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Shifting Paradigms on the Role of Connexin43 in the Skeletal Response to Mechanical Load

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Abstract

Gap junctions (GJs) are membrane-spanning channels that allow for the movement of small molecules across cell membranes. Connexin43 (Cx43) is the predominant GJ protein in bone. In vitro studies suggest that gap junctional intercellular communication (GJIC) sensitizes bone cells to mechanical signals. Additionally, mechanical signals detected by osteocytes are communicated to osteoblasts via GJIC, and osteocytic Cx43 hemichannels release anabolic factors, such as PGE_2 and ATP, in response to mechanical load. These findings and others have led to near consensus among researchers in the field that GJIC, hemichannels or connexins facilitate the anabolic response of bone to mechanical load and, in their absence, bone would be less sensitive to load. However, recent in vivo evidence suggests the opposite is true. Studies from our laboratory and others demonstrate that Cx43-deficient mice have an increased anabolic response to mechanical load and are protected against the catabolic effects of mechanical unloading. These developments suggest a paradigm shift in our understanding of connexins, GJIC, and mechanotransduction in bone. That is, inhibiting bone cell Cx43 expression or GJIC has a beneficial effect on bone's response to its mechanical environment, preserving bone during unloading and enhancing its formation during loading. Here, we review literature in support of this hypothesis and suggest a mechanism by which Cx43, through interaction with WNT/β-catenin signaling, moderates both arms of bone remodeling.

Keywords

BONE CELLS; GAP JUNCTIONS; ANIMAL MODELS; AGING

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Introduction

Gap junctions (GJs) are membrane-spanning channels that allow for the passage of small molecules between adjacent cells or between the cell and extracellular environment when unopposed as hemichannels.[1–4] An intercellular GJ is composed of two hemichannels termed connexons, which are in turn composed of six subunits termed connexins.[5] Connexins are typically named according to their molecular mass in kilodaltons. More than 20 mammalian connexins have been identified,[6] and the predominant connexin in skeletal tissue is Connexin43 (Cx43). However, bone cells also express Connexin45 (Cx45)[7] and Connexin46 (Cx46), but Cx46 is trapped in the Golgi and does not contribute to intercellular communication.[8] Although functional connexin redundancy has been identified to a very limited degree, in some cell types[9] the functional significance in bone cells is not known. Much work has focused on the role of Cx43 in the activity of osteoblasts and osteocytes,[10] but recent studies have also identified a role for Cx43 in the function of osteoclasts[11,12] and chondrocytes.[13] In addition to functioning in GJ channels or hemichannels, connexins, including Cx43, can also regulate cell behavior by interacting with intercellular signaling molecules.[14]

Cx43 has an integral role in the maintenance of skeletal homeostasis and the activity of mature bone cells.[15–17] However, Cx43 is also a critical factor during skeletal development, as demonstrated by humans with mutations in the Cx43 gene *GJA1*.[18] These individuals develop a disease called oculodentodigital dysplasia (ODDD), characterized by dental anomalies, including small teeth with enamel hypoplasia, syndactyly, craniofacial abnormalities, missing phalanges of the toes, and the development of a tubular bone morphology.[19] Several animal models have been developed in an attempt to recapitulate the clinical presentation of ODDD. Flenniken and colleagues developed a mouse model containing a point mutation in murine *Gja1* that results in production of a mutant protein that acts in a dominant negative fashion to disrupt assembly of GJs composed of Cx43. This model is able to recapitulate many of the classical features of ODDD but also demonstrates cortical thinning, reduced bone mineral density (BMD), and reduced bone strength.[20] Dobrowolski and colleagues inserted a human Cx43G138R point mutation into *Gja1*.[21] These transgenic mice had a skeletal phenotype characterized by reduced trabecular bone mass and cranial abnormalities. Finally, Watkins and colleagues utilized the *Dermo/Twist2* (DM1) promoter, which is expressed during endochondral ossification, to introduce an ODDD mutation into the chondro-osteogenic lineage.[22] These mice were significantly osteopenic, with cortical thinning, expanded marrow cavities, and reduced femur strength but no trabecular phenotype. Cx43 has also been shown to play a role in the development of limb patterning and growth in mice.[23] Inhibition of Cx43 expression, using antisense nucleotides in chick embryos, resulted in a significant decrease in bone formation, whereas zebrafish studies indicate a role for Cx43 in joint positioning.[24] Taken together, these studies demonstrate that Cx43 has a critical role both during skeletal development as well as in the function of mature bone cells.

One suggested role for GJs in mature bone is a contribution to mechanotransduction. For instance, mechanical signals regulate Cx43 levels and GJIC between bone cells;[25–30] GJIC sensitizes bone cell networks to diverse extracellular signals;[31–34] and mechanically

induced signals are communicated among bone cells via gap junctions.[10,35,36] This has led to near consensus among researchers in the field that GJIC, hemichannels, or connexins facilitate the anabolic response of bone to mechanical load.[1–4] Indeed, the paradigm that has emerged is that in the absence of connexins and/or GJIC, bone would lose sensitivity to the anabolic effects of mechanical load.[1–4] However, recent developments challenge this and suggest a new paradigm. Here, we describe this new paradigm, review the data supporting this paradigm, and suggest a mechanism underlying this paradigm. We begin with the role of connexins in bone cell differentiation.

Cx43 and Bone Cell Differentiation

Abundant data from in vitro studies have demonstrated that Cx43 expression and GJIC parallel osteoblastic differentiation, and inhibiting GJIC and Cx43 expression in osteoblastic cells, including MC3T3-E1, UMR-106, ROS 17/2.8, human primary culture osteoblastic cells, and murine calvarial cells, with pharmacological agents or genetic manipulation results in decreased expression of phenotypic characteristics of differentiated osteoblasts including alkaline phosphatase, osteocalcin, bone sialoprotein, and PTH responsiveness.[37–43] Furthermore, mineralized extracellular matrix synthesis was impaired in calvarial osteoblasts isolated from Cx43-deficient mice.[44] Additionally, Schiller and colleagues[45] showed that inhibiting GJIC induces the transdifferentiation of osteoblastic MC3T3-E1 cells and primary culture human osteoblastic cells to an adipocytic phenotype. More recently, Inose and colleagues used a microRNA approach to target Cx43, resulting in decreased alkaline phosphatase activity and *Bglap* (encodes Osteocalcin) expression, whereas restoration of Cx43 rescued osteoblastic differentiation.[46] Cx43 is also important in cell proliferation[47] and survival.[48] Gramsch and colleagues transfected osteoblastic UMR 106 cells with Cx43. At baseline, these cells have high Cx45 expression and low Cx43 expression. A seven-fold increase in proliferation and increased size of mineralized nodules were observed in cells with increased Cx43 expression.[47] Taken together, these studies strongly suggest that Cx43 and GJIC contribute to osteoblastic proliferation and differentiation in vitro.

In vivo studies suggest that GJs may be involved in cell signaling processes important to limb bud differentiation and skeletogenesis in embryonic mice[49] and contribute to cellular differentiation and intramembranous bone formation in developing chick mandible.[50,51] Furthermore, Cx43 null mice display impaired intramembranous bone formation, and osteoblastic cells from these animals express decreased levels of type 1 collagen, osteopontin, and osteocalcin,[52] suggesting a defect in osteoblastic maturation. Although these data strongly suggest that Cx43 contributes to osteoblastic differentiation, they do not exclude the possibility that other connexins or even pannexins also contribute to osteoblastic differentiation.[53]

In addition to modulating bone formation through osteoblastic maturation, Cx43 can also affect bone remodeling and osteoclast activity. Ransjo and colleagues found that Cx43 mRNA was upregulated during parathyroid hormone (PTH)-stimulated bone resorption in vitro, whereas Schilling and colleagues found increased Cx43 expression in the giant osteoclasts of patients with Paget's disease.[54–55] Consistent with this, inhibitors of GJIC

impair osteoclast formation,[55] degree of multinucleation,[54] and resorptive activity.[11,55,56] Ilvesaro and colleagues used Gap27, a synthetic connexin-mimetic peptide, to decrease GJIC and found a lower number of multinucleated, TRAP-positive osteoclasts, along with inhibition of osteoclastic pit formation.[56] We recently demonstrated that Cx43-deficient osteocytic MLO- Y4 cells have an increased receptor activator of nuclear factor-κB ligand (RANKL)/osteoprotegerin (OPG) ratio relative to wild-type (WT) osteocytes in vitro, a finding consistent with increased osteoclastic bone resorption in vivo.[12] Watkins and colleagues have shown that co-culture of osteoblasts and osteoclast precursors, both of which are Cx43 deficient, increases the number of osteoclasts per well relative to WT osteoblasts cultured with either WT or Cx43-deficient osteoclast precursors.[22] An increase in osteoclast number was also observed in Cx43-deficient osteoblasts cultured with WT osteoclast precursors, suggesting that osteoclastogenesis may be modulated by Cx43 expression in osteoblasts rather than osteoclast precursors.[22] Taken together, these data suggest that decreasing GJIC between osteoclast and themselves inhibits osteoclastogenesis, whereas decreasing Cx43 expression in osteoblast or osteocytes increases osteoclastogenesis. However, the relative contribution of each mechanism is not known. In any case, the emerging data suggest a critical role for Cx43 in both arms of bone remodeling.

In Vitro Signaling Through Cx43 Gap Junctions and Hemichannels

Apoptosis

Bisphosphonates are antiresorptive agents that maintain bone mass by inducing osteoclast apoptosis.[57] Conversely, osteocyte and osteoblast apoptosis is inhibited by bisphosphonates, a process that occurs via increased Cx43 hemichannel opening.[58,59] Interestingly, hemichannels are more important than complete GJ channels in cell survival, specifically in modulating the anti-apoptotic effects of bisphosphonates. Transfection of Cx43, but not other connexins, confers resistance to apoptosis in cells treated with bisphosphonates when cultured at a density low enough to prevent GJIC.[59] Bisphosphonates increase Cx43 interaction with Src leading to phosphorylation of Src and ERK1/2,[59] with hemichannel opening itself responsible for increased Src and ERK expression.[59] Upon ERK1/2 activation, osteoblast and osteocyte apoptosis is inhibited by the activation of an anti-apoptotic signaling cascade that includes activation of the kinase p90RSK, phosphorylation of BAD and C/EBPb, and the subsequent binding of C/EBPb to pro-caspases 1 and 8.[60] In addition to inhibiting osteoblast apoptosis, risedronate can enhance transcriptional activation of the Cx43 promoter and increase Cx43 protein expression, while concurrently stimulating osteoblastic differentiation.[61] Together, these data suggest that risedronate, and possibly other bisphosphonates, may modulate bone formation by promoting osteoblastic differentiation and inhibiting osteocyte apoptosis via Cx43.

Second messengers

Although the involvement of Cx43 and GJs in osteogenesis is clear, the small molecular signals that pass through the channels and how they affect osteoblastic differentiation is not well understood. However, previous studies demonstrated that changes in Cx43 expression

alter the osteoblastic response to fibroblast growth factor 2 (FGF2) signaling by modulating the transcriptional activity of Runx2, a process that is dependent on ERK and PKCd.[62–64] Furthermore, inhibition of phospholipase Cg1 (PLCg1) attenuates Cx43-mediated, FGF2-induced signaling through Runx2.[65] Interruption of this pathway inhibits PKCd nuclear accumulation and interaction with Runx2, leading to decreased Runx2 transcriptional activity of osteoblastic genes including *Col1a1*, Osteocalcin (*Bglap*), and *Osx* (encodes Osterix) in MC3T3 cells. These data suggest that transmission of inositol phosphates through Cx43 GJs are important in generating signals to activate PKCd and osteogenesis.

Cytosolic calcium also transfers between cells via GJIC and can affect downstream signaling including calmodulin and calcineurin. For instance, studies from our lab demonstrate that mechanically induced calcium signal activation of calcineurin mediates the effect of mechanical signals on mesenchymal stem cell proliferation.[66] Presumably, calcium signals arriving from osteocytic cells via GJIC would have a similar effect. Finally, recent evidence suggests that microRNA can pass from one cell to another via GJIC.[67] Although this has yet to be demonstrated specifically in bone cells, it offers yet another mechanism by which bone cells might communicate with one another.

In Vitro Studies of Cx43 and Mechanotransduction

The spatial localization of osteoblastic Cx43 in areas of contact between cells[40] and at the tips of the dendritic processes in osteocytes[35] suggests an important role for GJs in the bone cell response to biophysical stimuli, including mechanical loading. In vitro studies reveal that GJIC sensitizes bone cell networks to diverse extracellular signals including load-induced signals.[32,33] Furthermore, mechanical signals detected by osteocytes can be communicated to osteoblastic cells via GJIC.[10,35] Mechanical stimulation increased Cx43 expression[26,29] and phosphorylation[26] as well as Cx43-mediated GJIC.[26,29] Conversely, Thi and colleagues showed that shear stress decreased both Cx43 protein expression at the cell membrane and GJIC in MLO-Y4 osteocytic cells.[30] Cx43 protein localization at dendritic tips of osteocytes was decreased when low-magnitude shear stress (5 dynes/cm^2) was applied, relative to control cells. Cx43 expression decreased further as the magnitude of shear stress increased; however, differential effects on Cx43 and Cx45 mRNA expression were observed between low- and high-magnitude shear stresses. Cx43 mRNA was increased under low-magnitude shear stress and no change in Cx45 expression was observed, whereas Cx43 mRNA was decreased under high-magnitude shear stress, with a concomitant increase in Cx45 mRNA expression. These findings suggest a possible compensatory response by Cx45 as a result of Cx43 inhibition and indicate that the composition of GJs can be altered in response to specific mechanical stimuli.[30] However, although Cx45 upregulation may compensate for decreased Cx43 expression, the functional significance of this is unclear. Although the expression of Cx43 may vary under different mechanical stimulation conditions, there remains a clear role for Cx43 in the osteocytic response to these signals, as well as subsequent osteoblastic differentiation.[10] Taken together, these studies suggest that Cx43 and GJIC are critical to the anabolic response of bone to mechanical load.

In addition to the role of GJs in bone cell response to mechanical load, emerging evidence suggests that Cx43 hemichannels may also be a key component in the transduction of

osteogenic signals in response to mechanical stimulation.[68,69] Osteocytic MLO-Y4 cells cultured at low density exhibited enhanced hemichannel activity in response to fluid flow, a response attenuated by the inhibition of hemichannel activity or by inhibition of Cx43 expression.[70] Additionally, a recent study has demonstrated that direct mechanical perturbation via magnetic beads of integrin a5β1 leads to the opening of the Cx43 hemichannels.[2] Furthermore, estradiol increases Cx43 expression in osteocytic MLO-Y4 cells, and this increases the effect of fluid flow on MAP kinase activity.[71]

Mechanistically, hemichannel activity increases the release of adenosine triphosphate (ATP), which in turn stimulates the production of the bone anabolic agent prostaglandin E2 (PGE_2). Demonstrating the interdependent relationship between Cx43 and PGE_2 in response to mechanical stimulation, PGE_2 treatment stimulates Cx43 expression,[28] whereas Cx43 hemichannels are necessary for the release of PGE_2.[72] Moreover, inhibition of PGE_2 dramatically reduces Cx43 expression,[73] whereas Cx43 siRNA decreases ATP release from hemichannels and inhibition of ATP release attenuates PGE_2 production.[70] These data suggest a role for Cx43 hemichannels in the osteogenic response to mechanical stimulation via the release of anabolic PGE_2, ATP, or both, at least in vitro.

In Vivo Studies on Cx43 and Mechanotransduction

In vivo models

Aside from attempts to recapitulate the ODDD phenotype, numerous studies have focused on defining the physiological role of Cx43 in skeletal homeostasis through the creation of mouse strains with Cx43 deletion in specific cell populations (summarized in Table 1). This approach is necessary because global Cx43 knockout is perinatal lethal attributable to the development of severe cardiac dysfunction.[74] Castro and colleagues developed two mouse strains with an osteoblast/osteocyte-specific Cx43 deficiency (cKO), driven by either the 2.3-kb fragment of the a_1-collagen promoter (Col1-Cre) or the osteocalcin OG2 promoter (OCN-Cre).[75] The a_1-collagen promoter is expressed earlier in osteoblast differentiation than is the osteocalcin promoter.[76] Thus, using these different promoters, one can control the point in the osteoblastic lineage that *Gja1* expression is decreased. Because osteocytes are terminally differentiated osteoblasts, the result of using either of these promoters is an osteoblast and osteocyte-specific knockout. This approach avoids the development of skeletal deformities resulting from Cx43 ablation at earlier stages of maturation. Col1-Cre cKO mice have an osteopenic phenotype, with low BMD, cortical thinning, and expansion of the marrow cavity, much like their ODDD counterparts.[31,77] However, these mice do not possess cranial abnormalities or cardiac defects, highlighting the lack of effect on early development or systemic physiology. These mice also have reduced trabecular bone volume fraction (BV/TV), although the morphology of individual trabecular struts (ie, thickness, separation, number) is not different. Although OCN-Cre cKO mice have a similar osteopenic cortical bone phenotype, there is no difference in trabecular microstructure.[78,79] This suggests that the stage of the osteoblastic lineage at which Cx43 is deleted is critical to the bone compartments affected, with the relatively delayed deletion of Cx43 in OCN-Cre cKO mice resulting in a normal trabecular phenotype. It is possible that this is related to the greater rate of turnover of trabecular bone.[80] Col1-Cre cKO mice also weigh approximately

10% less than their WT littermates.[77] We have demonstrated that OCN-Cre mice weigh the same as WT and have a similar body composition (lean, fat, fluid mass) as assessed by ^{1}H-nuclear magnetic resonance (NMR).[78] A detailed assessment of Col1-Cre body composition may reveal differences in fat or lean mass, or it could be that early osteoblast deletion of Cx43 delays overall animal growth by an unknown mechanism. Watkins and colleagues used the DM1-Cre to ablate Cx43 from the chondro-osteogenic lineage. DM1-Cre cKO mice have low bone mass with delayed mineralization, a cortical osteopenic phenotype with characteristic cortical expansion and thinning, and no trabecular phenotype.[22] Finally, Bivi and colleagues created osteocyte-specific Cx43 knockout mice using the Dmp1 promoter (Dmp1-Cre) and found them to have the aforementioned cortical osteopenic phenotype with no effect on trabecular bone microstructural parameters or change in bone mass.[79]

Despite cortical expansion with increased cross-sectional moment of inertia, mechanical testing has generally demonstrated reduced mechanical strength of femurs from Cx43-deficient mice;[22] however, studies from our laboratory have demonstrated that although OCN-Cre mice have reduced femur strength at 2 months of age,[12] it is actually increased by 7 months.[78] It may be that the cortical phenotype of cKO mice changes with age, such that a confluence of morphological and material changes in bone results in an overall increase in strength.

The common morphological trait among in vivo models of Cx43 deficiency is cortical expansion and thinning, a phenomenon similar to that observed in human long bones with age.[81] In Cx43-deficient mice, bone formation and mineral apposition rate at the periosteal surface are increased, whereas osteoclast indices and serum bone resorption markers at the endocortical surface are also increased.[12,22,31,77–79] The result is the “tubular” phenotype initially described for patients with ODDD. Thus, understanding the role of Cx43 in skeletal homeostasis requires an understanding of its effect on both bone formation and resorption.

A major regulator of bone resorption is the action of RANKL on its receptor RANK on the surface of osteoclasts.[82] RANKL is a critical factor in the coupling of osteoblasts and osteoclasts, ensuring appropriate turnover of bone and maintenance of systemic mineral balance. Osteoblasts and stromal cells have long been thought of as an important source of RANKL, although recent studies have revealed that osteocyte-secreted RANKL may be the more critical regulator of homeostatic bone resorption.[83] The activity of RANKL is modulated by OPG, an endogenous decoy receptor for RANKL.[82] Thus, the RANKL/OPG ratio is a common means of expressing whether the overall balance of factors favors relatively increased or decreased osteoclast activity.

Cx43 deficiency results in an increased RANKL/OPG ratio. This has been demonstrated in vivo through measurement of whole bone mRNA levels of *Tnsfs11* (encodes RANKL) and *Tnsfs11b* (encodes OPG) from DM1-Cre cKO mice[22] and quantification, via immunohistochemistry, of RANKL+ and OPG+ osteocytes in femur cortical bone from Dmp1-Cre cKO mice. [79] In vitro, the *Tnsfs11/Tnsfs11b* ratio is increased in Cx43-deficient MLOY-4 osteocyte-like cells[12,79] and in primary calvarial cells isolated from Dmp1-Cre cKO mice.[79] Interestingly, although in vivo studies indicate that the increased

RANKL/OPG ratio is attributable to decreased OPG in individual osteocytes, with RANKL remaining unchanged, in vitro studies have shown it to be a result of a simultaneous decrease in OPG and increase in RANKL.[12,79] The reason for the difference is not certain. It is possible that RANKL expression in individual osteocytes is actually increased but cannot be detected by the techniques utilized, or it could be that confounding factors present in the in vivo bone milieu attenuate RANKL expression in the absence of Cx43. Furthermore, areas of increased osteoclast activity on the endocortical surface occur adjacent to sites of increased osteocyte apoptosis in cortical bone.[79] During osteocyte apoptosis, release of RANKL and apoptotic bodies occurs, which recruits osteoclasts and induces resorption.[84] This suggests that local osteocyte apoptosis contributes to the skeletal phenotype of Cx43-deficient mice. Regardless, Cx43 deficiency induces a net increase in the RANKL/OPG ratio, which most likely accounts for the increased endosteal resorption present in these mice.

Cx43 not only plays a role in bone resorption but also bone formation. The mechanism by which this occurs is not known; however, emerging evidence suggests an interaction with the Wnt/β-catenin pathway, one of the primary factors regulating bone formation.[85] Secreted Wnt ligand binds to a receptor complex formed by low-density lipoprotein receptor-related protein 5 or 6 (Lrp5/6) and frizzled. This membrane complex binds and inhibits Axin and GSK-3β, which prevents phosphorylation of β-catenin. Without Wnt signaling, phosphorylated β-catenin would be the target of ubiquitination and proteasomal degradation. Free β-catenin then translocates to the nucleus, where it associates with TCF/Lef transcription factors, resulting in increased expression of osteogenic genes including OPG,[85] cyclin D,[86,87] and at least some WNT genes.[88,89] β-catenin nuclear translocation also suppresses *Tnfsf11* expression.[90–93] Kramer and colleagues reported that homozygous deletion of β-catenin results in a severe fragile bone phenotype and premature death.[94] Indeed, the forearms of these mice cannot withstand mechanical loading. However, the same study showed that heterozygous mice that have lost only one β-catenin allele had only a modest bone phenotype. Interestingly, a recent study by Javaheri and colleagues[95] confirmed that heterozygous β-catenin-deficient mice had a modest bone phenotype, but this was largely confined to trabecular bone from female mice. More important, heterozygous β-catenin-deficient mice did not display an anabolic response to load. These results led the authors to suggest that a critical threshold of β-catenin is required for bone formation in response to mechanical loading. We will discuss this interesting concept within the context of the new paradigm we propose.

Sclerostin is an osteocyte-derived inhibitor of Wnt binding that suppresses bone formation and is responsive to mechanical signals; in general it is decreased during mechanical loading[96] and increased during mechanical unloading.[96,97] However, a recent study showed mechanical unloading resulted in increased expression of *Sost* (encodes sclerostin) in diaphyseal cortical bone but decreased expression in cancellous metaphyseal bone, suggesting site-specific differences in response to unloading.[98] Sclerostin expression is reduced in cortical bone of Cx43- deficient mice,[22,79,99] consistent with the increased osteoblastic bone formation in these mice. This may be the result of the significant Cx43 deficiency and related osteocyte apoptosis, as evidenced by the presence of empty lacunae and TUNEL staining throughout the cortical shell, in Dmp1-Cre and OCN-Cre cKO mice.

[79,99] Reduced sclerostin expression and osteocyte apoptosis were correlated with areas of increased bone formation at the periosteal surface. Interestingly, the trabecular compartment is unaffected by osteocyte apoptosis; however, this is consistent with a lack of effect of Cx43 deficiency on trabecular microstructure in these mice.[78,79] The remaining osteocytes, however, tend to have less OPG expression, suggesting that other mechanisms independent of osteocyte apoptosis are also important. Finally, Cx43 has been shown to bind β-catenin in cardiac myocytes,[100] mammary epithelial cells,[101] and seminiferous epithelium.[102] These mechanisms may also explain unexpected findings regarding the response of Cx43-deficient mice to mechanical signals.

Loading in vivo

Wolff's Law stipulates that bone structure is responsive to the physical force it experiences.[103] Practical examples of this can be found in the increased bone mass of the racquet arm of tennis players[104] or the development of increased bone density with resistance training.[105] As a result of the application of load, physical deformation of the bone matrix results in canalicular fluid flow,[106] direct mechanical strain,[107] electromagnetic effects,[34] vibration,[108] and microfractures.[109] These signals are then transduced to effector cells, which initiate bone formation and resorption at appropriate locations. In vitro evidence suggests that Cx43 is critical for sensitizing the skeletal system to mechanical signals. However, bone is a complicated biomechanical structure with many different and interconnected microenvironments that are difficult to reproduce in vitro. For example, osteocytes are normally embedded within the bone matrix and connected to surface osteoblasts and osteoclasts via canaliculi and dendritic processes. This complicated multicellular niche ultimately means that, although in vitro studies are critical to providing the direction future studies should take, eventually one must examine the role of Cx43 in bone in vivo using the aforementioned animal models.

Initial studies by Grimston and colleagues supported the hypothesis suggested by numerous in vitro studies, that the anabolic response to mechanical load is attenuated in Cx43-deficient mice.[110] The investigators subjected WT and Col1-Cre cKO mice to a three-point bending protocol. These mice experienced less of an increase in bone formation rate (BFR) and BMD in response to loading compared with WT mice. However, there are several important caveats to this investigation. First, the bending protocol used was less physiologic when compared with others, such as compression. In addition, the application of load in this study resulted in the production of copious amounts of woven bone. This suggests an injury response resulting from high strain (4665 με was used in these studies), rather than lamellar bone formation, which occurs during normal bone remodeling. In considering this limitation, a subsequent study from our group utilized a cantilever loading regimen of the tibia in OCN-Cre cKO mice.[12] In general, the response to loading is primarily manifested at the periosteal surface, and an active periosteum at baseline has the potential to confound assessment of the response to loading. Therefore, we initiated the loading protocol in mice at 6 months of age, having conducted dynamic histomorphometry on sentinel mice that indicated minimal baseline periosteal bone formation at this age. Load was applied such that the peak strain at the tibia midshaft was approximately 1300 με. In WT mice, this load was not enough to result in any detectable change in periosteal BFR, mineral apposition rate

(MAR), or mineralizing surface (MS/BS). Conversely, in OCN-Cre cKO mice, there was a 217% increase in periosteal BFR and 132% increase in MS/BS. These data were the first to suggest that, rather counterintuitively, Cx43 deficiency actually sensitizes bone to the effects of mechanical signals in vivo.

A subsequent study by Grimston and colleagues subjected relatively young (2-month-old) DM1-Cre cKO mice to an axial tibia loading protocol using physiological loads.[111] The investigators found that although 1200 με did not elicit any change in trabecular or cortical bone microstructural parameters in the tibia of WT mice, there were significant gains in bone mass in cKO mice. This included increased trabecular BV/TV and trabecular thickness, as well as increased cortical bone volume and total area. Similarly, 1900 με was required to achieve an increase in BFR and MS/BS in WT mice, whereas only 1200 με was required for cKO mice. BFR and MS/BS decreased at the endocortical surface in WT mice subjected to loading, and decreased by an even greater degree in cKO mice, indicating that Cx43 deficiency results in a loading response that is greater in magnitude than the response in WT mice. The increased response to loading in the tibia is surprising given the lack of trabecular phenotype in these mice.[22,111] These findings suggest that Cx43 has a critical role in the response of bone to acute loading. Compressive loading of the ulnae from Dmp1-Cre cKO mice confirmed these findings, with significantly greater bone formation response compared with WT.[112] However, the investigators did not see any effect of loading on histomorphometric parameters at the endocortical surface.

Taken together, emerging data suggest that Cx43 deficiency results in an increased anabolic response to mechanical loading. Thus, the long-held paradigm, developed largely from in vitro studies, that Cx43 contributes to and is necessary for the anabolic response to mechanical load is not supported by recent in vivo studies. Possible reasons that the extant paradigm is not supported by in vivo studies include the fact that most in vitro experiments were completed in two- rather than three- dimensional culture; the majority of in vitro studies focused on single cell types, usually osteoblasts,[113] and did not consider the results in the context of an interconnected multicellular (osteoblast/osteoclast/osteocyte) cyto-social network; and in vitro studies focused only on one mechanical signal, usually fluid flow-induced shear stress. In any case, the newly emerging paradigm is that Cx43 deficiency increases the sensitivity of bone to mechanical load. An adjunct to this new paradigm is that Cx43-deficient bone is less sensitive to unloading.

Unloading in vivo

Mechanical unloading is experienced during spaceflight,[114] bed rest,[115] and muscle paralysis.[116] Hindlimb suspension (HLS) is a widely used method to simulate microgravity in rodents on the ground.[117] Other models, such as botulinum toxin (botox)- induced immobilization have been introduced more recently.[118] Bone loss resulting from unloading is characterized by an imbalance of bone turnover: bone formation decreases while bone resorption increases.[119] The result is a net decline in bone mass, as much as 0.5% to 1.5% per month in astronauts aboard the International Space Station.[120] Decreased bone formation is primarily mediated by the production of sclerostin by osteocytes,[97] whereas increased bone resorption is mediated by increased sclerostin and RANKL.[121,122]

In a study of botox immobilization of the hindlimbs of heterozygous Col1 cKO mice, bone loss (as measured by bone mineral content) tended to occur at a slower rate in cKOs compared with WT, although recovery of both genotypes was similar and incomplete at 12 weeks.[123] A more comprehensive follow-up by Grimston and colleagues subjected homozygous Col1-Cre cKO mice to botox immobilization.[77] This resulted in a significant loss of trabecular BV/TV along with lower trabecular number and thickness in WT mice. The degree of trabecular bone loss was similar between WT and cKO. Conversely, the enlargement of the marrow area and thinning of the cortical shell induced by disuse in WT mice was significantly attenuated in cKO mice. The investigators also documented increased endocortical osteoclast number and surface in WT mice, with no change in cKO. These initial findings suggested that, unlike loading, Cx43 deficiency results in decreased sensitivity to mechanical unloading.

A subsequent study from our laboratory subjected OCN-Cre cKO mice to mechanical unloading via HLS.[78] In contrast to the findings of Grimston and colleagues, we observed attenuated trabecular bone loss in cKO mice. And, although BFR was essentially abolished in WT mice subjected to unloading, it was maintained near baseline levels in cKO mice. In a follow-up study, we confirmed our findings of attenuated trabecular bone loss.[99] This is interesting, given the lack of trabecular phenotype in OCN-Cre mice. Furthermore, trabecular bone was not preserved in Col1-Cre cKO mice with botox immobilization, and these mice do have an osteopenic trabecular phenotype.[77] As mentioned, there was also an increased anabolic response of trabecular bone to mechanical loading in these mice,[111] suggesting that Cx43 deficiency does indeed influence the response to mechanical signals, or the lack thereof, in trabecular bone.

Our group[99] and others[79,124] have demonstrated that accumulation of apoptotic osteocytes appears to have a key role in the baseline phenotype of Cx43-deficient mice. During unloading, we have demonstrated that there is a significant increase in whole bone mRNA levels of *Sost* and sclerostin-positive osteocytes in WT mice subjected to HLS.[99] cKO mice, however, did not experience this same increase. Similarly, serum levels of pro-collagen type 1 N-terminal propeptide (P1NP), a marker of bone formation, declined significantly in WT but not in cKO. Considering the resorptive arm of bone remodeling, we found that unloading induces significant increases in trabecular and cortical osteoclast number and surface, with no similar increase for cKO mice. mRNA levels of *Tnsfs11* (encodes RANKL) increased half as much during unloading of cKO mice compared with WT mice.

Increased osteocyte apoptosis could account for some of these findings. For example, in a series of experiments conducted by Tatsumi and colleagues, osteocytes were selectively ablated in mature mice.[125] Osteocyte ablation in this model is similar to the increased osteocyte apoptosis seen during Cx43 deficiency, albeit to a greater degree. These mice were completely resistant to the effects of HLS. They also did not exhibit increased levels of RANKL or sclerostin during unloading as seen in WT mice. In either case, the absence of mechanosensing osteocytes, which are also the primary source of sclerostin and RANKL, means that there is less potential to initially detect the unloading stimulus but also fewer cells to produce catabolic factors leading to bone loss. Thus, the reduced bone loss in

unloading combined with increased bone formation with loading suggests a vertical translation of the load:response curve. Interestingly, a similar vertical translation is observed in LRP5 gain of function mice.[126] Of course, increased apoptosis does not easily explain the results in the trabecular bone compartment, where osteocyte apoptosis is not increased. An explanation for this phenotype likely requires an additional mechanism, independent of osteocyte apoptosis.

Proposed Mechanism

The mechanism by which Cx43 deficiency leads to enhanced bone formation and resorption as well as enhanced response to load and decreased response to unload is not known. However, increased periosteal bone formation and increased osteoclastic bone resorption in Cx43-deficient mice suggest the intriguing possibility that Cx43 and/or GJIC serve as a negative regulator of osteoblast and osteoclast activity. We propose that in Cx43- deficient mice, osteocytes undergo apoptosis and the subsequent release of RANKL.[84] This leads to an increase in osteoclastic bone resorption. In the remaining Cx43-deficient osteocytes, there is an increased availability of β-catenin leading to increased release of osteogenic factors from osteocytes that activate osteoblastic bone formation (Fig. 1). The increased availability of β-catenin also leads to decreased RANKL,[90–93] but this is insufficient to counteract the increased RANKL resulting from apoptosis. The net result is high-turnover bone loss. In response to mechanical load, Cx43-deficient mice experience a decrease in release of osteocytic sclerostin that leads to a further increase in availability of osteocytic β-catenin, which further increases release of osteogenic factors and an inhibition of osteocytic RANKL. The decreased RANKL resulting from load is now sufficient to counteract the increased RANKL from osteocyte apoptosis. The result is an increase in osteoblastogenesis, decrease in osteoclastogenesis, and increased bone formation, relative to WT mice, in response to load. In response to unloading, osteocytes release more sclerostin, which, acting in an autocrine fashion, decreases availability of β-catenin, thus decreasing release of osteogenic factors and increasing expression of RANKL. However, in Cx43-deficient mice, this sclerostin-induced decrease in β-catenin availability is mitigated by less β- catenin being sequestered by Cx43. Thus, the catabolic response to unloading is mitigated in Cx43-deficient mice. The model we propose can also take into account the β-catenin “threshold” proposed by Javaheri and colleagues.[95] The β-catenin threshold was proposed to explain the modest basal bone phenotype, but lack of response to mechanical load, in heterozygous β-catenin- deficient mice. Our model predicts that Cx43, through its interaction with β-catenin, adjusts this threshold. This would suggest that osteocyte selective Cx43 and β-catenin (heterozygous) deficient mice would be more responsive to mechanical load than β-catenin (heterozygous) only deficient mice (Fig. 1).

It is possible that Cx43 regulates β-catenin and thereby both areas of bone remodeling, through GJIC, hemichannels, or both. However, we propose Cx43 acts in this manner independent of GJIC or hemichannels, but rather through a direct interaction with β-catenin. This concept is supported by several studies demonstrating that Cx43 is able to interact with signaling molecules such as β-arrestin,[127] kinase Src,[58,128] protein kinase C-d,[63] and zona occludens-1.[129] Cx43 itself is a target of Wnt/β-catenin,[130] which would allow for negative feedback regulation of an anabolic stimulus. Such a mechanism would mean that,

in the absence of Cx43, free β-catenin is present to a greater degree and bone cells are "primed" to respond to mechanical signals.[112]

Evidence supporting this potential mechanism is provided by a study from Bivi and colleagues.[112] The investigators found that protein levels of β-catenin were greater in ulnae isolated from Dmp1-Cre cKO mice compared with WT. In vitro, they show that Cx43-deficient MLOY-4 osteocyte-like cells have increased expression of β-catenin and its targets Axin2 and Sfrp4. However, the investigators were not able to detect a difference in expression of Wnt target genes cyclin-D1 or Smad-6. Furthermore, transcription of Wnt/β-catenin target Lef1, either at baseline or with inhibition of GSK-3β by lithium chloride, is similar in WT and Cx43-deficient MLOY-4 osteocyte-like cells. These findings suggest that the accumulation of β-catenin may have an effect on increasing sensitivity to load independent of transcription of classical genes associated with Wnt/β-catenin signaling. Supporting this mechanism, mice with haploinsufficiency of β-catenin have reduced sensitivity to mechanical load.[95]

Evidence for the potential of Cx43 as part of a negative feedback system of bone formation is also found in a study from Lozupone and colleagues, which showed that loading of rat metatarsal bones increased the incidence of osteocytic GJs.[131] In a later study, our laboratory demonstrated that expression of Cx43 by osteocytes is increased in areas of bone exposed to tension relative to areas exposed to compression or to control bone.[132] Thus, loading increases Cx43, which in turn sequesters β-catenin, which leads to a decrease in β-catenin stimulation of Cx43 expression, thus closing the negative feedback loop.

Cx43 in Bone Repair

Although the mechanism described above is attractive, it does not explain the effect of Cx43 deficiency in different contexts. For instance, recent work from our group highlights the possibility for divergent interaction of Cx43 with the Wnt/β-catenin/sclerostin signaling axis in the context of loading/unloading versus fracture healing. We have shown that osteoblast/osteocyte-specific Cx43-deficient mice heal with impaired bone formation, mechanical properties,[133,134] and attenuated β-catenin expression.[134] In both OCN-Cre (Fig. 2) and Col1-Cre cKO fractures, there is an increase in the number of sclerostin-positive osteocytes in the fracture callus relative to WT. A transient increase in *Sost* mRNA is also observed, concurrent with attenuated β-catenin expression.[134] Additionally, increases in GSK-3β mRNA and the active form of GSK-3β protein (phospho-Tyr216)[135] occur during the time period in which β-catenin expression is decreased. Increased GSK-3β activity in Cx43cKO fractures suggests that loss of Cx43 may negatively regulate bone formation during fracture repair by increasing activating phosphorylation events of GSK-3β through an as yet determined mechanism. These data are consistent with a preliminary study that suggests that loss of Cx43 decreases inactivating phosphorylation of GSK-3β (Ser9), thus increasing GSK-3β activity.[136] We have also shown that inhibition of GSK-3β activity with lithium chloride can rescue the impairments in bone formation and mechanical properties associated with Cx43 deficiency during fracture healing. This suggests that Cx43 may promote osteogenesis by inactivating GSK-3β and therefore stimulating β-catenin expression during fracture healing.

The disparate effects of Cx43 in loading/unloading and fracture repair are not completely surprising given that fracture repair is a recapitulation of development with healing occurring through endochondral ossification rather than the uncoupling of bone formation and resorption that occurs during unloading. Furthermore, vastly different cell populations including inflammatory cells and chondrocytes are involved in the fracture healing response relative to loading and unloading. Findings of impaired bone formation in Cx43-deficient fractures are supported by work from Grimston and colleagues, in which high-strain (4665 με) mechanical loading, causing an injury response, resulted in woven bone formation rather than lamellar bone.[110] This suggests that the osteogenic response may be differentially modulated by Cx43 during bone repair relative to anabolic mechanical loading. Additionally, impairments in Wnt/β-catenin signaling during fracture healing in Cx43-deficient mice raises the intriguing question of how Cx43 may modulate this important osteogenic signaling pathway, and as such is an important area of future research.

Future Studies

Future studies will need to further refine the connection between Cx43, β-catenin, and skeletal homeostasis. Findings of increased nuclear versus cytoplasmic fractions of β-catenin in primary osteocytes, MLOY-4, or IDG-SW3 osteocyte-like cells would support this hypothesis. In addition, it would be interesting to determine whether the phenotype and reduced mechanical sensitivity of β-catenin-deficient mice can be rescued by crossing with a Cx43-deficient strain. Finally, a comprehensive examination of the effect of loading and unloading on bone cell-specific Cx43-overexpressing mice would provide important insights.

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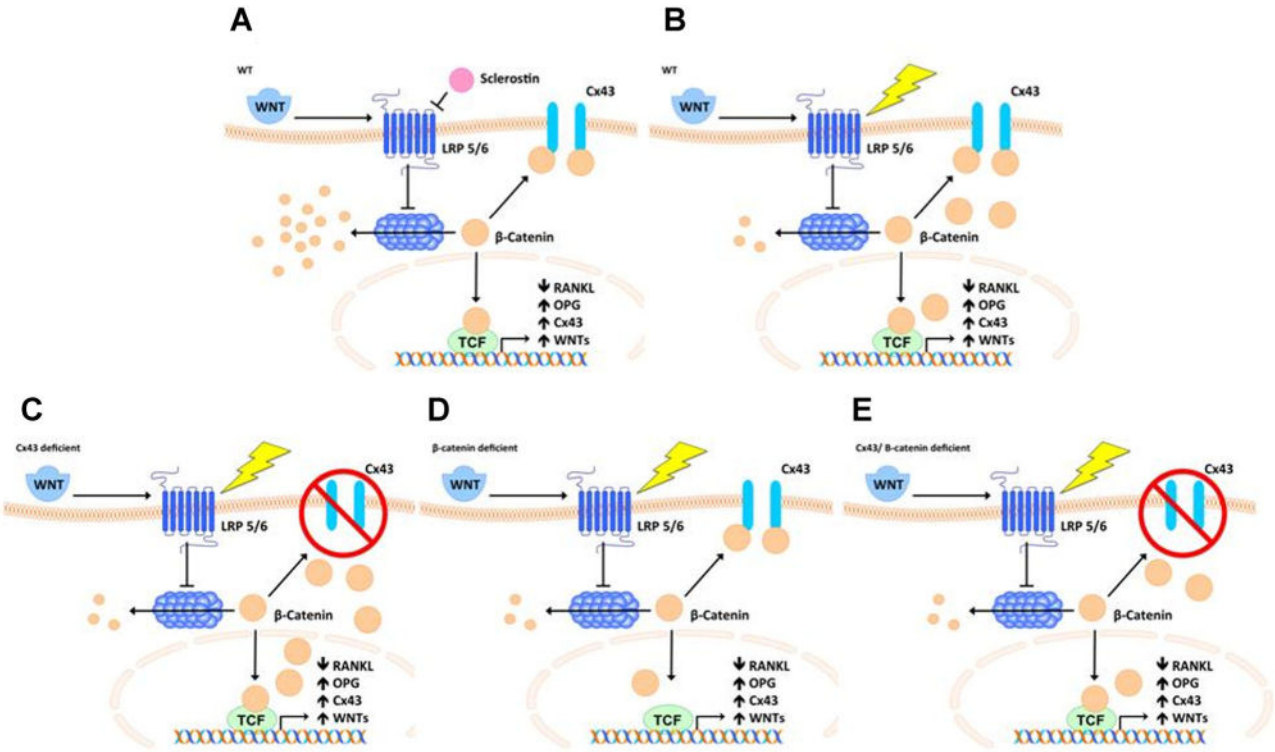

Fig. 1.

Proposed mechanism by which osteocytic Cx43, via β-catenin, regulates both bone formation and resorption in response to load. (*A*) WT osteocytic Cx43 binds β-catenin, decreasing its nuclear translocation. (*B*) Load (lightning bolt) in WT decreases sclerostin, thus increasing β-catenin, but this is governed by Cx43 functioning in a negative feedback loop. (*C*) In Cx43-deficient osteocytes, load-induced increases in β-catenin are amplified, resulting in further increase in expression of osteogenic genes, possibly WNTs, and decreased RANKL expression. (*D*) In β-catenin (heterozygous)-deficient osteocytes, β-catenin levels drop below the threshold needed for anabolic response to load. (*E*) In Cx43/β-catenin-deficient osteocytes, more β-catenin is made available, resulting in a modest, relative to Cx43-deficient, anabolic response to load.

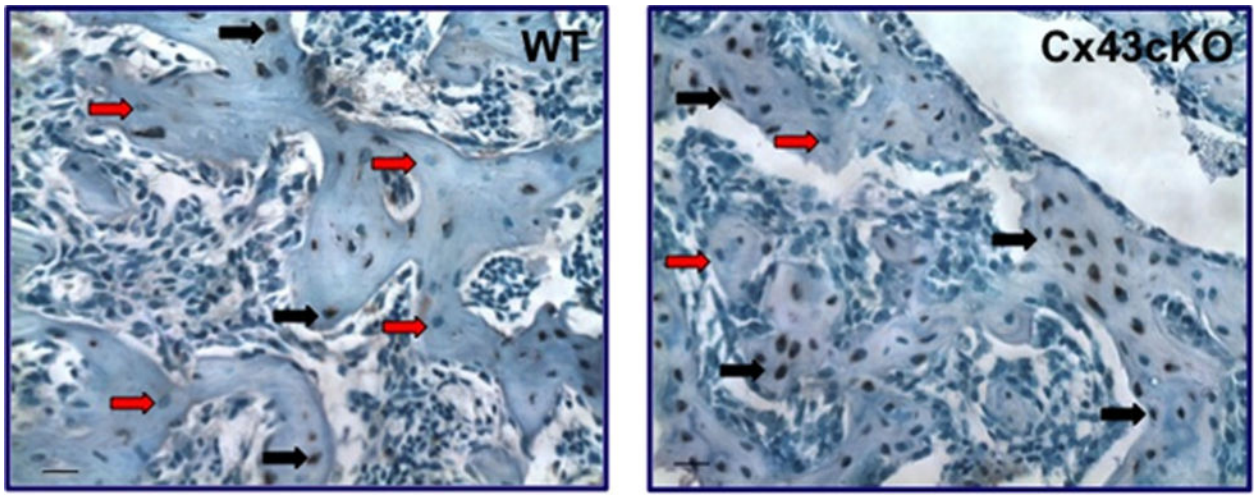

Fig. 2.

Sclerostin immunohistochemistry. An increase in the number of sclerostin+ osteocytes is observed in the fracture callus of OCN-Cre Cx43-deficient mice relative to WT at 21 days. Black arrows indicate sclerostin+ cells, red arrows indicate sclerostin− cells. Scale bar = 100 microns.

Table 1

Summary of Murine Models of Altered Cx43 Expression

Construct	Cells affected	Cortical phenotype	Trabecular phenotype	Loading response	Unloading response	Reference numbers
Global deletion	All	Cardiac defects, delayed ossification of axial and appendicular skeleton	?	?	?	(74)
Global ODDD mutation	All	Osteopenic: cortical thinning, reduced BMD, reduced strength	Osteopenic	?	?	(20,21)
DM1-Cre ODDD	Chondro-osteogenic lineage	Osteopenic: cortical thinning, reduced BMD, expanded marrow cavity, reduced strength	None	?	?	(22)
DM1-Cre cKO	Chondro-osteogenic lineage	Osteopenic: cortical thinning and marrow expansion, lower BMD	None	↑	?	(22)
Col1-Cre cKO	Early osteoblast/osteocyte	Osteopenic: attenuated response to anabolic PTH	Osteopenic	↑	↓	(31,75,77)
OCN-Cre cKO	Late osteoblast/osteocyte	Osteopenic: increased endosteal BFR, increased osteoclastic bone resorption, increased osteocyte apoptosis	None	↑	↓	(75,78,79)
Dmp1-Cre cKO	Osteocyte	No change in bone volume, increased cortical area, increased osteocyte apoptosis	None	↑	?	(79)
Global overexpression	All	Cardiac and neural tube defects; skeletal phenotypes have not been examined	?	?	?	(23)