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Roles of gap junctions and hemichannels in bone cell functions and in signal transmission of mechanical stress

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1. ABSTRACT

Gap junctions formed by connexins (Cx) play an important role in transmitting signals between bone cells such as osteoblasts and osteoclasts, cells responsible for bone formation and bone remodeling, respectively. Gap junction intercellular communication (GJIC) has been demonstrated to mediate the process of osteoblast differentiation and bone formation. Furthermore, GJIC propagates Ca^{2+} signaling, conveys anabolic effects of hormones and growth factors, and regulates gene transcription of osteoblast differentiation markers. GJIC is also implicated to regulate osteoclast formation, survival and apoptosis. Compared with other bone cells, the most abundant type are osteocytes, which express large amounts of connexins. Mechanosensing osteocytes connect and form gap junctions with themselves and other cells only through the tips of their dendritic processes, a relatively small percent of the total cell surface area compared to other cells. Recent studies show that in addition to gap junctions, osteoblasts and osteocytes express functional hemichannels, the un-opposed halves of gap junction channels. Hemichannels are localized at the cell surface and function independently of gap junctions. Hemichannels in osteocytes mediate the immediate release of prostaglandins in response to mechanical stress. The major challenges remaining in the field are how the functions of these two types of channels are coordinated in bone cells and what the asserted, distinct effects of these channels are on bone formation and remodeling processes, and on conveying signals elicited by mechanical loading.

2. INTRODUCTION

2.1. Gap Junctions in Bone Cells

Gap junctions are transmembrane channels, which connect the cytoplasm of adjacent cells. These channels permit molecules with molecular weights approximately less than 1 kD_a such as small metabolites, ions, and intracellular signaling molecules (i.e. calcium, cAMP, inositol triphosphate) to pass through. Gap junction channels have been demonstrated to be important in modulating cell signaling and tissue function in many organs, such as heart, liver, peripheral nerve, ovary, ear and lens of the eye (1-8). Gap junctions are formed by members of a family of sequentially and structurally related proteins known as connexins. Approximately twenty connexins have been identified and cloned from various tissues and cells (9-11). Six monomers of connexins are joined head-to-head across the extracellular "gap" between two adjacent cells to form intercellular channels. Connexins are membrane proteins, which consists of four conserved membrane spanning domains and two extracellular loop domains, sharing more than 95% homology. Sequences in the intracellular loop and especially, those in the carboxyl-terminus are divergent between connexins (12).

Three types of connexins, Cx43, Cx45 and Cx46, are expressed in bone tissues. Cx43, a ubiquitously expressed connexin, is identified in virtually all types of bone cells, including cultured osteoblasts from newborn rat calvaria (13), human bone marrow stromal cells, trabecular

bone osteoblasts (14), murine osteoblasts (15), primary osteocytes *in vivo* (16), mandibular bone and periodontal ligament cells of rat teeth (17), osteoblast-like MC3T3-E1 cells (18,19), osteocyte-like MLO-Y4 cells (20,21) and chondrocytes from articular and growth plate cartilage (22-24). Cx45 and Cx46 are expressed in several established osteoblast cell lines. Both Cx43 and Cx46 are expressed in osteoblast-like ROS17/2.8 cells, however, the latter connexin fails to assemble into multimeric complexes and to be expressed on the cell surface to form gap junction channels (25). Cx45 is expressed in UMR-102 and hFOB 1.19 osteoblastic, and MLO-Y4 osteocytic cell lines, but to a much lesser degree than Cx43 (21,26,27). However, we found that Cx45 protein is expressed in bone marrow, but not in osteoblasts, osteocytes and osteoclasts in the alveolar bone tissues of the tooth (28).

Functional gap junctions in osteoblasts were first demonstrated with electrical conductance and dye injection (29). Voltage-sensitive gap junction currents were detected in osteoblastic cells derived from calvarias of new-born rats with a single gap junction channel conductance of approximate 100 pS (30). Fluorescent dye injected into rat calvarial subperiosteal osteoblasts spreads to neighboring osteoblastic cells via GJIC. Using dye-transfer assays, it has also been observed that dye spreads rapidly between a numbers of odontoblasts (31) and osteoblasts (32). Microinjection of anti-Cx43 antibody in MC3T3-E1 cells blocks cell coupling (33). By using electron microcopy and histocytochemistry, the morphological proof of the existence of gap junction structures has also been obtained for periosteal fibroblasts, osteoblasts, and osteocytes *in vivo* (32,34-36). Interestingly, in addition to linear gap junctions, stacked, oval, and annual junction structures are found, especially within the osteocyte cytoplasm and in osteocyte cell processes within the canaliculi (36). However, the functional significance of these unusual gap junction structures inside the cells is still obscure. Together, connexins are richly expressed in bone cells and form functional gap junction channels.

2.2. Gap Junctions in Osteoblast Differentiation and Bone Formation

Gap junctions and Cx43 play essential roles in osteoblast differentiation and bone development in human and animal models *in vivo* (reviewed by Stains and Civitelli (37)). Cx43 mutations have been reported to cause the pleiotropic phenotypes of oculodentodigital dysplasia (ODDD) (38-41) and one of the mutations also leads to the skin disorder palmoplantar keratoderma (41). A mouse model of ODDD was identified from mutagenesis screening, which possesses a Cx43 mutation and displays similar bone defect phenotypes as human ODDD. A recent report shows that ODDD mutants of Cx43 form non-functional gap junctions and hemichannels, un-apposed halves of gap junction channels (see section 2.6) in C6 glioma cells (42). A mouse knockout model with a genetic deletion of a Cx43 gene has delayed ossification, craniofacial abnormalities, and misshapen ribs (43). Osteoblasts isolated from calvaria of Cx43-null mice display reduced differentiation and mineralization (43). The overall skeletal phenotypes of these knockout mice, however, are not as severe as human ODDD. A possible explanation could be that these analyses had to be conducted at the early developmental stages since these animals died prenatally caused by the blockade of ventricle outflow (4,44). More severe bone phenotypes might be expected if the assay were performed on adult animals. Knockdown of Cx43 using antisense oligonucleotides in chick leads to craniofacial abnormities (45-47), a phenotype remarkably analogous to ODDD. Moreover, in organ cultures of embryonic chick mandibular mesenchyme, bone formation is markedly reduced in the presence of the combined antisense oligonucleotides to Cx43 and Cx45, whereas bone formation is still evident when treated with antisense to either connexin (48), implying the compensating effect by these connexins. Two models of osteoblast-specific Cx43 gene deletion driven by either the osteocalcin OG2 promoter or collagen type I promoter were developed (49). These models will provide invaluable tools to investigate the specific role of Cx43 in osteoblasts and in the formation of bone in the adult skeleton. In another model system, recent studies show that mutations in the Cx43 gene cause sof, a mutant with a short fin, in zebrafish (50). This study suggests the crucial role of gap junctions in the regulation of bone size and growth.

Gap junctions have been demonstrated to play an important role in osteoblast differentiation and maintenance of a differentiated osteoblast phenotype *in vitro*. Gap junction inhibitors, antisense, and even Cx45 attenuate the stimulatory effect of Cx43 on differentiation of osteoprogenitors or osteoblasts, which is associated with a reduction in the expression of osteoblast differentiation markers, such as alkaline phosphatase, osteocalcin, and collagen type 1 and decreased mineralization (51-54). Expression of a dominant negative Cx43 construct in MC3T3-E1 cells leads to a decrease in alkaline phosphatase activity and calcium deposition (55). Interestingly, inhibition of GJIC induces the trans-differentiation of osteoblasts to an adipocytic phenotype *in vitro* (56). Conversely, overexpression of Cx43 promotes proliferation and differentiation of osteoblasts (52,57). Gap junction function and Cx43 expression are shown to parallel osteoblast differentiation (27). Promotion of osteoblast differentiation by gap junctions could be interpreted by its role in mediating intercellular passage of signaling factors that are elicited by differentiation-stimulating growth factors or hormones. Indeed, inhibition of gap junctions attenuates the effects of parathyroid hormone on osteoblast differentiation and mineralization (58, 59). In addition to osteoblasts, GJIC is also involved in the expression of alkaline phosphatase and type I collagen in bone marrow stromal cells when co-cultured with endothelial cells (51). A subsequent study from this group found that alkaline phosphatase activity is only increased by the direct contact of human osteoprogenitor cells with human vascular endothelial cell types, while a transformed human bone marrow endothelial cell line has no such effect (60). Disruption of GJIC attenuates this osteogenic effect. Both studies imply that heterotypic GJIC between two different types of cells is necessary to transmit the signals pertinent to osteoblast differentiation.

The role of gap junctions in osteoblast differentiation could be directly correlated to certain molecules passing through these channels. Cx43 and Cx45 have different gap junctional permeability in osteoblasts (26). Cx43 in osteoblast ROS17/2.8 cells mediated cell-cell coupling for both small ions and larger molecules, but Cx45 in UMR106 cells allowed passage only of small ions. This data implies the distinct roles of gap junction channels formed by these two connexins in passage of ions, signaling molecules and small metabolites in bone cells. Expression of Cx45 alters gap junctional permeability in osteoblastic cells expressing endogenous Cx43 (61). Overexpression of Cx45, which appears to function as a dominant negative, disrupts gap junctions formed by endogenous Cx43. This leads to a decrease in basal activities of the osteocalcin and type I collagen promoters (52). Both Cx43 and Cx45 interact with zonular occludens-1 (ZO-1) in osteoblast cells (62); however, the functional significance of this interaction in osteoblast differentiation is still unclear. Gap junctions have been reported to regulate gene transcription of osteoblastic differentiation markers. Stains and Civitelli (63) report that gap junctions regulate ERK/PI3 kinase (PI3K) signaling, which affects gene transcription through the gap junction-dependent osteocalcin connexin response element. Tbx, a developmental transcription regulatory factor, represses gene transcription of Cx43 both in osteoblasts and possibly in developing embryos (64). Mice deficient in Cx40, a Tbx-5-regulated connexin, display axial and appendicular skeletal malformations with similar phenotypes to Tbx5-deficient mice. Tbx5 exerts its role in bone growth and maturation by controlling gene expression of Cx40 (65).

2.3. Gap Junctions in Osteoclast Formation

In addition to its expression in osteoblast and osteocytes, Cx43 has also been identified in osteoclasts. An osteoclast is a multinucleated cell that degrades and reabsorbs bone. They are involved in the natural turnover of bone tissue along with osteoblasts. Osteoclasts arise from haemopoietic cells of the monocyte/neutrophil lineage. Gap junctions are suggested to play important functional roles in osteoclast precursor fusion to form multinucleated mature osteoclasts, communication in the bone multicellular unit in bone remodeling and osteoclast formation (66-68). Inhibitors of gap junctions, 18 α-glycyrrhetinic acid and oleamide, inhibited parathyroid hormone (PTH) and 1,25-$(OH)_2D_3$ (an active form of vitamin D_3)-stimulated osteoclastic pit formation, an assay for detection of osteoclast formation (68). The connexin-mimetic peptide GAP 27, that inhibits GJIC, decreases the numbers of TRAP-positive osteoclasts and increases the numbers of apoptotic osteoclasts (69). These experimental evidences suggest that GJIC is crucial for osteoclast formation and survival. Recently, the gap junction inhibitor carbenoxolone was shown to significantly inhibit osteoclastogenesis stimulated by PTH, prostaglandin E_2 (PGE_2) and 1,25$(OH)_2D_3$ in mouse bone marrow cultures (70), further implicating the role of GJIC in conveying the stimulating signals of these hormones on osteoclast formation and bone remodeling.

2.4. Gap Junction Regulation by Hormones, Growth Factors and Other Components

The functions and expression of gap junctions and Cx43 are regulated by hormones, and other signaling and regulatory molecules. PTH stimulates the expression of Cx43 in osteoblasts and osteocytes (71-74). The stimulatory effect of PTH appears to depend upon the developmental state of the osteoblasts along the osteoblastic differentiation pathway (74). Stable transfection antisense cDNA of Cx43 blocks the increase of cAMP elicited by PTH in ROS17/2.8 cells (59). Additionally, blocking of GJIC attenuates the stimulatory effect of PTH on osteoblast mineralization (58). Treatment with PGE_2, in most cases, increases GJIC and connexin expression in osteoblastic cell lines (59,71,72,74-78). Recently, an antiarrhythmic peptide analog rotigaptide (ZP123) has been shown to increase GJIC in human osteoblasts (79). Interestingly, this peptide also completely prevents the loss of femoral trabecular bone strength in ovariectomized rats. 17β-estradiol and 1,25$(OH)_2D_3$ do not affect Cx43 expression in UMR106 cells (74), but somehow cause cell uncoupling in primary osteocytes (80,81). In contrast to PTH and PGE_2, transforming growth factor β, osteogenin and bone morphogenetic protein-2 inhibit GJIC in MC3T3-E1 cells (82). pH plays a critical role in bone formation, by which alkaline pH supports mineral deposition while an acidic pH promotes mineral dissolution (83). GJIC is sensitive to pH changes in MC3T3-E1 cells and lower pH causes a reduction in cell coupling and the transcription rate of Cx43 (84,85). Elevated intracellular cAMP that increases GJIC induces an increase in the expression of osteocalcin, a marker for osteoblast differentiation and a decrease in alkaline phosphatase activity (73,86). ERK/PI3K signaling is suggested to functionally influence GJIC (87).

Components of the extracellular matrix (ECM) have direct impact on osteoblast differentiation, GJIC and connexins. ECM components, such as, type 1 collagen, fibronectin, vitronectin, are present during specific stages of bone development and have been shown to have different roles in supporting adhesion of mechanically strained osteoblasts (88). Modified extracellular matrix molecule, sulfated hyaluronan, enhances the expression of Cx43 and N-cadherin, further resulting in remarkable induction of the alkaline phosphatase activity in rat osteoblast cells (89). Hydroxy apatite (HA) microspheres, which enhance the differentiation of osteoblasts, increase GJIC in osteoblasts (90).

2.5. Gap Junctions in Ca^{2+} Signaling and Propagation in Bone Cells

Ca^{2+} signaling evoked by hormones, growth factors and mechanical stimulation in bone cells is thought to be crucial for bone formation and remodeling. Acting through the ERK signaling pathway in primary calvarial osteoblasts, extracellular Ca^{2+} induces COX-2 transcription and PGE_2 production (91). Gap junction-dependent and independent mechanisms have been proposed to modulate

the Ca^{2+} wave propagation in bone cells. In Cx43-expressing ROS17/2.8 cells, calcium waves are propagated by GJIC and influx of extracellular calcium. However, in Cx45-predominantly expressed UMR106 cells, the propagation of Ca^{2+} is mediated through gap junction-independent pathway, by which calcium waves are transmitted by activation of purinergic receptors, causing a release of intracellular calcium stores (92). In cultured primary osteoblasts and in bone marrow stromal cells, elevated intracellular Ca^{2+} reduces intercellular coupling (72,81). Extracellular Ca^{2+} influx through L-type Ca^{2+} channels triggers the surge of intracellular Ca^{2+}, which is transmitted via Cx43 gap junction channels (93). GJIC is also demonstrated to mediate intercellular signaling in isolated chondrocytes (22,23,94) and paired chondrocytes *in situ* (24). Gap junctions mediate the propagation of intracellular Ca^{2+} waves in mechanically stimulated articular chondrocytes (95) and between chondrocytes and synovial cells (96,97). Recent evidence from long-term culture of human osteoblast-like cells shows that in less differentiated cells, purinergic receptor P2Y-mediated intracellular calcium waves are primarily involved, but as cells differentiate in culture, gap junction-mediated intracellular Ca^{2+} become more prominent (98). Gap junctions provide a direct transmitting pathway for intracellular Ca^{2+} propagation, which results in organizing the cells as a group of cells as a syncytium for the coordinated action of bone formation and remodeling.

2.6. Gap Junction Hemichannels

In addition to being the major components of gap junction channels, connexins have recently been shown to exist and function in the form of un-apposed halves of gap junction channels called hemichannels (99-101). These channels are localized at the cell surface, independent of physical contact with adjacent cells. Hemichannels, like gap junction channels, display relatively low substrate selectivity and permit molecules with molecular weights less than 1 kD_a to pass through. However, the function of hemichannels is very different from gap junctions; the former mediate communication between cells and the extracellular matrix, while the latter is involved in the communication between adjacent cells. An atomic force microscopic study reveals the structures of Cx43 hemichannels as randomly distributed individual particles and clusters, showing a lack of preferential orientation in a lipid membrane (102). Extracellular domains of these undocked hemichannels are structurally different from connexons in docked gap junctional plaques. Hemichannels appear to provide a mechanism for ATP and NAD^+ release, which raises intracellular Ca^{2+} levels and promotes Ca^{2+} wave propagation in astrocytes, bone cells, epithelial cells, and retinal cells (103-111). Hemichannels in astrocytes are shown to be involved in the release of the neurotransmitter glutamate (112). Existence of functional hemichannels formed by Cx43 has been reported in neural progenitors, neurons (113,114), astrocytes (115,116), and heart (117). These hemichannels are regulated by voltage, protein kinase C, extracellular Ca^{2+}, and retinoic acid (106,118,119). Electrophysiological studies demonstrate the opening of hemichannels formed by Cx45 when the external Ca^{2+} concentration is reduced (120). The direct interaction of Ca^{2+} with Asp residues is responsible for preventing voltage-gated opening of Cx32 hemichannels. Disruption of the binding site, which is linked to a hereditary peripheral neuropathy, causes complete Ca^{2+} deregulation of hemichannels (118). Hemichannels are reported to regulate cell volume in response to the change in extracellular physiological calcium (121). Metabolic inhibition also induces opening of Cx43 hemichannels in cortical astrocytes and isolated ventricular myocytes (122), and intriguingly, GJIC is reduced (123). Hemichannels are demonstrated to exist in osteoblasts and osteocytes (109,124,125). These hemichannels expressed in bone cells appear to function as essential transducers of anti-apoptotic effects of bisphosphonates (124,125). We reported that hemichannels formed by Cx43 serve as the pathway for the exit of elevated intracellular PGE_2 in osteocytes induced by fluid flow shear stress (126,127). However, the physiological roles of hemichannels *in vivo* remain largely unknown.

2.7. Gap Junctions and Hemichannels in Mechanosensing Bone Osteocytes

The skeleton adapts to mechanical usage (128) and mechanical loading promotes bone formation and remodeling. In general, most bone cells may be involved in mechanosensing since several types of cells have been shown to be sensitive to mechanical stress (129). However, several arguments have been raised in favor of osteocytes as the mechanosensory cells (130-132). If osteocytes are the cells responsible for bone modeling and remodeling, key issues are how mechanical loading is sensed, how these signals are conveyed to other non-sensing cells and how these signals are translated into chemical signals. The application of force to bone results in several potential stimuli for osteocyte function including hydrostatic pressure and fluid flow shear stress. Over four decades, various theoretical and experimental studies argue that flow of interstitial fluid driven by extravascular pressure as well as by the applied cyclic mechanical loading is likely the stress-related factor that informs bone cells about mechanical loading and stimulates bone formation. *In vivo* experiments have demonstrated the circulation of interstitial fluid in the lacuno-canalicular network by mechanical loading (133-135). Furthermore, bone cells including osteoblasts and osteocytes are more sensitive to fluid flow shear stress than mechanical strain, stretching and compression (136-140). Theoretical models and other experimental evidence also suggest that osteocytes are the most likely mechosensor cells in the bone and shear stress induced by fluid flow shear stress is the major mechanism of mechanical loading (130-132,141-146). A cable model predicts that the diffusion time for the spread of current along the membrane of the osteocytic processes is about 0.03 seconds, close to the same predicted pore pressure relaxation time for the draining of the bone fluid into the osteonal canal (147). It has been hypothesized that the bending of primary cilia of an osteocyte by extracellular fluid sends signals into cells through gap junctions (148). Osteocytes have been shown to be more sensitive with respect to the release of PGE_2 following both hydrostatic compression and fluid flow treatment than osteoblasts. However, fluid flow shear stress is even more effective

than hydrostatic compression in eliciting this response in osteocytes (138). It has been found that mechanical forces applied to bone cause fluid flow through the canaliculi surrounding the osteocyte that is probably responsible for the deformation of the cell membrane (141,149,150). Fluid flow shear stress is likely to stimulate chemical responses, and the signaling molecules generated are likely to be transmitted between cells by gap junction channels connected through the extensive networks of dendritic processes, and through hemichannels between osteocytes and the extracellular matrix. In turn, the signaling cascade activated in this fashion leads to the expression of important regulatory molecules crucial for modulating bone formation and remodeling.

Osteocytes are dispersed throughout the mineralized matrix. The mature osteocyte is described as a stellate or star-shaped cell with a large number of slender, cytoplasmic processes radiating in all directions but perpendicular to the bone surface. These cell processes pass through the bone in thin canals called canaliculi. It has been hypothesized that matrix-embedded osteocytes emit signals that stimulate osteoblast differentiation into osteocytes (32,131,151). The cell processes of osteocytes are connected with each other and the lining cells via gap junctions (34,35,152), thereby allowing direct cell-to-cell coupling. The rapid intracellular passage of ions and signaling molecules mediated by gap junction channels and the extracellular signaling by hemichannels renders them suitable for sensing mechanical strain and later transmitting these mechanical signals into biochemical events. Therefore, gap junctions and hemichannels in osteocytes appear to have essential, distinctive roles in transmitting the signals elicited by mechanical stimulation to other bone cells and the extracellular matrix, and further promote bone formation and remodeling. In osteocyte-like MLO-Y4 cells and primary osteocytes, we and others have shown that Cx43 is the major connexin expressed (153,154) and expression patterns are similar in these two types of cells (20,154). In addition to Cx43, Cx45 was also detected in MLO-Y4 cells (21). However, only Cx43, but not Cx45, was detected in osteocytes of alveolar bone in the tooth (28). Yellowley and coworkers (155) have shown that MLO-Y4 cells can couple through gap junctions to osteoblast-like MC3T3-E1 cells detected by the passage of calcein between cells and the mechanically induced calcium response.

2.8. Gap Junctions and Hemichannels in Mechanical Signal Transduction of Bone

There are multiple molecular sensory molecules that sense and somehow concert mechanical stimulation into biological signals in bone cells (for recent review, see Rubin *et al.* (156)). It has been postulated that mechanical usage of bone results in strain-induced signals that are sensed by the mechanostat and GJIC defines the mechanostat set point (157). Gap junctions through the propagation of intracellular signals contribute to mechanotransduction in bone and contribute to the regulation of bone cell differentiation (158). Few studies have been conducted examining the expression and regulation of Cx43 in response to mechanical stimulation using *in vivo* animal models. By using a rat experimental tooth movement model, Su *et al.* (17) reported that Cx43 is increased in osteoclasts and periodontal ligament cells in compression zones, and in osteoblasts and osteocytes in tension zones of the periodontal ligament. They also observed high expression of Cx43 mRNA in osteocytes. Our studies using a mouse tooth movement model show the similar stimulation of Cx43 mRNA expression by mechanical loading in osteoblasts and lining cells in bone formation sites and osteoclasts in resorption sites (28). However, there is no discernible increase of Cx43 mRNA in osteocytes, but in contrast, level of Cx43 protein in alveolar osteocytes is highly up-regulated in response to mechanical loading. This discrepancy between their studies and our work could be caused by the different mechanical models used, variation of tooth movement applied, and duration of loading regimes. These *in vivo* studies suggest that expression of Cx43 in bone is highly responsive to mechanical loading.

Several *in vitro* studies have been conducted to determine the relationship between mechanical stress and function and expression of gap junctions and connexins in bone cells. Mechanical loading through cyclical stretching enhances the phosphorylation of Cx43 and GJIC in osteoblastic cells, implying the formation of functional gap junction channels (159). Mechanical stimulation by magnetic field exposure inhibits osteoblast growth through a mechanism independent of gap junction coupling, while the alteration in alkaline phosphatase activity appears to be stimulated by the induced electric field in a gap junction-dependent manner (160). Mechanical stress causes the release of PGE_2, which is shown to be dependent upon gap junctions (161,162). A dominant negative mutant of Cx43 diminishes fluid flow-induced release of PGE_2, but not Ca^{2+} responses (161). Consistently, the fluid flow-induced PGE_2 response of ROS17/2.8 cells is gap junction-mediated and independent of intracellular Ca^{2+} (162). Gap junction activities and accumulation of Cx43 protein in osteocytes are altered by mechanical loading (20,21,87). Oscillating fluid flow has been shown to up-regulate GJIC in MLO-Y4 cells by an ERK1/2 MAP kinase-dependent mechanism (87). We have shown that fluid flow stimulates GJIC and increases Cx43 expression in osteocyte-like MLO-Y4 cells (20). Recently, we found that in addition to gap junctions, Cx43 forming hemichannels mediate the biological responses elicited by fluid flow (127). Fluid flow increased surface expression of Cx43 and induced the rapid opening of hemichannels, which in turn mediated the release of PGE_2 in MLO-Y4 cells. All this evidence suggests that signals generated by mechanical stimulation are likely to be transmitted between bone cells through gap junction channels, and between cells and the extracellular matrix through hemichannels. The role of hemichannel opening in response to mechanical stress is postulated in a model diagram (Figure 1).

Prostaglandin increases the skeletal response to mechanical loading (163). Prostaglandin released by bone cells is generally thought to be a skeletal anabolic agent as these agents can increase bone mass in animals (164-166). A recent report shows that fluid flow induced PGE_2 release

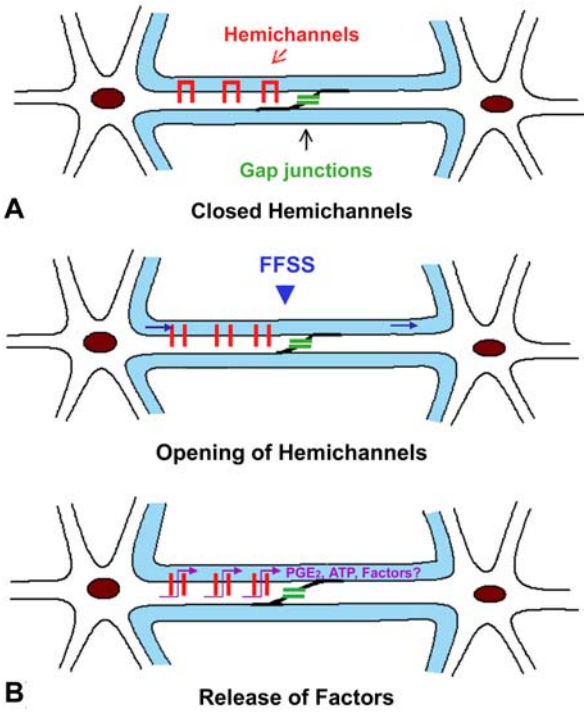

Figure 1. Model diagram for the role of hemichannels under fluid flow shear stress in osteocytes. Hemichannels are expressed on the plasma membrane away from cell-cell junction regions. (A) In the absence of mechanical stress, hemichannels remain closed, whereas gap junctions are kept open. (B) Fluid flow shear stress induces the opening of hemichannels (upper panel). PGE_2, possibly ATP and other responding physiological factors are released into canaliculi to mediate biological responses elicited by mechanical stress (lower panel).

by MLO-Y4 cells is reduced by the degradation of the glycocalyx on the cell surface, a hypothesized mechanosensor in osteocytes (167). We show that PGE_2 released via Cx43-hemichannels in response to fluid flow functions in an autocrine fashion to activate EP_2 receptor signaling, including increased intracellular cAMP and activated protein kinase A, which in turn, stimulates gap junction function and Cx43 expression (76,77,127). These data suggest that hemichannels are likely to provide an important novel means, distinct from gap junctions, for modulating the immediate biological responses of osteocytes to mechanical stress.

2.9. Gap Junction Hemichannel Regulation

The experimental evidences revealing the regulatory mechanisms of connexin-forming hemichannels have started to emerge. Dephosphorylation of Cx43 due to ATP depletion and activation of Ca^{2+}-dependent protein phosphatases are proposed to induce the opening of hemichannels (168). The opening of hemichannels by ATP-depletion supports a role for hemichannels in causing injury in epithelial cells in general and in renal-tubule cells (169,170). Liposomes in which nonphosphorylated Cx43 is constituted show greater hemichannel permeability than those phosphorylated by mitogen-activated protein kinase (171). Moreover, hemichannels formed by Cx43(S368A), a PKC site mutant, remains preferentially open (172). Oxidation of Cx43 by enhanced generation of reactive oxygen-derived species also promotes the opening of hemichannels (168). Conversely, a free-radical scavenger (Trolox) inhibits opening of Cx43 hemichannels in metabolically inhibited astrocytes (123,168), implying the involvement of redox potential in the opening of hemichannels. A recent study by Retamal *et al.* (173) shows that metabolic inhibition increases the levels of Cx43 on the cell surface and induces dephosphorylation and nitrosylation of Cx43.

We recently found that the opening of hemichannels is likely to be modulated by the association of Cx43 with α5 integrin (our unpublished result, (174)). Previous evidence suggests the involvement of integrins in regulation of GJIC and Cx43 expression (175-177). Interaction of α3β1 with laminin 5 promotes gap junction coupling (175). Cx43 and Cx26 expression and distribution, formation of gap junction plaques and GJIC are regulated by matrix proteins (177). Previous immunohistochemical studies have shown the co-localization of Cx43 with cell and substrate adhesion molecules during intramembranous bone formation (178). However, the role of integrin and adhesion molecules in regulation of hemichannels has not been elucidated. It is likely that integrins and cell or substrate adhesion molecules serve as molecular tethers that sense mechanical stress and transducer the effects on gap junctions and hemichannels. Integrins are reported to be mechanical sensors on the cell surface (179,180) and have been proposed to be the candidate mechanosensors in bone cells (181). Fluid flow stimulates pathways that are regulated by integrin binding to the extracellular matrix (182). Among various isotypes of integrins, α5 and β1 integrins are expressed in virtually all cell types in bone (183-185) and cartilage (186), and are found to induce responses to mechanical stimuli (181). Integrins, α2, α5 and β1 induce ERK activation in osteocytic cells (187). The potential association between integrin and connexins in bone cells, particularly, in osteocytes, may provide a novel, effective mechanism in the process of mechanotransduction mediated by gap junctions and hemichannels.

3. CONCLUSION AND FUTURE PERSPECTIVES

In summary, ample experimental evidences support the notion that gap junction and hemichannels provide important signaling pathways in all major bone cells and play crucial regulatory roles in the various phases of bone modeling and remodeling processes. These channels pass the signals generated by growth factors, hormones, mechanical stress and other physiological factors between cells and their extracellular environments, and convey the anabolic or catabolic effects of these factors on bone cells.

One of important future directions will involve in the elucidation of the specific role of hemichannels, distinct

from gap junctions, and their regulatory components *in vivo*. At present time, this research has been hampered by a lack of specific dominant negative connexin mutants that can specifically inhibit function of hemichannels, but not gap junctions or vice versa. With the identification of these mutants, the distinct roles of hemichannels as well as gap junctions in bone cells are likely to be revealed. Another line of possible future research will be on the investigation of the roles of gap junctions and hemichannels in osteocytes, the most abundant cell type in normal human bone. Little is known about the function of this cell type compared to any other bone cells. As a mechanosensor cell, it is not clear how osteocytes transduce signals generated by mechanical stress via gap junctions and hemichannels, subsequently convert mechanical stress to chemical signals, and convey these signals between osteocytes and the extracellular matrix, which in turn regulate the bone remodeling process. This avenue of research will increase our understanding of the mechanistic roles of osteocytes in sensing mechanical stress, and in bone biology.

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