.e. Runx2 and Sp7/Osterix), matrix production (i.e. collagen type 1-alpha 1 chain (Col1a1), gamma-carboxyglutamic acid-containing protein (Bglap)/osteocalcin) collagen processing and crosslinking (i.e. lysyl oxidase), osteoclastogenic factors (i.e. OPG and the RANKL/OPG ratio) and sclerostin [45,51–53,62,75–78]. Notably in most of these examples, increasing Cx43 production enhances signaling and the expression of osteoblast genes, while decreasing Cx43 attenuates osteoblast differentiation. However, in vivo experiments on the role of Cx43 in bone have shown that context matters. For example, during mechanical loading, loss of Cx43 increases the anabolic response of bone, whereas during mechanical unloading, Cx43 deletion can diminish bone loss and even in this context this view is somewhat oversimplified, as these responses can differ in periosteal osteoblasts and endosteal osteoblasts [52,78–81]. A few in vitro studies highlight the context dependence of the role of Cx43 in osteoblast/osteocyte function. Loss of Cx43 has been shown to reduce the inhibitory effects of endothelin-1 on osteoblasts [82,83], perhaps by disrupting calcium-dependent signaling events [84]. Similarly, loss of Cx43 increases  $\beta$ -catenin levels in cultured osteocytes, enhancing their mechano-responsiveness [85].

Interestingly, mounting in vitro and in vivo evidence demonstrates that heterogeneous cell-to-cell coupling can influence bone cell function, with osteocytes [86–88], fibroblasts [89], endothelial cells [90–93] and megakaryocytes [90] influencing osteoblast function when interconnected by Cx43-containing gap junctions. In addition, osteoblastic Cx43 expression has been shown to contribute to the maintenance of the hematopoietic niche [94] and to even indirectly regulate skeletal muscle function [95].

Beyond the indirect control of osteoclasts by the osteoblast/osteocyte RANKL/OPG/Cx43 axis, little is known about the direct role of connexins in bone-resorbing osteoclasts.

Osteoclasts also express Cx43 [96]. Blocking of Cx43 in human and rodent osteoclasts in vitro can impact osteoclast fusion and function in vitro [96–98]. Furthermore, strong expression of Cx43 is observed in the giant osteoblasts found in patients with Paget's disease and giant cell tumors of bone, suggesting a role for Cx43 in the formation of these cells [97]. In contrast, a clear role for Cx37 in osteoclastogenesis has been shown [17]. Osteoclasts derived from Cx37 knockout mice are smaller, with fewer nuclei and exhibit markedly reduced expression of a host of osteoclast markers as well as increased Notch signaling.

### 3.2. In vivo studies

**Genetically modified mouse models**—Mice with global deletion of Cx43, which results in perinatal death, exhibit delayed ossification due to dysfunction of osteoblastic cells [16,49]. Mice with deletion of Cx43 in cells of the osteoblastic lineage are viable and exhibit a bone phenotype that is progressively more profound, as the cells in which the connexin is deleted are less differentiated [51,75,78,99]. Thus, deletion of Cx43 from osteochondroprogenitors using the *Dermo1/Twist2* promoter to target Cre recombinase expression leads to decreased bone mass and reduced length of long bones [51]. Deletion in committed osteoblast progenitors using Cre recombinase under the control of the 2.3kb fragment of the collagen 1a1 gene also results in reduced bone mineral density [75], but mice are not shorter than their littermates expressing Cx43 [95]. This suggests that Cx43 expression in osteochondroprogenitors, but not in committed osteoblasts, is required for normal long bone growth. Deletion of Cx43 from mature osteoblasts, using the human osteocalcin promoter to drive Cre expression, or from osteocytes only, using the 8kb fragment of the dentin matrix protein 1 promoter, does not affect either bone mineral density or longitudinal growth [52,53,99].

A recent study shows that deletion of Cx43 from osteoblast progenitors using the osterix-Cre mice results in delayed intramembranous ossification [74]. However, it has been shown that mice expressing the osterix-Cre show a defect in craniofacial bone development even in the absence of any floxed gene [100] and, therefore, the presence of Cre under the control of the osterix promoter might contribute to the intramembranous ossification defect. Nevertheless, mice with Cx43 deletion in osterix-expressing cells exhibit a similar cortical bone phenotype [74] as other mice with bone cell-specific Cx43 deletion, as will be detailed below.

Further support for a role of Cx43 in osteoblast differentiation was provided by studies in which microRNA 206 (miR-206) was overexpressed under the control of the 2.3 kb fragment of the collagen1a1 promoter [101]. These animals exhibit decreased mass of trabecular and cortical bone and low levels of Cx43. In addition, in vitro experiments showed that the decrease in alkaline phosphatase induced by miR-206 expression is reversed by transfection with Cx43, which is a target of miR-206. While the decrease in Cx43 protein levels might be responsible in part for the phenotype of miR-206 transgenic mice, other genes are involved, because the phenotype of miR-206 transgenic mice is more profound than those in which Cx43 was deleted using the same promoter [75].

Despite the difference in bone mass and size, all animal models of Cx43 deletion in cells of the osteoblastic lineage exhibit increased periosteal bone formation and endocortical bone

resorption, resulting in widening of the marrow cavity and the external perimeter of long bones, albeit the effect is more profound when the connexin is deleted earlier in the osteoblastic lineage [51,53,74,78,79]. These mice display increased cortical osteocyte apoptosis [52,53,99], periosteal bone formation and a high RANKL/OPG ratio, which favors osteoclast differentiation [51,53,78], resulting in increased bone resorption. Loss of osteocytic Cx43 reproduces many of the features of Cx43 deletion in cortical bone [53], suggesting a fundamental role of osteocytes in the gap junction network. However, the increasing severity of the skeletal phenotype in models where Cx43 is deleted in less mature osteoblastic cells implies that Cx43 plays a role in osteoblast function as well. In addition to its role in bone mass and cortical bone structure, osteocytic Cx43 might play a role in regulating intracortical bone resorption and the removal of surrounding bone matrix by osteocytes, a process known as osteocytic osteolysis [5], as suggested by the increased osteocytic lacunar size in mice lacking Cx43 in osteoblasts and osteocytes [102].

Mutations in the Cx43 gene associated with oculodentodigital dysplasia (ODDD) [103] and craniometaphyseal dysplasia [104] result in skeletal manifestations in humans. These abnormalities have been reproduced in 2 animal models in which mutated Cx43 genes (i.e. G138R and G60S) are expressed in all cells and tissues [105–107]. In addition, expression of the G138R-Cx43 in osteochondroprogenitors recapitulates the skeletal phenotype of mice with global expression of the mutated gene [51]. Recent studies showed that, besides the decreased bone mass, mice expressing G60S-Cx43, a dominant-negative mutant that disrupts gap junction assembly and function, exhibit changes in the bone marrow with progressive bone marrow atrophy and increased adipocytes [107,108]. These phenotypic changes were not reported for mice carrying the G138R-Cx43 ODDD mutation, which does not alter gap junction assembly, but that forms communication-deficient gap junction plaques [105] or in any of the mouse models of bone-specific deletion of Cx43. The mechanism by which mutated G60S Cx43 increases adipogenesis remains to be determined.

Recent evidence shows that Cx43 expression in osteoblastic cells not only controls bone mass and structure, but also skeletal muscle growth and function [95]. Thus, mice in which Cx43 was deleted using the 2.3kb fragment of the *Col1a1* promoter to target Cre expression exhibit lower weight and strength of fast twitching muscles than littermate controls expressing Cx43. These effects of osteoblastic Cx43 have been linked to a decrease in undercarboxylated glu-osteocalcin. Indeed, glu-osteocalcin promotes myotube formation *in vitro* and rescues the effect of Cx43 deletion on muscle mass and strength *in vivo*. The decrease in muscle mass is associated with reduced body weight without changes in body size. On the other hand, mice lacking Cx43 in mature osteoblasts or in osteocytes do not exhibit reduced body weight [52,53,99], suggesting that the regulation of muscle development by Cx43 is restricted to its expression in immature osteoblasts.

In addition to the regulation of osteoclast differentiation through Cx43 expression in osteoblasts and osteocytes [51,53,78], the gap junction protein is also required for osteoclast differentiation in a cell-autonomous manner. This was reported recently in a study in which Cx43 was deleted using the human cathepsin K promoter to target Cre expression to pre-osteoclasts [109]. These mice exhibit fewer osteoclasts on the trabecular bone of the distal

femur and increased cortical thickness and reduced trabecular spacing, suggesting reduced osteoclastic bone resorption [109].

As in mice, deletion of Cx43 in other vertebrates also results in a bone phenotype, with limb [110,111] and face [112] abnormalities in chicken. Like in mice, complete deletion of Cx43 in zebrafish is lethal, but mutation of Cx43 results in the so-called short-fin phenotype, with short bony segments and reduced cell proliferation in the fin skeleton [113]. Recent studies showed that hyaluronan and proteoglycan link protein 1a (Hapln1a) and semaphorin3d both work downstream of Cx43 to regulate cell proliferation and joint formation in zebrafish fin bones [114].

**Bone-acting stimuli and Cx43 in bone cells**—Cx43 not only is required for bone development and structure under basal (i.e. non-stimulated) conditions, as evidenced by the genetic manipulations, but also for the response of the skeleton to bone-acting stimuli. In particular, the bone-protecting bisphosphonate drugs [115] preserve osteoblast and osteocyte viability by opening Cx43-based hemichannels [116,117] and the survival effect of the drugs is abolished in mice lacking Cx43 in osteocytes [99]. Intermittent administration of PTH is the only Food and Drug Administration (FDA)-approved treatment to increase bone mass [118]. Cx43 expression appears to be required to obtain a full anabolic response to intermittent PTH administration in mice, as PTH-induced mineral appositional rate, a measure of the work of osteoblast teams, is reduced in mice lacking Cx43 in osteoblastic cells [75]. On the other hand, deletion of Cx43 in osteocytes does not impair the ability of the hormone to increase bone mass, but PTH does not increase endocortical bone formation or mechanical properties in cortical bone of mice lacking osteocytic Cx43, suggesting that part of the effect of the hormone required the expression of Cx43 in osteocytes [119].

In vitro studies showed that Cx43 expression is required for the response of osteoblastic and osteocytic cells to mechanical stimulation [120]. Based on this finding, it was hypothesized that the response to mechanical loading is eliminated, or at least reduced, in the absence of Cx43 in osteoblasts and/or osteocytes. However, work of several groups showed that, instead, bone formation induced by mechanical loading is enhanced in mice in which Cx43 was deleted from osteochondroprogenitors [80], from mature osteoblasts and osteocytes [78] or from osteocytes only [85]. This suggests that Cx43 restrains the response to loading likely through its function in osteocytes. It has been proposed that Cx43 sequesters  $\beta$ -catenin, thereby inhibiting Wnt signaling, known to increase bone formation [85]. Thus, in the absence of Cx43,  $\beta$ -catenin is free and Wnt signaling is increased. This primes osteoblastic cells to respond to mechanical signals by a yet to be identified mechanism. However, the detailed molecular pathway that mediates this inhibitory effect of Cx43 on bone formation induced by mechanical forces remains to be determined [120].

Osteoblastic Cx43 also participates in the response to lack of mechanical forces as demonstrated by reduced bone loss and a lack of increase in osteoclasts in mice lacking Cx43 in osteoblasts and osteocytes subjected to unloading by tail suspension compared to control mice expressing Cx43 [52,79]. In addition, absence of Cx43 in osteoblasts and osteocytes impairs fracture healing by a combination of decreased bone formation and bone resorption [121]. Interestingly, the effect of Cx43 deletion in fracture healing appears to be



opposite to that of normal bone, since the RANKL/OPG ratio is decreased, while the levels of the Wnt inhibitor sclerostin (i.e. the product of the *Sost* gene) are increased and  $\beta$ -catenin is decreased in the fracture [122]. In addition, the effect of Cx43 deletion on fracture healing can be reversed by increasing  $\beta$ -catenin stability [122]. These pieces of evidence raise the possibility that Cx43 has distinct roles in bone acquisition/maintenance versus bone healing.

Aging also affects Cx43 expression and function. Thus, osteoblastic cells from old rats (i.e. 12-months old and 24/28-months old) exhibit decrease gap junction communication in response to PTH or cholera toxin, compared to cells from younger (i.e. 4-months old) animals [123]. On the other hand, no changes in Cx43 expression in osteoblastic cells isolated from young versus old rats were found. Similar results were shown in mice, with no change in mRNA levels for the gene when it was measured in vertebral bone from 6-months old and 24-months old mice [124]. However, in this case, PTH increased the levels of the Cx43 gene in both young and old mice. A more recent study showed that Cx43 protein expression is decreased in regenerated bone from aged (i.e. 21-months old) rats compared to mature (6-months old) animals, which is associated with reduced nitric oxide and prostaglandin E2 production following mechanical stimulation [125]. The old bones also exhibit decreased lacunar and osteocyte density. Whether the decrease in osteocyte viability and the accumulation of empty lacunae with old age results from decreased Cx43 protein expression or function remains to be determined.

**Channel versus cytoplasmic domains: what we know about the function of Cx43 domains in bone cells?**—Most of the studies in which the role of Cx43 in bone was investigated using genetic tools involved complete deletion of the molecule, precluding the possibility to understand the role of the different connexin domains in the overall phenotype [3]. Although the Cx43 variants associated with ODDD result from point mutations in particular amino acids, there is not always a direct relationship between the site of the mutation and the consequence in channel activity, ability to form gap junction channels versus hemichannels, or in the interaction and regulation of signaling molecules through the C-terminus domain [103].

A recent study described the skeletal phenotype of 2 mouse models with mutated Cx43 expressed in osteocytes. In particular, in 1 model with the point mutation R67W, Cx43 has the ability to form functional hemichannels, but not gap junction channels, while in the other model, with a deletion of amino acids 130–136 (i.e. 130–136), Cx43 lacks channel permeability [126] and therefore cannot form either functional hemichannels or gap junction channels [127]. Mice expressing the 130–136 mutant exhibit increased bone mass, whereas R67W mice are indistinguishable from wild-type controls. No changes in cancellous bone was observed in either mice, but cortical bone was affected in 130–136 Cx43 animals, resulting in a phenotype that resembles that of mice with complete deletion of Cx43 from osteocytes [53,77]. Similarly, cortical osteocyte apoptosis was increased in mice expressing the mutated 130–136 Cx43 in osteocytes [127] as in mice lacking Cx43 in these cells [53]. On the other hand, 130–136 Cx43 animals, but not mice lacking Cx43 from osteocytes, display increased osteocyte apoptosis in cancellous bone. This discrepancy could be due to the purported low levels of Cx43 in cancellous bone, which could not be decreased further in mice lacking Cx43 in osteocytes. On the other hand, overexpression of 130–136 Cx43

might be toxic for osteocytes in this bone compartment. The expression of the anti-osteoclastogenic cytokine OPG is decreased in 130–136 Cx43 mice as in mice with deletion of Cx43 in osteocytes. However, whereas the former also have reduced levels of the pro-osteoclastogenic cytokine RANKL, the animal model with complete deletion of Cx43 exhibits high RANKL expression [3].

We recently analyzed the bone phenotype [119] of mice expressing a copy of a truncated form of Cx43 lacking the C-terminus tail (i.e. Cx43<sup>245</sup>) in all cells and tissues [128]. Even though these mice still express a copy of the full length connexin, they exhibit decreased cancellous bone volume due to deficient osteoblast function, suggesting that truncated Cx43 acts as a dominant negative, similar to the ODDD mutant [107]. This bone phenotype differs from the lack of effect of deleting Cx43 in mature osteoblasts and osteocytes [3] or of expressing channel-deficient Cx43 in osteocytes [127] in the cancellous bone compartment.

The current evidence for the role of the Cx43 domains in bone cells is shown in Table 1. Overall, Cx43-based channel function in osteocytes appears to be required for proper cortical bone acquisition and material strength, whereas the C-terminus domain is required to reach normal cancellous bone volume and osteoblast function. On the other hand, absence of channel activity increases bone mass and osteoblast function in cancellous bone, and the lack of the C-terminus domain results in increased cortical bone strength. In summary, Cx43 domains exert distinct functions depending on the bone compartment study, controlling bone accrual and bone cell function and survival [53,119,127].

**Cx37, a recently found bone connexin that regulates bone mass**—In addition to Cx43, studies showed that Cx37 is also expressed in osteoblasts, osteocytes and osteoclasts [17,18]. Global deletion of Cx37 result in increased bone mass, an effect that is more pronounced in male than in female mice [17]. Consistent with this gender-specific effect of Cx37 deletion, men, but not women, carrying the Cx37-319P allele exhibit higher total body, lumbar spine, femoral neck and trochanter bone mineral density compared to individuals carrying the Cx37-319S allele in a Japanese population [129]. The murine high bone mass phenotype is due to decreased osteoclast differentiation, resulting in decreased osteoclast number and bone resorption without altering osteoblast differentiation or function [17].

## 4. Pannexins in bone

### 4.1. In vitro studies

Panx1 and 3 are expressed by osteoblasts [33], but their role is only beginning to be uncovered. Panx3 is a direct target of the osteoblastogenic transcription factor Runx2 and its expression increases during osteoblast differentiation [130]. Overexpression of Panx3 promotes the osteogenic differentiation of C2C12 cells in culture, including inducing the expression of Sp7/Osterix and osteocalcin [37]. Conversely, short hairpin RNA-mediated knockdown of Panx3 inhibits osteoblastogenesis. In addition, ex vivo adenoviral transduction of newborn mouse metatarsals with a Panx3 expression construct enhanced osteoblastogenesis and increased bone length. Panx3 carries out this function by serving not only as a direct channel between the cytoplasm and extracellular space, but perhaps by also

acting as a calcium channel in the endoplasmic reticulum whose function converges on Akt signaling networks. Furthermore, Panx3-based channel activity can promote osteogenic differentiation through increased  $\beta$ -catenin activity and the attenuation of protein kinase A-signaling permitting cell cycle exit and subsequent differentiation [38]. While it has been suggested that Panx3 may function as a gap junction-like channel for direct cell-to-cell communication [131], this is based on the use of relatively non-selective inhibitors that impact both gap junction and pannexin channel functions. Indeed, a recent paper has suggested that many of the hemichannel activities ascribed to Cx43 may be rather due to pannexin activities [132]. Panx1 is virtually unstudied in osteoblast or osteocytes and neither Panx1 nor 3 have been reported in osteoclasts.

#### 4.2. In vivo studies

In vitro studies have shown that pannexins are involved in osteoblast differentiation and function as described above. However, in vivo evidence for the role of pannexins in bone cell biology is lacking. Even though mice with global deletion of Panx1 and 3 have been generated [133], their bone phenotype or lack thereof has not been reported. Nevertheless, a preliminary study presented as an abstract showed that the increase in RANKL, a cytokine required for osteoclast differentiation, which occurs in the area surrounding apoptotic osteocytes following microdamage is abolished in mice lacking Panx1 [134].

### 5. Connexins in cartilage, ligaments and tendons

#### 5.1. In vitro studies

**Cartilage**—Cx43 is expressed in both articular chondrocytes as well as growth plate chondrocytes [135], but its role is quite intriguing, as these cells are generally functioning in virtual isolation free from many direct cell-to-cell contacts. While these cells can form classic gap junction in culture [19,136], it is generally thought that the role of Cx43 in chondrocytes may be related to hemichannel activity [137–140]. Cyclic loading of articular chondrocytes has been shown to open Cx43-based hemichannels and leads to the release of ATP, which may be part of a mechanotransduction pathway [137,138]. Furthermore, fluid flow shear stress has been shown to activate Cx43-based hemichannels in cultured chondrocytes, which in turn affects extracellular prostaglandin E2 levels [139], a model that closely mimics observations made in osteocytes [65,67]. Analogous to the role of Cx43 in osteoblasts, inhibition of Cx43 has been shown to restrict the differentiation of mesenchymal stem cells down the chondrogenic lineage, although in contrast to osteoblasts, this effect is independent on cell-to-cell contacts [140].

An interesting association has been made between Cx43 levels and osteoarthritis-related changes in the joint. In vitro, Cx43 is increased by the pro-inflammatory cytokine interleukin 1 in chondrocytes [141]. In synovial cells [141,142], Cx43 can increase calcium wave propagation between cells [136] and can as such influence signaling cascades and osteoarthritis-associated gene expression [143–145]. In vivo data implicating Cx43 in osteoarthritis pathology will be discussed below.

**Tendon**—Tendon cells express Cx43 and Cx32, forming a 3-dimensional network in vivo [27]. In tenocytes exposed to mechanical load, Cx43 and Cx32 have opposing functions [29]. Cyclic tensile loading increases collagen secretion, an effect that is reduced by knockdown of Cx32 expression, but increased by knockdown of Cx43 expression. This may be consistent with a recent report showing downregulation of Cx43 protein expression following static tensile load [146]. However, loss of gap junctional coupling among tenocytes during aging has also been implicated in the reduced potential of specific tendons to repair [147]. Despite evidence that Cx43 inhibits tenocyte repair capacity, interleukin 1 increases Cx43 expression in tenocytes, where it may play a role in cell survival following strain [148].

**Ligament**—Ligamentous fibroblasts express Cx43, Cx40, Cx45 and Cx32 [23–25]. As in the other skeletal tissues, it seems that mechanical strain opens Cx43-based hemichannels in ligament cells leading to the release of ATP [149]. In addition, an increase in Cx43 expression is associated with pathologic mineralization of the posterior longitudinal ligament in the cervical spine [150]. Ligamentous cells isolated from patients with an ossified posterior longitudinal ligament have increased expression Cx43 and of osteogenic genes, including osteocalcin, alkaline phosphatase and collagen I, relative to control non-ossified posterior longitudinal ligament cultures [150]. Furthermore, these changes in osteoblast gene expression can be inhibited by Cx43-targeting small interfering RNA. In addition, these ossified posterior longitudinal ligament cells have a more robust osteogenic response to mechanical strain, which depends on Cx43 expression [151]. Indeed, Cx43 expression in ligament cells appears to be enhanced by mechanical strain [151,152]. Like in many other skeletal tissues, Cx43 influences ERK signaling in ligamentous fibroblasts and can regulate osteogenic genes during cyclic mechanical tension [153].

## 5.2. In vivo studies

**Cartilage**—Little is known about the role of connexins in cartilage in vivo. Cx43 is expressed by growth plate and articular chondrocytes [135] and chondrocytes in the meniscus [154] in vivo. Human chondrocytes also express Cx45, Cx32 and Cx46 [20]. Direct cell-to-cell communication has been shown in the superficial zone of articular chondrocytes [155]. A paradigm challenging study has even shown that chondrocytes in situ may form long cell processes, 5 to 150 microns in length, that permit communication through gap junction channels [156]. Increased presence of gap junction plaques and Cx43 expression have been found in synovial lining cells obtained from the knee of patients with osteoarthritis [157]. Similarly, Cx43 production is increased in the cartilage of the knee and femoral head [20] and in the shoulders of patients with osteoarthritis, suggesting that the molecule might have a role in the pathogenesis of this disease [158]. Consistent with this, Cx43 levels in osteoarthritic cartilage correlate with the expression of several pro-inflammatory and catabolic factors [158]. In line with this notion, silencing of Cx43 expression protected against inflammation and joint destruction in a model of rheumatoid arthritis in rats [159].

**Tendon**—The expression of Cx43 and Cx32 has been studied in fetal and adult tendons obtained from horse [28], rat [27,160] and sheep [30]. These studies show that the 2

connexins are present throughout tendon development, although with a different pattern of expression and localization. Their level is high in fetal life and decreases in adult tissue, and these changes accompany the decrease in the proliferation index and cellularity of the tendons, as well as the change from round to elongated cell nuclei [27,28,30,160]. This suggests that Cx43 and Cx32 could be involved in the maturation of the tendon. However, further studies are required to confirm this possibility.

**Ligaments**—As for tendons, little is known on the role of connexins in ligaments in vivo. A study showed that Cx43 is increased in periodontal ligaments following experimental tooth movement [23]. Increases in Cx43 expression were found in spinal ligaments with ectopic bone formation [150]. Silencing Cx43 production inhibits the increase in osteoblastic genes after mechanical stimulation [151], suggesting that Cx43 plays a role in the progression of ectopic spinal ligament ossification.

## 6. Pannexins in cartilage, ligaments and tendons

### 6.1. In vitro studies

Beyond its expression in these tissues, very little is known about how pannexins can impact the function of these cells. Panx3 is both necessary and sufficient to induce chondrogenic differentiation of ATDC5 and primary chondrocytes in vitro [39]. Furthermore, Panx3 expression in chondrocytic cells reduces intracellular cAMP levels, protein kinase A activity and diminishes the proliferative response to cAMP, suggesting that Panx3 regulates the switch from proliferation to chondrocyte differentiation [39].

In periodontal ligament cells, Panx1 interacts with P<sub>2</sub>X<sub>7</sub> receptors as part of mechanical strain responsive mechanisms for ATP release [44]. Furthermore, this complex is involved in the secretion of interleukin 1 $\beta$ , perhaps through the regulation of vesicular secretion [44]. However, detailed molecular mechanisms still remain to be defined.

### 6.2. In vivo studies

To date, there are no reported studies in which pannexins were manipulated in cartilage, ligaments or tendons. Although studies with global deletion of pannexins do not report any gross abnormalities in these tissues, the possibility that pannexins are involved in the development or function of these tissues cannot be ruled out.

## 7. Conclusions

Extensive research has shown the fundamental role of Cx43 expression in osteoblast and osteocyte differentiation, intracellular signaling and in bone acquisition and maintenance, as well as in the response to stimuli that affect bone mass and strength. Furthermore, recent studies began to examine the domains of connexins that are responsible for their effect on bone, thereby uncovering a complex role of Cx43 and its cytoplasmic C-terminus and transmembrane channel domains depending on the bone envelope investigated. In addition to Cx43, recent studies show that Cx37 also is involved in bone homeostasis, yet in this case controlling osteoclast differentiation and bone resorption. On the other hand, little is known about the role of connexins in other skeletal tissue. Similarly, the role of pannexins in bone,

cartilage, tendon and ligaments is beginning to be revealed. Understanding the dynamics of cell-to-cell signaling via connexins and pannexins will enable the development of novel therapeutic strategies to optimize the musculoskeletal system and/or to enhance the effectiveness of current therapeutic agents by manipulating connexin and pannexin expression or activity.

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## Abbreviations

<b>ATP</b>	adenosine triphosphate
<b>Bglap</b>	gamma-carboxyglutamic acid-containing protein
<b>BMP</b>	bone morphogenetic protein
<b>cAMP</b>	cyclic adenosine monophosphate
<b>Col1a1</b>	collage type 1-alpha 1 chain
<b>CTX</b>	carboxy-terminal collagen crosslinks fragments
<b>Cx</b>	connexin
<b>ERK</b>	extracellular signal regulated kinases
<b>FDA</b>	Food and Drug Administration
<b>FGF2</b>	fibroblast growth factor 2
<b>Hapln1a</b>	hyaluronan and proteoglycan link protein 1a
<b>miR</b>	microRNA
<b>nd</b>	not determined
<b>ODDD</b>	oculodentodigital dysplasia
<b>OPG</b>	osteoprotegerin
<b>Ot</b>	osteocyte
<b>P1NP</b>	total procollagen type 1 N-terminal propeptide
<b>Panx</b>	pannexin
<b>PKC</b>	protein kinase C
<b>PTH</b>	parathyroid hormone
<b>RANKL</b>	receptor activator of nuclear factor kappa-B ligand
<b>Runx2</b>	Runt-related transcription factor 2
<b>Sp</b>	specificity protein

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Table 1

Skeletal effect of altering Cx43 gene expression or its domains in osteocytes

Mice	Ot-Cx43 cKO [53]	Ot- 130–136 Cx43 [127]	Ot-R67W Cx43 [127]	Cx43 245 (global) [119]
bone mass	-	↑	-	-
cancellous bone area/tissue area	-	-	-	↓
cancellous bone formation	nd	↑	-	↓
cancellous osteocyte apoptosis	-	↑	-	-
cortical osteocyte apoptosis	↑	↑	-	-
cortical bone area/tissue area	↓	↓	-	-
cortical thickness	-	↑	-	↑
marrow cavity area	↑	↑	-	-
cortical bone material strength	↓	↓	↓	-
cortical bone mechanical strength	-	↑/-	-	↑
periosteal bone formation	↑	↑	↑	-
endocortical bone formation	-	-	-	-
OPG levels in bone/osteocytes	↓	↓	-	nd
RANKL levels in bone/osteocytes	↑	↓	-	nd
circulating resorption marker	-(CTX)	-(CTX)	↑ (CTX)	-(CTX)
circulating formation marker	-(osteocalcin)	-(PINP)	↑ (PINP)	-(osteocalcin)

CTX, carboxy-terminal collagen crosslinks fragment; nd, not determined; OPG, osteoprotegerin; Ot, osteocyte; PINP, total procollagen type I N-terminal propeptide; RANKL, receptor activator of nuclear factor kappa-B ligand.