Published in final edited form as:
Clin Rev Bone Miner Metab. 2010 December 1; 8(4): 189–200. doi:10.1007/s12018-011-9084-8.

Gap Junctions and Biophysical Regulation of Bone Cells

Shane A. J. Lloyd and
Division of Musculoskeletal Sciences, Department of Orthopaedics and Rehabilitation, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033, USA

Henry J. Donahue
Division of Musculoskeletal Sciences, Department of Orthopaedics and Rehabilitation, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033, USA

Shane A. J. Lloyd: slloyd@hmc.psu.edu; Henry J. Donahue: hdonahue@hmc.psu.edu

Abstract

Communication between osteoblasts, osteoclasts, and osteocytes is integral to their ability to build and maintain the skeletal system and respond to physical signals. Various physiological mechanisms, including nerve communication, hormones, and cytokines, play an important role in this process. More recently, the important role of direct, cell–cell communication via gap junctions has been established. In this review, we demonstrate the integral role of gap junctional intercellular communication (GJIC) in skeletal physiology and bone cell mechanosensing.

Keywords

Gap junctions; Connexins; Mechanosensing; Mechanotransduction; Cell-to-cell communication; Intercellular communication; Osteocytes

Introduction

Aside from the skeletal system, gap junctions (GJs) have a well-established role in the physiology of various systems including the heart [1], nervous system [2], gonads [3], and inflammatory response [4]. GJs are membrane-spanning channels that allow passage of small molecules such as calcium ions, inositol phosphates, and nucleotides, from one cell to another [5]. Each GJ is comprised of two hexameric hemichannels termed connexons that in turn are comprised of six subunits termed connexins (Cxs) [6]. Over twenty mammalian Cxs have been identified and are typically named according to their molecular mass in kilodaltons [7]. Cxs are comprised of four conserved membrane-spanning domains and two small extracellular loop domains that contain over 95% homology between subtypes [5, 6]. There is also a large intracellular loop and intracellular amino- and carboxyterminal ends, the latter of which has a particularly divergent structure [8, 9].

The major cell types involved in bone turnover are bone-forming osteoblasts, bone-resorbing osteoclasts, and osteocytes, which are terminally differentiated osteoblasts. Osteocytes are embedded within the bone matrix and are connected to each other and to the bone surface via canaliculi and cellular processes. The osteocyte is considered to be the

Correspondence to: Henry J. Donahue, hdonahue@hmc.psu.edu.

putative mechanosensor of the skeletal system [10]. Osteoblasts and osteocytes have been shown to primarily express the most abundant Cx, Connexin43 (Cx43); however, osteoblasts also express lower amounts of Connexin45 (Cx45) [11]. Connexin46 (Cx46) is also expressed by these cells, although it has been found to remain trapped in the Golgi, never actually participating in intercellular communication [12]. In addition, osteoclasts and cartilage-forming chondrocytes express Cx43 [13–15]. The structure of connexins determines the specific molecules it is able to transmit [6]. For example, Cx43 allows for the diffusion of negatively charged signal molecules <1.2 kD, while Cx45 forms a smaller pore, permitting diffusion of molecules <0.3 kD with a preference for positively charged molecules [16, 17].

Aside from allowing for direct cell-to-cell communication, Cxs can also assemble as homomeric or heteromeric hemichannels at the cell surface [18]. Rather than forming a channel with an adjacent cell, these hemichannels act as cellular pores, allowing for the passage of small molecules with selectivity dictated by their constituent connexins. Hemichannels have been shown to release of PGE_2, ATP, and other factors in response to mechanical stimulation [19, 20]. More recent studies have revealed a role of Cxs in gap junction- and hemichannel-independent cellular mechanisms [21]. Regulation of factors involved in cell growth by the c-terminus of Cx43 and regulation of factors in cell cycle progression have been proposed as potential mechanisms for this action [5]. Regardless of the particular structure or location of Cxs, their critical role in a wide variety of physiological mechanisms from cell to organism is clear.

Gap Junctions in Skeletal Development

GJIC plays a key role in the function of osteoblasts, osteoclasts, and osteocytes in the maintenance of skeletal homeostasis in the mature organism. However, Cxs also serve an integral role in the proper development of the skeletal system itself [22]. This concept is demonstrated rather vividly by the genetic disease oculodentodigital dysplasia (ODDD), a disorder identified in individuals with mutations in the gene for Cx43, *GJA1* [23]. Individuals with ODDD have a skeletal phenotype characterized by dental anomalies, including small teeth with enamel hypoplasia, syndactyly, and missing phalanges of the toes [24]. Other disease features, such as glaucoma, iris deformities, seizures, and muscle weakness, highlight the varied systemic contribution of Cx43 to human physiology [25].

Flenniken et al. [26] developed a transgenic mouse model that was able to mimic many of the classical features of ODDD, as well as decreased bone mass and mechanical strength. These mice contain a point mutation, unrelated to human ODDD, which results in the production of a mutant protein that acts in a dominant-negative fashion to disrupt GJ assembly. Work by Dobrowolski et al. [27] involved insertion of a human Cx43G138R point mutation into the mouse *GJA1* gene to create a transgenic animal with cortical bone thinning and cranial abnormalities consistent with ODDD. In addition, previous mouse studies suggest that Cx43 has a role to play in the development of limb patterning and growth [28]. A Cx43 null-mutant mouse displayed delayed enchondral and endo-osteal ossification in the cranial vault, although the axial and appendicular skeleton was essentially normal at birth [29]. Studies in chick embryos have revealed that blocking Cx43 expression with antisense nucleotides results in a significant decrease in bone formation [30], while studies in mutant zebrafish have suggested a role of Cx43 in joint location [31]. Taken together, a picture of GJIC as a critical mediator of skeletal development is coming in to focus.

Osteoblast Differentiation

GJIC has been identified as playing a key role in the differentiation and proliferation of bone-forming osteoblasts [9, 32]. In vivo studies suggest that GJs may be involved in cell

signaling processes important to limb bud differentiation and skeletogenesis in embryonic mice [33] and cellular differentiation and intramembranous bone formation in the developing chick mandible [34]. Indeed, Cx43 null mice display impaired intramembranous bone formation and osteoblastic cells from these animals express decreased levels of type 1 collagen, osteopontin, and osteocalcin [29], suggesting a defect in osteoblastic maturation. Several in vitro studies from our laboratory [35, 36] and others [37–39] demonstrated that Cx43 expression and GJIC parallel osteoblastic differentiation. It was also found that inhibition of GJIC and Cx43 expression in osteoblastic cells (e.g., 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 the decreased expression of phenotypic characteristics of differentiated osteoblasts, including alkaline phosphatase, osteocalcin, bone sialoprotein, and PTH responsiveness. Additionally, Schiller et al.[32] showed that inhibition of GJIC induces the trans-differentiation of both osteoblastic MC3T3-E1 cells and primary culture human osteoblastic cells into an adipocytic phenotype. Conversely, there are at least two documented studies suggesting that GJIC is related to decreased osteoblastic differentiation [40, 41]. Additional studies may help to clarify these discrepancies, but a fundamental involvement of gap junctional communication in these processes is undisputed.

GJIC also functions in the development of osteoblasts through interaction with other cellular mechanisms and non-bone cells. Recent studies by Inose et al. [42] have shown that the microRNA miR-206, an miRNA previously thought to be muscle specific, is expressed during osteoblast differentiation and that Cx43 is a target. Overexpression of miR-206 resulted in decreased osteoblast differentiation, which was rescued by Cx43 expression, while knockdown promoted differentiation. Development of a transgenic mouse model expressing miR-206 in osteoblasts revealed an osteopenic phenotype similar in many respects to that of Cx43 knockouts. Studies by Geneau et al. [43] suggest that the presence of Cx43 is required for the effect of endothelin-1, a peptide that has been shown to inhibit the mineralization of mouse pre-osteoblast MC3T3-E1 cells and osteoblast differentiation [44]. Previous studies demonstrated similar mitigation of ET-1's ability to elicit calcium influx in calvarial osteoblastic cells from $Cx43^{+/-}$ mice [43] and a decrease in ET-1's inhibitory effects in a human cell line deficient in Cx43 [45]. The role of Cx43 in the inhibition of osteoblastic cell differentiation was also highlighted by recent studies from Clobvacco and colleagues, which demonstrated that osteoblasts and megakaryocytes (MKs) can communicate via Cx43 and that MKs inhibit osteoblast differentiation in vitro when cultured for extended durations. GJIC was also found to inhibit the MK-mediated juxtacrine enhancement of osteoblast proliferation, but did not appear to alter MK-mediated reductions in osteoblast differentiation.

There has also been much research focusing on the role of ATP in skeletal physiology, ATP stimulates the proliferation of endothelial and smooth muscle cells [46] through a mechanism involving cytosolic calcium mobilization [47]. Additionally, ATP increases intracellular calcium levels in osteoblastic cells expressing the appropriate repertoire of purinergic receptors and cytosolic calcium increases the activity of extracellular regulated kinases, suggesting it may stimulate osteoblastic cell proliferation [48]. Indeed, two reports suggest that ATP stimulates the proliferation of osteoblastic MG-63 [49] and HOBIT [50] cells. Thus, the bulk of published data suggest that ATP contributes to osteoblastic proliferation while GJIC contributes to osteoblastic differentiation.

Osteoclast Differentiation

While much work has focused on the role of gap junctional communication in the differentiation, proliferation, and activity of osteoblasts, there has been relatively little focus

on its role in osteoclasts. Early studies identified that osteoclasts do indeed express Cx43 and that GJ communication channels form between osteoclasts and the overlying osteoblasts [51]. Later studies by Ilvesaro et al, [52] demonstrated that rat osteoclasts cultured on bovine bone slides express Cx43 along the basolateral membrane. Furthermore, this group utilized the gap junction inhibitor heptanol and a pit formation assay to establish that Cx43 is required for osteoclast activity and that blocking GJIC results in a decrease in both osteoclast number and activity. Studies using a more specific inhibitor, the connexin-mimetic peptide Gap27, confirmed that intercellular communication via Cx43 is essential for the activity of osteoclasts [53]. It is now clear that connexins and gap junctions play a key role in the maturation of mature, multi-nucleate osteoclasts from hematopoietic cells of the mono-cyte/macrophage lineage as well as the bone-resorbing function of osteoclasts that occurs as a part of normal bone turnover [54, 55]. It is interesting to note that studies utilizing sections of human bone found lower expression of Cx43 in mature versus precursor osteoclasts, which may mean that it is more important to cellular function in earlier stages of differentiation [14]. Blockage of GJIC with heptanol resulted in a dose-dependent decrease in multi-nuclearity in these cells, suggesting that GJIC has a role in the fusion and size of mature osteoclasts. Because of the well-established interest in osteoclast function in the context of pathological bone loss, it is certain that refining the role of Cx43 in osteoclast development and function may reveal its potential as a pharmacologic target.

Role of Gap Junctions in Mechanosensing

One of the fundamental tenants of bone biology is that bone form follows function. First proposed by the orthopaedic surgeon Julius Wolff in 1892, Wolff's Law stipulates that the physical characteristics of bone (i.e., strength, bone mass) are dependent upon the physical forces and stress exerted upon it [56]. The classic example is the increased bone mineral content found in the racquet arm of a tennis player [57]. Of course, it is one thing to acknowledge the manifestations of Wolff's Law, it is another to understand the fundamental mechanisms that underlie it.

Bone is a complicated biomechanical structure, with forces of gravity, physical activity, etc. ultimately producing a complex array of biophysical signals that must be detected and transduced into an appropriate cellular response [58]. As a result of the application of load, bone matrix deformation creates a complex, non-uniform bio-physical environment within tissue [59] consisting of fluid flow [60], direct mechanical strain [61], electromagnetic effects [62], vibration [63], and microdamage [64]. These diverse biophysical signals affect the level or activity of various markers of bone cell activity and differentiation. These factors include alkaline phosphatase in the case of electric fields [65], cytosolic Ca^{2+} mobilization, NF-κB activation, Cx protein expression and phosphorylation, osteopontin mRNA expression, and prostaglandin E2 (PGE_2) production in the case of fluid flow [66–69], and osteopontin mRNA expression during substrate stretch [70]. In the presence of fluid flow and electric fields, the effects are more pronounced in cells displaying relatively more abundant GJIC. It has been demonstrated that GJIC-deficient cells are dramatically less responsive to parathyroid hormone (PTH) [71], fluid flow [68], and electric fields [62] than are normal osteoblastic cells. Thus, one can see that GJIC contributes to the responsiveness of osteoblastic cells to diverse extracellular signals.

There is abundant evidence suggesting that GJIC contributes to mechanotransduction in the musculoskeletal system. Banes et al. [72] demonstrated that equibiaxial strain upregulates Cx43 expression in tendon cells, while cyclic stretch has been shown to increase Cx43 protein expression and GJIC in osteoblastic cells [73]. Our laboratory [69] and others [74] have demonstrated that fluid flow increases GJ expression and function in osteocytic MLO-Y4 cells. Conversely, studies conducted by Thi and colleagues suggest that fluid flow

decreases GJ function and expression in MLO-Y4 cells. Results of these studies highlight the apparently complicated role of Cx43 and emphasize the need for additional examination of this issue.

In vivo, Lozupone et al. [75] demonstrated that mechanical loading of rat metatarsal bones increased the incidence of osteocytic GJs. In a later study, our laboratory demonstrated that expression of Cx43 by osteocytes is increased in the areas of bone exposed to tension relative to areas exposed to compression or to control bone [76]. The localization of GJs within the bone cell syncytium, their regulation by biophysical signals, and their contribution to bone cell responsiveness to extracellular signals suggest that GJs contribute to the integration and amplification of biophysical signals within osteocytic-osteoblastic networks.

Gap Junctions and ATP

In addition to GJIC, another mechanism by which mechanical signals could be amplified and integrated throughout the osteocyte–osteoblast network is through stimulation of the release of autocrine factors such as extracellular nucleotides. ATP induces a broad spectrum of cell responses, including calcium mobilization, muscle cell proliferation, and endothelial cell adhesion and proliferation [77, 78]. Extracellular nucleotides also act as important signaling molecules for cell-cell communication in the skeletal system [79] and play an important role in bone remodeling [80]. ATP and UTP are known to exert their effects via a family of specific receptors termed P2 purinergic receptors [81]. P2 purinergic receptors are expressed in the osteoblastic cells of rat and human origin [82]. Several studies have demonstrated that extracellular nucleotides, such as ATP and ADP, increase osteoblastic cell proliferation and differentiation through specific P2 purinergic receptors [49, 83].

Accumulating evidence suggests that, in many types of cells, mechanical signals induce the release of nucleotides, including ATP and UTP, from one cell, which then activate purinergic P2 receptors in adjacent cells. An intriguing possibility is that GJs contribute to bone cell mechano-transduction via GJIC and nucleotide release via GJ hemichannels. Several studies demonstrated mechanically induced ATP release through hemichannels, including studies involving astrocytes [84], glioma cells [85], and chondrocytes [86]. However, it is less clear whether hemichannels function in a similar manner in bone cells. Romanello et al. [87] demonstrated that a human osteoblastic cell line has functional hemichannels, but that these hemichannels are not involved in ATP release. Jorgensen et al. [88] reported that they were unable to detect functional hemichannels in UMR-106-01 cells overexpressing Cx43 or in ROS 17/2.8. A small percentage of primary culture human osteoblastic cells did express functional hemichannels but only in low extracellular calcium. Studies from our laboratory demonstrate that fluid flow stimulates the release of ATP from osteoblastic MC3T3-E1 cells, but this was not inhibited by blocking hemichannels [89]. It is clear that additional work is needed to delineate the role of ATP and hemichannels in mechanosensing.

Gap Junctions and PGE_2

In addition to ATP, the contribution of the paracrine release of PGE_2 from bone cells is considered to be important because of its established role in the bone cell response to mechanical loading [90]. Studies by Civitelli et al. [91, 92] revealed that PTH and PGE_2 enhance GJIC by inducing redistribution of Cx43 to the cell membrane and increasing *GJA1* expression. A study in our laboratory demonstrated that fluid flow activates Cx43 hemichannels in osteocyte-like MLO-Y4 cells, but not MC3T3 cells, resulting in both ATP and PGE_2 release [93]. A study by Siller-Jackson et al. [94] determined that the release of PGE_2 was dependent on the magnitude of shear stress and that this response could be

blocked by a hemichannel-specific antibody and not gap junctions or other channels. Saunders et al. [95] looked at MC3T3-E1 and found that PGE_2 release, but not calcium release, following oscillatory fluid flow was dependent on the presence of intact GJIC.

Cx43 hemichannel opening in response to fluid flow shear stress has been found to be adaptive to the magnitude and duration of exposure, with increased PGE_2 release occurring in cells subjected to interval fluid flow shear stress versus continuous [19]. Fluid shear stress-induced release of PGE_2 may be dependent on the calcium-mediated release of ATP and its action on P2 receptors [96]. Such a mechanism is supported by the finding that calvarial osteoblasts isolated from mice with a null mutation in the P2X7 nucleotide receptor release less PGE_2 in response to fluid flow than their wildtype counterparts [97]. MC3T3-E1 osteoblasts and MLO-Y4 osteocytic cells were also found to have suppressed PGE_2 release in response to P2X7 blockade and enhanced PGR_2 production with application of an agonist. In vivo, loading studies in rats have revealed that cyclooxygenase-2-specific inhibitors and indomethacin are able to inhibit strain-induced lamellar bone formation [98]. The physiological importance of prostaglandins in the maintenance of bone mass has been well established, and these studies highlight an important role of GJIC in this process.

RANK/RANKL/OPG

Bone turnover is one of the key mechanisms leading to efficient homeostatic maintenance of the skeletal system. The action of osteoblasts and osteoclasts are coordinated so that older, damaged bone is degraded and replaced with new bone matrix that is later mineralized. One of the key pathways involved in coordinating this mechanism is the receptor activator of NF-kappaB (RANK)/RANK Ligand (RANKL)/osteoprotegerin (OPG) axis [99]. RANKL is secreted by stromal cells such as osteoblasts, which in turn bind to RANK on the surface of osteoclasts, inducing osteoclast differentiation, proliferation, and activation. To date, very little research has examined the role of GJIC in the function of osteoclasts or the RANK/RANKL/OPG axis. Studies by Matemba et al. [100] utilizing the GJ inhibitor carbenoxolone (CBX) revealed that while inhibition of GJIC did not affect the expression of mRNA for RANK/RANKL/OPG in mouse bone marrow cultures, it did inhibit RANKL-mediated osteoclastogenesis. These results suggest that GJIC has a role to play downstream of the RANK/RANKL/OPG axis in osteoclasts. It is clear that much additional work remains in characterizing the relationship between GJIC and this critical skeletal signaling axis.

Sclerostin/Wnt/β-Catenin

The canonical Wnt/β-catenin pathway is a critical signaling cascade required for both the formation and maintenance of bone [101]. 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 initiates a signaling cascade that prevents β-catenin from undergoing constitutive proteosomal degradation. Increased levels of β-catenin in the cytoplasm result in its translocation to the nucleus where it can associate with other factors to control target gene expression [102]. Wnt/β-catenin signaling is required for osteoblast differentiation and activity, as evidenced by $LRP5^{-/-}$ knockout mice, which have low bone mass and reduced sensitivity to mechanical loading [103]. Conversely, mice that overexpress Lrp 5 have increased bone mass and Cx43 expression [104]. In MLO-Y4 osteocytic cells, pulsating fluid flow results in the increased expression of Wnt/β-catenin components and targets such as Wnt3a, c-jun, CD44, and Cx43 [105]. These effects were blocked by the nitric oxide (NO) inhibitor L-NG-nitroarginine methyl ester (L-NAME), suggesting that NO may play a role in this mechanism. In addition, cerebellar formation defects found in Wnt-1 knockout mice are reversed by Cx43 overexpression [106]. These findings are consistent with earlier work, showing that Cx43 is a functional target of Wnt signaling in the brain [107]. In the

heart, Wnt signaling has been demonstrated as an important modulator of Cx43-mediated intercellular communication [108]. Additionally, lithium chloride has been shown to activate Cx43 expression in skeletal myoblasts via the Wnt/β-catenin pathway [109]. Sclerostin is a soluble protein secreted almost exclusively by osteocytes that binds Lrp 5/6 and decreases Wnt/β-catenin signaling by preventing proteasome complex formation [110]. Sclerostin knockout mice have a high bone mass phenotype and exhibit increased expression of Cx43 [111]. These results highlight the relationship between the Wnt/β-catenin signaling pathway and GJIC and suggest that manipulation of one may result in effects in the other. The net result, however, appears to be consistent, whereby increases in Wnt signaling or Cx43 expression lead to an increase in bone mass. Whether these two mechanisms act in parallel or series requires further study.

Mechanotransduction: In Vitro

While accumulating research suggests that GJIC has an important role in transducing mechanical signals throughout bone cell networks, most of this evidence has been obtained from experiments that examined osteoblastic cells. This approach has been taken despite the fact that the majority of cells in bone are osteocytes. In the cellular milieu of the bone microenvironment, osteocytes are the best candidates for detecting and coordinating responsiveness to mechanical signals and communicating these signals to osteoblastic cells [112]. In addition, several studies suggest that osteocytes regulate osteoclastic cell activity and mesenchymal stem cell differentiation [113, 114]. Despite this, the involvement of mechanical signals in the osteocytic cell regulation of other bone cells has not been investigated. We demonstrated that GJIC contributes to mechanically induced osteocytic wave propagation from osteocytic MLO-Y4 cells to osteoblastic MC3T3-E1 cells [115], but few studies have examined the physiological consequence of mechanical signals detected by osteocytes and communicated with osteoblasts.

A study in our laboratory attempted to address this issue by exploring the effects of fluid slow shear stress in a co-culture model utilizing MC3T3-E1 osteoblasts and MLO-Y4 osteocytes [116]. By separating the cell types with a porous membrane, it was possible to expose one to shear stress while shielding the other [117]. Cellular processes traversed the pores in the membrane, resulting in connections that were shown to include GJs. Osteocytes exposed to shear stress on the order of 4.4 dyn/cm^2 induced a rapid increase in the level of alkaline phosphatase activity of the shielded osteoblasts. This transmission of mechanical signals from osteocytes to osteoblasts was inhibited by the gap junction blocker alpha-glycyrrhetinic acid. Furthermore, this effect was not seen when other types of mesenchymal cells were exposed to fluid shear and was dependent on physical contact of the two cell types.

Biophysical signals may also regulate proliferation and differentiation. Unfortunately, the evidence supporting this hypothesis is distinctly contradictory. As regards proliferation, several studies demonstrated that physiological levels of substrate strain (up to 3,500 μstrain) increase osteoblastic cell proliferation [118, 119]. On the other hand, a similar number of studies [120, 121] demonstrated that substrate deformation actually decreases osteoblastic cell proliferation. Of the studies that have examined the effect of fluid flow on osteoblast proliferation, two found that steady fluid flow inducing shear stresses within the physiological range (5–20 dynes/cm^2) did not increase proliferation [122, 123], while two others found that pulsatile flow did increase proliferation [123, 124]. Additionally, long-term (i.e., 7 days) pulsatile flow was not found to affect proliferation [125]. Studies examining the effects of mechanical signals on osteoblastic differentiation are also inconsistent. Several studies demonstrated that mechanical load, including substrate deformation, hydrostatic pressure, fluid flow, or hypergravity, increase the expression or

synthesis of markers of differentiation including osteocalcin, osteopontin, type I collagen, and alkaline phosphatase [126–129]. Conversely, a number of studies have demonstrated that exposure to similar load protocols results in decreases in the same markers of osteoblastic differentiation [130–133].

This contradictory evidence, as regards the effect of mechanical signals on osteoblastic proliferation and differentiation, may be because several different bone cell lines were utilized, as well as the use of variable loading regimes. This emphasizes the importance of future work designed to examine, in detail, the effect of a physiologically relevant load-induced signal on well-characterized osteoblastic cells that can be stimulated to differentiate in vitro. Additionally, when differentiation was examined in these studies, only a relatively small number of phenotypic markers of osteoblastic differentiation were examined. However, the fully mature (differentiated) osteoblastic phenotype reflects changes in the expression of many genes and proteins. Thus, it is difficult to define a mature osteoblastic cell based on only a few phenotypic characteristics (i.e. alkaline phosphatase activity, type 1 collagen, osteocalcin, or even in vitro mineralization). This challenge is emphasized by the existence of subclones of osteoblastic MC3T3-E1 cells that express alkaline phosphatase but do not mineralize and other subclones that mineralize but do not express alkaline phosphatase activity [134]. There is a need to define changes in osteoblastic differentiation based on patterns of gene or protein expression, rather than changes in the expression of a small number of genes or proteins.

Mechanotransduction: In Vivo

The complex physical structure and microenvironments of bone, with osteocytes embedded within the bone matrix and osteoblasts and osteoclasts on the surface, make in vitro models difficult to translate. One of the most physiologically applicable means to study the role of GJs in bone biology is to develop transgenic models that are lacking the GJ proteins of interest. The ubiquitous nature of GJIC, however, makes the development of a global null knockout challenging. For example, mice with a global Cx43 knockout die prior to birth due to severe cardiac dysfunction [135]. A solution developed by Castro and colleagues is an osteoblast- and osteocyte-specific knockout of the Cx43 gene *GJA1* using the Cre-loxP method [136, 137]. Two models were developed, using Cre-mediated replacement of a “floxed” Cx43 allele with a LacZ reporter gene. In this case, Cre recombinase expression in osteoblasts was driven the osteocalcin OG2 promoter or the Colalpha1(I) promoter. Using this model, the Cre enzyme is expressed prior to birth within cells that have already begun to differentiate into osteoblasts [138]. Because osteocytes are terminally differentiated osteoblasts, the result is an osteoblast- and osteocyte-specific post-natal knockout. This transgenic approach avoids skeletal deformities that might be expected from Cx43 ablation at an earlier stage of development.

Studies involving tissue-specific Cx43 knockout mice have found them to be relatively osteopenic, with a low peak bone mass as a result of reduced osteoblast differentiation and function. Whole-body density as measured by dual-energy X-ray absorptiometry (DEXA) revealed lower bone density in knockouts beginning at 1 month of age and continuing to at least 6 months [139]. Bone mineral content data from this study parallel the DEXA findings. Measurements via microcomputed tomography revealed a 40% reduction in trabecular bone. There was also observed a 50% reduction in osteoblast number, with no change in osteoclast number. Administration of parathyroid hormone (PTH) to Cx43 knockout animals resulted in an attenuated response compared with wildtype littermates, with a failure to increase mineral apposition rate. At the cellular level, there was an increase in osteoblast number following PTH administration, but not an increase in activity. These results suggest the

defect may be the result of ineffective hormonal activation related to Cx43, rather than complete ablation of PTH response.

A structural analysis of cortical bone from Cx43-deficient mice showed them to have thinner cortical bone, with a larger marrow area and total cross-sectional area—changes consistent with that found during normal skeletal aging [140]. During aging, endocortical resorption is compensated for by periosteal bone apposition, with the result being thinner cortical bone and larger diaphyseal cross-sections [141, 142]. These knockout mice also had a reduced anabolic response to in vivo mechanical loading at the tibia endocortical surface as assessed via three-point bending. These effects are consistent with an osteoblast defect, whereby these bone-building cells are unable to properly initiate a response to increased mechanical load. It is important to note, however, that the anabolic response to mechanical loading was not completely abolished in knockout mice. Which may imply that Cx43 is either not essential for mechanoresponsiveness or that compensatory mechanisms exist in the absence of Cx43.

Studies by Su et al. [76] investigated the expression of Cx43 in a rat model of experimental tooth movement. In their studies, osteoclasts and fibroblasts were found to express Cx43 in areas of compression, whereas osteocytes and osteoblasts had increased Cx43 expression in areas of tension. A follow-up study in mice found that mechanical loading induced differential expression of Cx43 in alveolar bone induced by tooth movement [143]. Cx43 mRNA expression was observed in osteoblasts and lining cells at bone formation sites and osteoclasts at resorption sites. While Cx43 mRNA expression was not increased in osteocytes, protein expression was increased and punctate staining suggested the formation of gap junctions between osteocytes and the presence of hemichannels. These studies suggest that bone cells may be differentially responsive to different types of mechanical load in regard to their expression of Cx43. These differences may contribute to a coordinated bone remodeling response in a complex mechanical environment with aspects of tension and compression.

Unloading

Along with mechanical loading, the physiological response to unloading is also a mechanical situation where emerging research suggests a role of GJIC. Practical examples of unloading-induced bone loss include that experienced during spaceflight [144], bed rest [145], or as a result of neurologic injury [146]. A study by Lang et al. [147] of astronauts who spent 4–6 months on the international space station demonstrated an average bone loss of 0.5–1.5% per month. These losses were not completely recovered a year after returning to Earth [148]. Understandably, due to the high cost, there is a limited data set of the skeletal response to true micro-gravity. The development of ground-based models of microgravity has thus been an important objective of the National Aeronautics and Space Administration (NASA) and its research partners. The ground-based model of unloading called hindlimb suspension is the standard for simulating microgravity in rodents on the ground [149]. More recent models, including botulinum toxin-induced immobilization, have also been proposed [150]. In a study of botox immobilization of the hindlimbs of heterozygous Cx43-deficient mice, bone loss (as measured by BMC) tended to occur at a slower rate in knockouts compared with wildtype equivalents [151], although recovery of both genotypes was similar and incomplete at 12 weeks. More comprehensive studies are required in order to further explore the response of Cx43 knockout mice to hindlimb unloading and immobilization.

In a rather elegant series of experiments conducted by Tatsumi et al. [152], osteocytes were selectively ablated in mature mice. At baseline, these mice demonstrated a significant decrease in bone formation, trabecular bone, and mechanical strength. Interestingly, these

mice were completely resistant to the effects of hindlimb suspension, experiencing no significant bone loss and not exhibiting the increased levels of RANKL or sclerostin seen in their wildtype counterparts. Similar to this study, Lin et al. [111] have demonstrated that sclerostin knockout mice are also resistant to the effects of hindlimb suspension, experiencing no loss of cortical or trabecular bone, as well as no changes in BMD, bone formation rate, or expression of factors in the Wnt/β-catenin pathway. The investigators postulate that sclerostin is thus the essential mediator of unloading-induced bone loss. As previously mentioned, sclerostin is an antagonist of the Wnt/β-catenin pathway that is exclusively produced by osteocytes. Because Cx43 expression is a target of Wnt/β-catenin signaling [107] and Cx43 has a well-established role in mechanotransduction, it is likely that GJIC plays a role in the phenotype observed in these transgenic animals and in response to mechanical unloading.

Clinical Aspects

With an aging population, osteoporosis has become a significant health problem in our society [153]. The bone loss and decrease in skeletal strength experienced by post-menopausal women is a major health concern, with the 1-year mortality rate following hip fracture on the order of 20–35% [154, 155]. The skeletal phenotype of Cx43-deficient mice is similar to that seen in aging, with thinner cortical bone with a larger marrow and cross-sectional area [140]. These changes may make the Cx43-deficient mouse an ideal model for studying the effects of aging and pharmacological countermeasures.

Bisphosphonates are the frontline drugs for the treatment of postmenopausal osteoporosis [156]. These drugs not only decrease osteoclast development, recruitment, and activity [157] but also promote the survival of osteocytes and osteoblasts [158]. In vitro studies by Plotkin et al. [159] found that the ERK-activating/anti-apoptotic effects of bisphosphonates are dependent on Cx43. Alendronate was found to open Cx43 hemichannels, which in turn activated src kinase and ERKs. While it is thought that bisphosphonates bind to the hydroxyapatite of bone and reach the osteoclast during resorption, the results of this study suggest that bisphosphonates may enter the cell via Cx43. Studies with truncated versions of the Cx43 protein suggest that direct or indirect interaction with the pore-forming region of the hemichannel may be responsible for its opening. Additional studies by this group found that the non-aminobisphosphonate etidronate required Cx43 expression to prevent etoposide-induced apoptosis [160]. Etidronate was unable to prevent apoptosis in Cx43-lacking HeLa cells or primary osteoblasts isolated from Cx43-deficient mice, while apoptosis was abolished in Cx43-transfected HeLa cells and primary osteoblasts from wildtype mice. In vivo studies by Plotkin et al. [161] confirmed that alendronate is ineffective in preventing glucocorticoid-induced osteoporosis in Cx43-deficient mice.

Conclusions

Research to date has exposed the fundamental role of GJIC in many aspects of human biology. From the nervous and cardiovascular system to the immune response and bone physiology, connexins have been established as critical components of various physiological mechanisms. In the skeletal system, connexins and GJIC have a role in mechanotransduction, signal propagation, and sensitization to hormones and are required for the pharmacological effects of a variety of drugs and agents. From a basic science perspective, it will be necessary to more precisely define the phenotype of Cx43-deficient mice and to elucidate the precise contribution of Cx43 to their observed response to mechanical loading and unloading. In addition, new frontiers lie with the development of so-called connexin-mimetic agents. These agents have shown to be active in the brain and heart and have shown potential as therapeutic interventions for cancer. As we are able to better

refine the precise role that GJs play in the development of pathological and age-related bone loss, it may be possible to develop therapeutic agents to modulate bone connexins in order to prevent bone loss, preserve strength, and reduce fracture risk. Regardless of the clinical applications, however, it is clear that research in the future will continue to refine the vital, albeit complex, role of connexins in skeletal homeostasis.

References

1. Dasgupta C, Martinez AM, Zuppan CW, Shah MM, Bailey LL, Fletcher WH. Identification of connexin43 (alpha1) gap junction gene mutations in patients with hypoplastic left heart syndrome by denaturing gradient gel electrophoresis (DGGE). Mutat Res. 2001; 479(1–2):173–186. [PubMed: 11470490]
2. Omori Y, Mesnil M, Yamasaki H. Connexin 32 mutations from X-linked Charcot–Marie–Tooth disease patients: functional defects and dominant negative effects. Mol Biol Cell. 1996; 7(6):907–916. [PubMed: 8816997]
3. Vitale ML, Akpovi CD, Pelletier RM. Cortactin/tyrosine-phosphorylated cortactin interaction with connexin 43 in mouse seminiferous tubules. Microsc Res Tech. 2009; 72(11):856–867. [PubMed: 19725064]
4. Scheckenbach KE, Crespin S, Kwak BR, Chanson M. Connexin channel-dependent signaling pathways in inflammation. J Vasc Res. 2010; 48(2):91–103. [PubMed: 20926890]
5. Jiang JX, Siller-Jackson AJ, Burra S. Roles of gap junctions and hemichannels in bone cell functions and in signal transmission of mechanical stress. Front Biosci. 2007; 12:1450–1462. [PubMed: 17127393]
6. Maeda S, Tsukihara T. Structure of the gap junction channel and its implications for its biological functions. Cell Mol Life Sci. 2010
7. Willecke K, Eiberger J, Degen J, Eckardt D, Romualdi A, Guldenagel M, et al. Structural and functional diversity of connexin genes in the mouse and human genome. Biol Chem. 2002; 383(5):725–737. [PubMed: 12108537]
8. Goodenough DA, Goliger JA, Paul DL. Connexins, connexons, and intercellular communication. Annu Rev Biochem. 1996; 65:475–502. [PubMed: 8811187]
9. Stains JP, Civitelli R. Cell-cell interactions in regulating osteo-genesis and osteoblast function. Birth Defects Res C Embryo Today. 2005; 75(1):72–80. [PubMed: 15838921]
10. Rochefort GY, Pallu S, Benhamou CL. Osteocyte: the unrecognized side of bone tissue. Osteoporos Int. 2010; 21(9):1457–1469. [PubMed: 20204595]
11. Stains JP, Civitelli R. Cell-to-cell interactions in bone. Biochem Biophys Res Commun. 2005; 328(3):721–727. [PubMed: 15694406]
12. Koval M, Harley JE, Hick E, Steinberg TH. Connexin46 is retained as monomers in a trans-Golgi compartment of osteoblastic cells. J Cell Biol. 1997; 137(4):847–857. [PubMed: 9151687]
13. Knight MM, McGlashan SR, Garcia M, Jensen CG, Poole CA. Articular chondrocytes express connexin 43 hemichannels and P2 receptors—a putative mechanoreceptor complex involving the primary cilium? J Anat. 2009; 214(21):275–283. [PubMed: 19207989]
14. Schilling AF, Filke S, Lange T, Gebauer M, Brink S, Baranowsky A, et al. Gap junctional communication in human osteoclasts in vitro and in vivo. J Cell Mol Med. 2008; 12(6A):2497–2504. [PubMed: 18266960]
15. Donahue HJ, Guilak F, Vander Molen M, McLeod RJ, Rubin CT, Grande DA, et al. Chondrocytes isolated from mature articular cartilage retain the capacity to form functional gap junctions. J Bone Min Res. 1995; 10(9):1359–1364.
16. White TW, Bruzzone R, Paul DL. The connexin family of intercellular channel forming proteins. Kidney Int. 1995; 48(4):1148–1157. [PubMed: 8569076]
17. Elfgang C, Eckert R, Lichtenberg-Frate H, Butterweck A, Traub O, Klein RA, et al. Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. J Cell Biol. 1995; 129(3):805–817. [PubMed: 7537274]
18. Evans WH, De Vuyst E, Leybaert L. The gap junction cellular internet: connexin hemichannels enter the signalling limelight. Biochem J. 2006; 397(1):1–14. [PubMed: 16761954]

19. Burra S, Jiang JX. Connexin 43 hemichannel opening associated with prostaglandin E(2) release is adaptively regulated by mechanical stimulation. Commun Integr Biol. 2009; 2(3):239–240. [PubMed: 19641742]
20. Song D, Liu X, Liu R, Yang L, Zuo J, Liu W. Connexin 43 hemichannel regulates H9c2 cell proliferation by modulating intracellular ATP and [Ca^{2+}]. Acta Biochim Biophys Sin (Shanghai). 2010; 42(7):472–482. [PubMed: 20705586]
21. Jiang JX, Gu S. Gap junction- and hemichannel-independent actions of connexins. Biochim Biophys Acta. 2005; 1711(2):208–214. [PubMed: 15955305]
22. Stains JP, Civitelli R. Gap junctions in skeletal development and function. Biochim Biophys Acta. 2005; 1719(1–2):69–81. [PubMed: 16359941]
23. Paznekas WA, Karczeski B, Vermeer S, Lowry RB, Delatycki M, Laurence F, et al. GJA1 mutations, variants, and connexin 43 dysfunction as it relates to the oculodentodigital dysplasia phenotype. Hum Mutat. 2009; 30(5):724–733. [PubMed: 19338053]
24. Gorlin RJ, Miskin LH, St GJ. Oculodentodigital dysplasia. J Pediatr. 1963; 63:69–75. [PubMed: 13949300]
25. Gutmann DH, Zackai LH, McDonald-McGinn DM, Fischbeck KH, Kamholz J. Oculodentodigital dysplasia syndrome associated with abnormal cerebral white matter. Am J Med Genet. 1991; 41(1):18–20. [PubMed: 1659191]
26. Flenniken AM, Osborne LR, Anderson N, Ciliberti N, Fleming C, Gittens JE, et al. A Gjal missense mutation in a mouse model of oculodentodigital dysplasia. Development. 2005; 132(19): 4375–4386. [PubMed: 16155213]
27. Dobrowolski R, Sasse P, Schrickel JW, Watkins M, Kim JS, Rackauskas M, et al. The conditional connexin43G138R mouse mutant represents a new model of hereditary oculodentodigital dysplasia in humans. Hum Mol Genet. 2008; 17(4):539–554. [PubMed: 18003637]
28. Meyer RA, Cohen MF, Recalde S, Zakany J, Bell SM, Scott WJ Jr. et al. Developmental regulation and asymmetric expression of the gene encoding Cx43 gap junctions in the mouse limb bud. Dev Genet. 1997; 21(4):290–300. [PubMed: 9438343]
29. Lecanda F, Warlow PM, Sheikh S, Furlan F, Steinberg TH, Civitelli R. Connexin43 deficiency causes delayed ossification, craniofacial abnormalities, and osteoblast dysfunction. J Cell Biol. 2000; 151(4):931–944. [PubMed: 11076975]
30. Minkoff R, Bales ES, Kerr CA, Struss WE. Antisense oligo-nucleotide blockade of connexin expression during embryonic bone formation: evidence of functional compensation within a multigene family. Dev Genet. 1999; 24(1–2):43–56. [PubMed: 10079510]
31. Sims K Jr. Eble DM, Iovine MK. Connexin43 regulates joint location in zebrafish fins. Dev Biol. 2009; 327(2):410–418. [PubMed: 19150347]
32. Schiller PC, D'Ippolito G, Brambilla R, Roos BA, Howard GA. Inhibition of gap-junctional communication induces the trans-differentiation of osteoblasts to an adipocytic phenotype in vitro. J Biol Chem. 2001; 276(171):14133–14138. [PubMed: 11278824]
33. Lo CW. The role of gap junction membrane channels in development. J Bioenerg Biomembr. 1996; 28(4):379–385. [PubMed: 8844335]
34. Minkoff R, Rundus VR, Parker SB, Hertzberg EL, Laing JG, Beyer EC. Gap junction proteins exhibit early and specific expression during intramembranous bone formation in the developing chick mandible. Anat Embryol. 1994; 90(3):231–241. [PubMed: 7818094]
35. Li Z, Zhou Z, Yellowley CE, Donahue HJ. Inhibiting gap junctional intercellular communication alters expression of differentiation markers in osteoblastic cells. Bone. 1999; 25(5):661–666. [PubMed: 10593410]
36. Donahue HJ, Li Z, Zhou Z, Yellowley CE. Differentiation of human fetal osteoblastic cells is partially dependent on gap junctional intercellular communication. Am J Physiol (Cell). 2000; 278:C315–C322.
37. Chiba H, Sawada N, Oyamada M, Kojima T, Nomura S, Ishii S, et al. Relationship between the expression of the gap junction protein and osteoblast phenotype in a human osteoblastic cell line during cell proliferation. Cell Struct Funct. 1993; 18(6):419–426. [PubMed: 8033223]

38. Schiller PC, D'Ippolito G, Balkan W, Roos BA, Howard GA. Gap-junctional communication is required for the maturation process of osteoblastic cells in culture. Bone. 2001; 28(4):362–369. [PubMed: 11336916]
39. Schiller PC, Roos BA, Howard GA. Parathyroid hormone up-regulation of connexin 43 gene expression in osteoblasts depends on cell phenotype. J Bone Miner Res. 1997; 12(12):2005–2013. [PubMed: 9421233]
40. Huang W, Rudkin GH, Carlsen B, Ishida K, Ghasri P, Anvar B, et al. Overexpression of BMP-2 modulates morphology, growth, and gene expression in osteoblastic cells. Exp Cell Res. 2002; 274(2):226–234. [PubMed: 11900483]
41. Romanello M, Moro L, Pirulli D, Crovella S, D'Andrea P. Effects of cAMP on intercellular coupling and osteoblast differentiation. Biochem Biophys Res Commun. 2001; 282(5):1138–1144. [PubMed: 11302734]
42. Inose H, Ochi H, Kimura A, Fujita K, Xu R, Sato S. A microRNA regulatory mechanism of osteoblast differentiation. Proc Natl Acad Sci USA. 2009; 106(49):20794–20799. [PubMed: 19933329]
43. Geneau G, Defamie N, Mesnil M, Cronier L. Endothelin1-induced Ca(2+) mobilization is altered in calvarial osteoblastic cells of Cx43(±) mice. J Membr Biol. 2007; 217(1–3):71–81. [PubMed: 17568972]
44. Hiruma Y, Inoue A, Shiohama A, Otsuka E, Hirose S, Yamaguchi A, et al. Endothelins inhibit the mineralization of osteoblastic MC3T3-E1 cells through the A-type endothelin receptor. Am J Physiol. 1998; 275(4 Pt 2):R1099–R1105. [PubMed: 9756539]
45. Niger C, Geneau G, Fiorini C, Defamie N, Pointis G, Mesnil M, et al. Endothelin-1 inhibits human osteoblastic cell differentiation: influence of connexin-43 expression level. J Cell Biochem. 2008; 103(1):110–122. [PubMed: 17486593]
46. Burnstock G. Purinergic signaling and vascular cell proliferation and death. Arterioscler Thromb Vasc Biol. 2002; 22(3):364–373. [PubMed: 11884276]
47. Albert JL, Boyle JP, Roberts JA, Challiss RA, Gubby SE, Boarder MR. Regulation of brain capillary endothelial cells by P2Y receptors coupled to Ca^{2+}, phospholipase C and mitogen-activated protein kinase. Br J Pharmacol. 1997; 122(5):935–941. [PubMed: 9384512]
48. English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S, et al. New insights into the control of MAP kinase pathways. Exp Cell Res. 1999; 253(1):255–270. [PubMed: 10579927]
49. Nakamura E, Uezono Y, Narusawa K, Shibuya I, Oishi Y, Tanaka M, et al. ATP activates DNA synthesis by acting on P2X receptors in human osteoblast-like MG-63 cells. Am J Physiol Cell Physiol. 2000; 279(2):C510–C519. [PubMed: 10913018]
50. Pines A, Romanello M, Cesaratto L, Damante G, Moro L, D' Andrea P, et al. Extracellular ATP stimulates the early growth response protein 1 (Egr-1) via a protein kinase C-dependent pathway in the human osteoblastic HOBIT cell line. Biochem J. 2003; 373(Pt 3):815–824. [PubMed: 12729460]
51. Jones SJ, Gray C, Sakamaki H, Arora M, Boyde A, Gourdie R, et al. The incidence and size of gap junctions between the bone cells in rat calvaria. Anat Embryol. 1993; 187:343–352. [PubMed: 8390141]
52. Ilvesaro J, Vaananen K, Tuukkanen J. Bone-resorbing osteoclasts contain gap-junctional connexin-43. J Bone Miner Res. 2000; 15(5):919–926. [PubMed: 10804022]
53. Ilvesaro J, Tavi P, Tuukkanen J. Connexin-mimetic peptide Gap 27 decreases osteoclastic activity. BMC Musculoskelet Disord. 2001; 2(1):10. [PubMed: 11747476]
54. Ilvesaro J, Tuukkanen J. Gap-junctional regulation of osteoclast function. Crit Rev Eukaryot Gene Expr. 2003; 13(2–4):133–146. [PubMed: 14696962]
55. Ransjo M, Sahli J, Lie A. Expression of connexin 43 mRNA in microisolated murine osteoclasts and regulation of bone resorption in vitro by gap junction inhibitors. Biochem Biophys Res Commun. 2003; 303(4):1179–1185. [PubMed: 12684060]
56. Ahn AC, Grodzinsky AJ. Relevance of collagen piezoelectricity to "Wolff's Law": a critical review. Med Eng Phys. 2009; 31(7):733–741. [PubMed: 19286413]
57. Kontulainen S, Sievanen H, Kannus P, Pasanen M, Vuori I. Effect of long-term impact-loading on mass, size, and estimated strength of humerus and radius of female racquet-sports players: a

peripheral quantitative computed tomography study between young and old starters and controls. J Bone Miner Res. 2003; 18(2):352–359. [PubMed: 12568413]

58. Turner CH, Warden SJ, Bellido T, Plotkin LI, Kumar N, Jasiuk I, et al. Mechanobiology of the skeleton. Sci Signal. 2009; 2(68) pt3.
59. Jacobs CR, Temiyasathit S, Castillo AB. Osteocyte mechanobiology and pericellular mechanics. Annu Rev Biomed Eng. 2010; 12:369–400. [PubMed: 20617941]
60. Riddle RC, Donahue HJ. From streaming-potentials to shear stress: 25 years of bone cell mechanotransduction. J Onhop Res. 2009; 27(2):143–149.
61. Sato K, Adachi T, Ueda D, Hojo M, Tomita Y. Measurement of local strain on cell membrane at initiation point of calcium signaling response to applied mechanical stimulus in osteoblastic cells. J Biomech. 2007; 40(6):1246–1255. [PubMed: 16887125]
62. Vander Molen MA, Donahue HJ, Rubin CT, McLeod KJ. Osteoblastic networks with deficient coupling: differential effects of magnetic and electric field exposure. Bone. 2000; 27(2):227–231. [PubMed: 10913915]
63. Castillo AB, Alam I, Tanaka SM, Levenda J, Li J, Warden SJ, et al. Low-amplitude, broad-frequency vibration effects on cortical bone formation in mice. Bone. 2006; 39(5):1087–1096. [PubMed: 16793358]
64. Chapurlat RD. Bone microdamage. Osteoporos Int. 2009; 20(6):1033–1035. [PubMed: 19340506]
65. McLeod KJ, Donahue HJ, Levin PE, Fontaine MA, Rubin CT. Electric fields modulate bone cell function in a density-dependent manner. J Bone Miner Res. 1993; 8(8):977–984. [PubMed: 8213260]
66. Jacobs CR, Yellowley CE, Davis BR, Zhou Z, Donahue HJ. Differential effect of steady versus oscillating flow on bone cells. J Biomech. 1998; 31:969–976. [PubMed: 9880053]
67. Kurokouchi K, Jacobs CR, Donahue HJ. Oscillating fluid flow inhibits TNF-alpha-induced NF-kappa B activation via an Ik-appa B kinase pathway in osteoblast-like UMR 106 cells. J Biol Chem. 2001; 276(16):13499–13504. [PubMed: 11096064]
68. Saunders MM, You J, Zhou Z, Li Z, Yellowley CE, Kunze EL, et al. Fluid flow-induced prostaglandin L2 response of osteoblastic ROS 17/2.8 cells is gap junction-mediated and independent of cytosolic calcium. Bone. 2003; 32(4):350–356. [PubMed: 12689677]
69. Alford, Al; Jacobs, CR.; Donahue, HJ. Oscillating fluid flow regulates gap junction communication in osteocytic MLO-Y4 cells by an ERK1/2 MAP kinase dependent mechanism. J Bone Miner Res. 2002 (in press).
70. You J, Yellowley CE, Donahue HJ, Zhang Y, Chen Q, Jacobs CR. Substrate deformation levels associated with routine physical activity are less stimulatory to bone cells relative to loading-induced oscillatory fluid flow. J Biomech Eng. 2000; 122(4):387–393. [PubMed: 11036562]
71. Vander Molen MA, Rubin CT, McLeod KJ, McCauley LK, Donahue HJ. Cap junctional intercellular communication contributes to hormonal responsiveness in osteoblastic networks. J Biol Chem. 1996; 271(21):12165–12171. [PubMed: 8647809]
72. Banes, AJ.; Tsuzaki, M.; Yang, X.; Faber, J.; Bottlang, M.; Pederson, D., et al., editors. Equibiaxial strain activates AP-1 and CRE transcription factors but not NF-KB or SSRE and upregulates Cx43 mRNA in tendon cells in vitro; 44th Annual meeting of the orthopaedic research society; New Orleans, LA. 1998.
73. Ziambaras K, Lecanda F, Steinberg TH, Civitelli R. Cyclic screech enhances gap junctional communication between osteoblastic cells. J Bone Miner Res. 1998; 13(2):218–228. [PubMed: 9495514]
74. Cheng B, Zhao S, Luo J, Sprague E, Bonewald LF, Jiang JX. Expression of functional gap junctions and regulation by fluid flow in osteocyte-like MLO-Y4 cells. J Bone Miner Res. 2001; 16(2):249–259. [PubMed: 11204425]
75. Lozupone E, Palumbo C, Favia A, Ferretti M, Palazzini S, Cantatore FP. Intermittent compressive load stimulates osteo-genesis and improves osteocyte viability in bones cultured “in vitro”. Clin Rheumatol. 1996; 15(6):563–572. [PubMed: 8973865]
76. Su M, Borke JL, Donahue HJ, Li Z, Warshawsky NM, Russell CM, et al. Expression of connexin 43 in rat mandibular bone and periodontal ligament (PDL) cells during experimental tooth movement. J Dent Res. 1997; 76(7):1357–1366. [PubMed: 9207768]

77. Communi D, Boeynaems JM. Receptors responsive to extra-cellular pyrimidine nucleotides. Trends Pharmacol Sci. 1997; 18(3):83–86. [PubMed: 9133777]

78. Boarder MR, Hourani SM. The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. Trends Pharmacol Sci. 1998; 19(3):99–107. [PubMed: 9584626]

79. Jorgensen NR, Henriksen Z, Sorensen OH, Eriksen EF, Civitelli R, Steinberg TH. Intercellular calcium signaling occurs between human osteoblasts and osteoclasts and requires activation of osteoclast P2X7 receptors. J Biol Chem. 2002; 277(9):7574–7580. [PubMed: 11756404]

80. Dixon SJ, Sims SM. P2 purinergic receptors on osteoblasts and osteoclasts: potential targets for drug development. Drug Dev Res. 2000; 49:187–200.

81. Lambrecht G. Agonists and antagonists acting at P2X receptors: selectivity profiles and functional implications. Naunyn Schmiedebergs Arch Pharmacol. 2000; 362(4–5):340–350. [PubMed: 11111828]

82. Schofl C, Cuthbertson KS, Walsh CA, Mayne C, Cobbold P, von zur Muhlen A, et al. Evidence for P2-purinoceptors on human osteoblast-like cells. J Bone Miner Res. 1992; 7(5):485–491. [PubMed: 1615757]

83. Bowler WB, Dixon CJ, Halleux C, Maier R, Bilbe G, Fraser WD, et al. Signaling in human osteoblasts by extracellular nucleotides Their weak induction of the c-fos proto-oncogene via Ca^{2+} mobilization is strongly potentiated by a parathyroid hormone/cAMP-dependent protein kinase pathway independently of mitogen-activated protein kinase. J Biol Chem. 1999; 274(20):14315–14324. [PubMed: 10318853]

84. Stout CE, Costantin JL, Naus CC, Charles AC. Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. J Biol Chem. 2002; 277(12):10482–10408. [PubMed: 11790776]

85. Cotrina ML, Lin JHC, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, et al. Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci USA. 1998; 95(26):15735–15740. [PubMed: 9861039]

86. Graff RD, Lazarowski ER, Banes AJ, Lee GM. ATP release by mechanically loaded porcine chondrons in pellet culture. Arthritis Rheum. 2000; 43(7):1571–1579. [PubMed: 10902762]

87. Romanello M, Pani B, Bicego M, D' Andrea P. Mechanically induced ATP release from human osteoblastic cells. Biochem Biophys Res Commun. 2001; 289(5):1275–1281. [PubMed: 11741333]

88. Jorgensen NR, Teilmann SC, Henriksen Z, Civitelli R, Sorensen OH, Steinberg TH. Activation of L-type calcium channels is required for gap junction-mediated intercellular calcium signaling in osteoblastic cells. J Biol Chem. 2003; 278(6):4082–4086. [PubMed: 12446698]

89. Genetos DC, Geist DJ, Donahue HJ, Duncan RL. Fluid flow-induced ATP secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. J Bone Miner Res. 2004 (in press).

90. Klein-Nulend J, Bacabac RG, Mullender MG. Mechanobiology of bone tissue. Pathol Biol (Paris). 2005; 53(10):576–580. [PubMed: 16364809]

91. Civitelli R, Ziambaras K, Warlow PM, Lecanda F, Nelson T, Harley J, et al. Regulation of connexin43 expression and function by prostaglandin L2 (PGE2) and parathyroid hormone (PTH) in osteoblastic cells. J Cell Biochem. 1998; 68(1):8–21. [PubMed: 9407310]

92. Jiang JX, Cherian PP. Hemichannels formed by connexin 43 play an important role in the release of prostaglandin E(2) by osteocytes in response to mechanical strain. Cell Commun Adhes. 2003; 10(4–6):259–264. [PubMed: 14681026]

93. Genetos DC, Kephart CJ, Zhang Y, Yellowley CE, Donahue HJ. Oscillating fluid flow activation of gap junction hemichannels induces ATP release from MLO-Y4 osteocytes. J Cell Physiol. 2007; 212(1):207–214. [PubMed: 17301958]

94. Siller-Jackson AJ, Burra S, Gu S, Xia X, Bonewald LF, Sprague E, et al. Adaptation of connexin 43-hemichannel prostaglandin release to mechanical loading. J Biol Chem. 2008; 283(39):26374–26382. [PubMed: 18676366]

95. Saunders MM, You J, Trosko JE, Yamasaki H, Li Z, Donahue HJ, et al. Gap junctions and fluid flow response in MC3T3-E1 cells. Am J Physiol Cell Physiol. 2001; 281(6):C1917–C1925. [PubMed: 11698250]

96. Genetos DC, Geist DJ, Liu D, Donahue HJ, Duncan RL. Fluid shear-induced ATP secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. J Bone Miner Res. 2005; 20(1):41–49. [PubMed: 15619668]

97. Li J, Liu D, Ke HZ, Duncan RL, Turner CH. The P2X7 nucleotide receptor mediates skeletal mechanotransduction. J Biol Chem. 2005; 280(52):42952–42959. [PubMed: 16269410]

98. Forwood MR. Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading in vivo. J Bone Miner Res. 1996; 11(11):1688–1693. [PubMed: 8915776]

99. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys. 2008; 473(2):139–146. [PubMed: 18395508]

100. Matemba SF, Lie A, Ransjo M. Regulation of osteoclastogenesis by gap junction communication. J Cell Biochem. 2006; 99(2):528–537. [PubMed: 16639710]

101. Liu F, Kohlmeier S, Wang CY. Wnt signaling and skeletal development. Cell Signal. 2008; 20(6): 999–1009. [PubMed: 18164181]

102. Kramer I, Halleux C, Keller H, Pegurri M, Gooi JH, Weber PB, et al. Osteocyte Wnt/beta-catenin signaling is required for normal bone homeostasis. Mol Cell Biol. 2010; 30(12):3071–3085. [PubMed: 20404086]

103. Sawakami K, Robling AG, Ai M, Pitner ND, Liu D, Warden SJ, et al. The Wnt co-receptor LRP5 is essential for skeletal mechanotransduction but not for the anabolic bone response to parathyroid hormone treatment. J Biol Chem. 2006; 281(33):23698–23711. [PubMed: 16790443]

104. Robinson JA, Chatterjee-Kishore M, Yaworsky PJ, Cullen DM, Zhao W, Li C, et al. Wnt/beta-catenin signaling is a normal physiological response to mechanical loading in bone. J Biol Chem. 2006; 281(42):31720–31728. [PubMed: 16908522]

105. Santos A, Bakker AD, Zandieh-Doulabi B, Semeins CM, Klein-Nulend J. Pulsating fluid flow modulates gene expression of proteins involved in Wnt signaling pathways in osteocytes. J Orthop Res. 2009; 27(10):1280–1287. [PubMed: 19353691]

106. Melloy PG, Kusnierczyk MK, Meyer RA, Lo CW, Desmond ML. Overexpression of connexin43 alters the mutant phenotype of midgestational wnt-1 null mice resulting in recovery of the midbrain and cerebellum. Anat Rec A Discov Mol Cell Evol Biol. 2005; 283(1):224–238. [PubMed: 15678491]

107. van der Heyden MA, Rook MB, Hermans MM, Rijksen G, Boonstra J, Defize LH, et al. Identification of connexin43 as a functional target for Wnt signalling. J Cell Sci. 1998; 111(Pt 12):1741–1749. [PubMed: 9601103]

108. Ai Z, Fischer A, Spray DC, Brown AM, Fishman GI. Wnt-1 regulation of connexin43 in cardiac myocytes. J Clin Invest. 2000; 105(2):161–171. [PubMed: 10642594]

109. Du WJ, Li JK, Wang QY, Hou JB, Yu B. Lithium chloride regulates connexin43 in skeletal myoblasts in vitro: possible involvement in Wnt/beta-catenin signaling. Cell Commun Adhes. 2008; 15(3):261–271. [PubMed: 18972237]

110. Poole KE, van Bezooijen RL, Loveridge N, Hamersma H, Papapoulos SE, Lowik CW, et al. Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. FASLB J. 2005; 19(13):842–844.

111. Lin C, Jiang X, Dai Z, Guo X, Weng T, Wang J, et al. Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/beta-catenin signaling. J Bone Miner Res. 2009; 24(10):1651–1661. [PubMed: 19419300]

112. Duncan RL, Turner CH. Mechanotransduction and the functional response of bone to mechanical strain. Calcif Tissue Int. 1995; 57(5):344–358. [PubMed: 8564797]

113. Heino TJ, Hentunen TA, Vaananen HK. Osteocytes inhibit osteo-clastic bone resorption through transforming growth factor-beta: enhancement by estrogen. J Cell Biochem. 2002; 85(1):185–197. [PubMed: 11891862]

114. Heino TJ, Hentunen TA, Vaananen HK. Conditioned medium from osteocytes stimulates the proliferation of bone marrow mesenchymal stem cells and their differentiation into osteoblasts. Exp Cell Res. 2004; 294(2):458–468. [PubMed: 15023534]

115. Yellowley CE, Li Z, Zhou Z, Jacobs CR, Donahue HJ. Functional gap junctions between osteocytic and osteoblastic cells. J Bone Miner Res. 2000; 15(2):209–217. [PubMed: 10703922]

116. Taylor AF, Saunders MM, Shingle DL, Cimbala JM, Zhou Z, Donahue HJ. Mechanically stimulated osteocytes regulate osteoblastic activity via gap junctions. Am J Physiol Cell Physiol. 2007; 292(1):C545–C552. [PubMed: 16885390]

117. Riddle RC, Taylor AF, Donahue HJ. Fluid flow assays. Methods Mol Biol. 2008; 455:335–345. [PubMed: 18463829]

118. Kaspar D, Seidl W, Neidlinger-Wilke C, Beck A, Claes L, Ignatius A. Proliferation of human-derived osteoblast-like cells depends on the cycle number and frequency of uniaxial strain. J Biomech. 2002; 35(7):873–880. [PubMed: 12052389]

119. Fong KD, Nacamuli RP, Loboa EG, Henderson JH, Fang TD, Song HM, et al. Equibiaxial tensile strain affects calvarial osteoblast biology. J Craniofac Surg. 2003; 14(3):348–355. [PubMed: 12826806]

120. Akhouayri O, Lafage-Proust MH, Rattner A, Laroche N, Caillot-Augusseau A, Alexandre C, et al. Effects of static or dynamic mechanical stresses on osteoblast phenotype expression in three-dimensional contractile collagen gels. J Cell Biochem. 1999; 76(2):217–230. [PubMed: 10618639]

121. Tanaka SM, Li J, Duncan RL, Yokota H, Burr DB, Turner CH. Effects of broad frequency vibration on cultured osteoblasts. J Biomech. 2003; 36(1):73–80. [PubMed: 12485640]

122. Bannister SR, Lohmann CH, Liu Y, Sylvia VL, Cochran DL, Dean DD, et al. Shear force modulates osteoblast response to surface roughness. J Biomed Mater Res. 2002; 60(1):167–174. [PubMed: 11835172]

123. Jiang GL, White CR, Stevens HY, Frangos JA. Temporal gradients in shear stimulate osteoblastic proliferation via ERK1/2 and retinoblastoma protein. Am J Physiol Endocrinol Metab. 2002; 283(2):E383–E389. [PubMed: 12110546]

124. Kapur S, Baylink DJ, Lau KH. Fluid flow shear stress stimulates human osteoblast proliferation and differentiation through multiple interacting and competing signal transduction pathways. Bone. 2003; 32(3):241–251. [PubMed: 12667551]

125. Nauman EA, Satcher RL, Keaveny TM, Halloran BP, Bikle DD. Osteoblasts respond to pulsatile fluid flow with short-term increases in PGL(2) but no change in mineralization. J Appl Physiol. 2001; 90(5):1849–1854. [PubMed: 11299276]

126. Owan I, Burr DB, Tumer CH, Qiu J, Tu Y, Onyia JE, et al. Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanical strain. Am J Physiol Cell Physiol. 1997; 42(3):C810–C815.

127. Yoshikawa T, Peel SA, Gladstone JR, Davies JE. Biochemical analysis of the response in rat bone marrow cell cultures to mechanical stimulation. Biomed Mater Eng. 1997; 7(6):369–377. [PubMed: 9622104]

128. Harter LV, Hruska KA, Duncan RL. Human osteoblast-like cells respond to mechanical strain with increased bone matrix protein production independent of hormonal regulation. Endocrinology. 1995; 136(2):528–535. [PubMed: 7530647]

129. Matsuda N, Morita N, Matsuda K, Watanabe M. Proliferation and differentiation of human osteoblastic cells associated with differential activation of MAP kinases in response to epidermal growth factor, hypoxia, and mechanical stress in vitro. Biochem Biophys Res Commun. 1998; 249(2):350–354. [PubMed: 9712699]

130. Hillsley MV, Frangos JA. Alkaline phosphatase in osteoblasts is down-regulated by pulsatile fluid flow. Calcif Tissue Int. 1997; 60(1):48–53. [PubMed: 9030480]

131. Brighton CT, Strafford B, Gross SB, Leatherwood DF, Williams JL, Pollack SR. The proliferative and synthetic response of isolated calvarial bone cells of rat to cyclic mechanical strain. J Bone Joint Surg Am. 1991; 73(3):320–331. [PubMed: 1848246]

132. Ozawa H, Imamura K, Abe E, Takahashi N, Hiraide T, Shibasaki Y, et al. Effect of a continuously applied compressive pressure on mouse osteoblast-like cells (MC3T3-E1) in vitro. J Cell Physiol. 1990; 142(1):177–185. [PubMed: 2298821]

133. Stanford CM, Stevens JW, Brand RA. Cellular deformation reversibly depresses RT-PCR detectable levels of bone-related mRNA. J Biomech. 1995; 28(12):1419–1421. [PubMed: 8666582]

134. Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH, Franceschi RT. Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. J Bone Miner Res. 1999; 14(6):893–903. [PubMed: 10352097]

135. Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, et al. Cardiac malformation in neonatal mice lacking connexin43. Science. 1995; 267(5205):1831–1834. [PubMed: 7892609]

136. Nagy A. Cre recombinase: the universal reagent for genome tailoring. Genesis. 2000; 26(2):99–109. [PubMed: 10686599]

137. Castro CH, Stains JP, Sheikh S, Szejnfeld VL, Willecke K, Theis M, et al. Development of mice with osteoblast-specific connexin43 gene deletion. Cell Commun Adhes. 2003; 10(4–6):445–450. [PubMed: 14681055]

138. Dacquin R, Starbuck M, Schinke T, Karsenty G. Mouse alpha1(I)-collagen promoter is the best known promoter to drive efficient Cre recombinase expression in osteoblast. Dev Dyn. 2002; 224(2):245–251. [PubMed: 12112477]

139. Chung DJ, Castro CH, Watkins M, Stains JP, Chung MY, Szejnfeld VL, et al. Low peak bone mass and attenuated anabolic response to parathyroid hormone in mice with an osteoblast-specific deletion of connexin43. J Cell Sci. 2006; 119(Pt 20):4187–4198. [PubMed: 16984976]

140. Grimston SK, Brodt MD, Silva MJ, Civitelli R. Attenuated response to in vivo mechanical loading in mice with conditional osteoblast ablation of the connexin43 gene (Gjal). J Bone Miner Res. 2008; 2(6):879–886. [PubMed: 18282131]

141. Beck TJ, Looker AC, Ruff CB, Sievanen H, Wahner HW. Structural trends in the aging femoral neck and proximal shaft: analysis of the third national health and nutrition examination survey dual-energy X-ray absorptiometry data. J Bone Miner Res. 2000; 15(12):2297–2304. [PubMed: 11127194]

142. Smith RW Jr. Walker RR. Femoral expansion in aging women: implications for osteoporosis and fractures. Science. 1964; 145:156–157. [PubMed: 14171549]

143. Gluhak-Heinrich J, Gu S, Pavlin D, Jiang JX. Mechanical loading stimulates expression of connexin 43 in alveolar bone cells in the tooth movement model. Cell Commun Adhes. 2006; 13(1–2):115–125. [PubMed: 16613785]

144. Keyak JH, Koyama AK, LeBlanc A, Lu Y, Lang TF. Reduction in proximal femoral strength due to long-duration spaceflight. Bone. 2009; 44(3):449–453. [PubMed: 19100348]

145. Spector ER, Smith SM, Sibonga JD. Skeletal effects of long-duration head-down bed rest. Aviat Space Environ Med. 2009; 80(5 Suppl):A23–A28. [PubMed: 19476166]

146. Lee TQ, Shapiro TA, Bell DM. Biomechanical properties of human tibias in long-term spinal cord injury. J Rehabil Res Dev. 1997; 34(3):295–302. [PubMed: 9239622]

147. Lang T, LeBlanc A, Evans H, Lu Y, Genant H, Yu A. Cortical and trabecular bone mineral loss from the spine and hip in long-duration spaceflight. J Bone Miner Res. 2004; 19(6):1006–1012. [PubMed: 15125798]

148. Lang TF, Leblanc AD, Evans HJ, Lu Y. Adaptation of the proximal femur to skeletal reloading after long-duration space-flight. J Bone Miner Res. 2006; 21(8):1224–1230. [PubMed: 16869720]

149. Morey-Holton ER, Globus RK. Hindlimb unloading rodent model: technical aspects. J Appl Physiol. 2002; 92(4):1367–1377. [PubMed: 11895999]

150. Warner SE, Sanford DA, Becker BA, Bain SD, Srinivasan S, Gross TS. Botox induced muscle paralysis rapidly degrades bone. Bone. 2006; 38(2):257–264. [PubMed: 16185943]

151. Grimston SK, Silva MJ, Civitelli R. Bone loss after temporarily induced muscle paralysis by Botox is not fully recovered after 12 weeks. Ann N Y Acad Sci. 2007; 1116:444–460. [PubMed: 17584988]

152. Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, et al. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. Cell Metab. 2007; 5(6):464–475. [PubMed: 17550781]

153. Lin JT, Lane JM. Osteoporosis: a review. Clin Orthop Relat Res. 2004; 425:126–134. [PubMed: 15292797]

154. da Costa JA, Ribeiro A, Bogas M, Costa L, Varino C, Lucas R, et al. Mortality and functional impairment after hip fracture—a prospective study in a Portuguese population. Acta Reumatol Port. 2009; 34(4):618–626. [PubMed: 20852575]

155. Goldacre MJ, Roberts SE, Yeates D. Mortality after admission to hospital with fractured neck of femur: database study. BMJ. 2002; 325(7369):868–869. [PubMed: 12386038]

156. Favus MJ. Bisphosphonates for osteoporosis. N Engl J Med. 2010; 363(21):2027–2035. [PubMed: 21083387]

157. Hughes DE, Wright KR, Uy HL, Sasaki A, Yoneda T, Roodman GD, et al. Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo. J Bone Miner Res. 1995; 10(10): 1478–1487. [PubMed: 8686503]

158. Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T. Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. J Clin Invest. 1999; 104(10):1363–1374. [PubMed: 10562298]

159. Plotkin LJ, Bellido T. Bisphosphonate-induced, hemichannel-mediated, anti-apoptosis through the Src/ERK pathway: a gap junction-independent action of connexin43. Cell Commun Adhes. 2001; 8(4–6):377–382. [PubMed: 12064622]

160. Plotkin LI, Manolagas SC, Bellido T. Dissociation of the pro-apoptotic effects of bisphosphonates on osteoclasts from their anti-apoptotic effects on osteoblasts/osteocytes with novel analogs. Bone. 2006; 39(3):443–452. [PubMed: 16627025]

161. Plotkin LI, Lezcano V, Thostenson J, Weinstein RS, Manolagas SC, Bellido T. Connexin 43 is required for the anti-apoptotic effect of bisphosphonates on osteocytes and osteoblasts in vivo. J Bone Miner Res. 2008; 23(11):1712–1721. [PubMed: 18597631]