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Cell-Cell Communication in the Osteoblast/Osteocyte Lineage

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Abstract

Skeletal development (bone modeling) and its maintenance in post-natal life in response to local and systemic stimuli (bone remodeling) require coordinated activity among osteoblasts (bone forming cells), osteocytes (cells embedded in bone) and osteoclasts (bone resorbing cells), in order to meet the needs of structural integrity, mechanical competence and maintenance of mineral homeostasis. One mechanism of cell-cell interaction is via direct cell-cell communication via gap junctions. These are transmembrane channels that allow continuity of cytoplasm between communicating cells. The biologic importance of connexin43 (Cx43), the most abundant gap junction protein in the skeleton is demonstrated by the skeletal malformations present in oculodentodigital dysplasia (ODDD), a disease linked to Cx43 gene (*GJA1*) mutations, and by the low bone mass and osteoblast dysfunction in *Gja1* ablated mice. The presence of Cx43 is required for osteoblast differentiation and function, and by forming either gap junctions or “hemichannels” Cx43 allows participation of cell networks to responses to extracellular stimuli, via propagation of specific signals converging upon connexin sensitive transcriptional units. Hence, Cx43 is involved in skeletal responsiveness to anabolic signals, as those provided by parathyroid hormone and physical load, the latter function probably involving osteocyte-osteoblast communication.

Keywords

connexin; bone; osteoblast; osteocyte

Introduction

The skeleton is a dynamic system, and after full development (bone modeling) it must be constantly remodeled to maintain structural integrity, mechanical competence as well as mineral homeostasis. Control of bone modeling and remodeling requires a tightly orchestrated interplay among osteoblasts (bone forming cells), osteocytes (cells embedded in bone) and osteoclasts (bone resorbing cells). One mechanism of cell-cell interaction is via direct cell-cell communication by gap junctions, transmembrane channels that allow continuity of cytoplasm between communicating cells [1;2]. This mode of cell-cell communication is of particular importance in the skeleton, where a large variety of systemic and locally generated signals (physical load in particular) must be detected, transduced into biologic signals and transmitted to cells at specific locations to permit bone resorption and formation to occur where necessary. A large body of work has been generated in the last 10-15 years on gap junctional

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communication and connexin biology in bone. This review examines the current status of knowledge as well as the emerging challenges and controversies in this area of research, in the backdrop of milestone findings that demonstrate the critical role of connexins in skeletal biology.

Connexins and Cell-Cell Communication

Connexins are integral membrane proteins composed of four transmembrane domains, two small extracellular loops, an intracellular loop and intracellular amino- and carboxyl-ends (see [1;2] for reviews). This superfamily includes more than 20 genes, which are well conserved among vertebrates [3]. Connexins are expressed in many cells, where they form gap junctions, transmembrane channels that allow aqueous continuity between two cells. Each gap junctional hemichannel, or connexon, is composed of a hexameric array of connexin monomers. Docking of two hemichannels on juxtaposed cells forms a transcellular channel called gap junction [4]. Gap junctions permit diffusion of ions, metabolites and small signaling molecules (e.g., cyclic nucleotides and inositol derivatives) from cell to cell, and depending upon the connexin (s) participating in the hexamer, the resulting gap junction channels will exhibit specific charge and size permeability. For example, Cx43 permits the diffusion of relatively large signal molecules <1.2 kDa molecular mass, with a preference for negatively charged molecules. In contrast, Cx45 forms a smaller pore, permitting diffusion of molecules <0.3kDa, with a preference for positively charged molecules. Importantly, Cx43 and Cx45 can assemble in either homomeric or heteromeric connexons, and in the resultant Cx43/Cx45 heteromeric channel, the biochemical properties of Cx45 dominate and chemical and electrical coupling among cells is similar to Cx45 homomeric channels [5-7]. Connexons can be active even without pairing with apposing connexons, thereby functioning as gap junction “hemichannels” [8;9]. This configuration provides an alternative mechanism for connexin function, by forming a membrane channel of large permeability. As it will be discussed below, hemichannels have been shown to regulate the release of ATP and PGE₂ in response to mechanical stimulation [10], and to be involved in the response to pharmacologic agents active on bone cells [11].

In the skeletal system, gap junctions are present in all bone cells, but they are particularly abundant among osteoblasts and osteocytes. They are also present at the tip of osteocyte dendritic processes and between this processes and osteoblasts (Fig. 1) [12-14]. Recent data have proven in ex-vivo calvaria organ cultures that gap junctions allow cell-cell communication among the osteocytic network, and that such communication is regulated by parathyroid hormone (PTH) and pH [15]. Connexins are involved in many aspects of bone cell function, including control of osteoblastic cell proliferation [16;17] differentiation [18-20] and survival [21].

Connexins in Skeletal Development

The most abundant connexin family member present in the skeleton is Cx43 [22-26]. Other connexins are also expressed in bone, specifically Cx45 and Cx46, though their functions in these cells remain unknown [22;23]. Cx40 is also present in developing limbs ribs and sternum [27], but its expression in the adult skeleton has never been documented. Earlier studies using antisense nucleotide strategies demonstrated severe limb malformations upon inhibition of Cx43 gene (*Gja1*) expression [28;29]. However, the biological importance of Cx43 in skeletal development has been established by work in human and mouse genetics. Initial observations in mice with germline null mutation of *Gja1* (*Gja1*^{-/-}) revealed hypomineralization of craniofacial bones and a severe delay in ossification of the axial and appendicular skeleton [20]. Skeletal elements of both neural crest and mesoderm origin are affected by the ossification defects, including the cranial vault, clavicles, ribs, vertebrae and limbs. These defects are attended by a reduced osteogenic differentiation and mineralization potential *Gja1*^{-/-}

osteoblasts [20]. Closely similar cellular abnormalities were also found in mice with an osteoblast specific deletion of *Gja1* [30], although these animals do not exhibit the craniofacial malformations nor the ossification defects of the germline null mutation, findings consistent with post-developmental *Gja1* gene deletion in this model obtained using a fragment of the $\alpha_1(I)$ collagen promoter [30;31].

The demonstration of a genetic linkage of the human disease oculodentodigital dysplasia (ODDD) to the *GJA1* locus offers the strongest evidence for a critical role of Cx43 in skeletal development. To date, at least 24 distinct point mutations of *GJA1* have been identified in subjects with this disease [32-34]. Although other organs are affected, ODDD patients exhibit unique skeletal abnormalities, including widened mandible alveolar ridge, dentition abnormalities, such as microdontia, anodontia, and enamel hypoplasia, but also cranial hyperostosis [35;36]. Other typical findings are syndactyly of hands and feet, broad tubular bones, and/or hypoplasia or aplasia of the middle phalanges [36]. Based upon the inheritance pattern (autosomal dominant), Cx43 mutants found in ODDD would be expected to have a dominant negative action on wild type Cx43. Cell biology studies have confirmed this premise. While most ODDD mutant proteins can assemble into gap junction plaques, they do not form functional channels [37]; and some of them inhibit Cx43-mediated gap junctional intercellular communication (GJIC) [38].

Additional insights on ODDD pathogenesis and on the role of Cx43 in skeletal biology can be garnered by two mouse models of ODDD that have been recently reported. A first one, identified from a random N-ethyl N-nitrosourea mutagenesis screen, carries a heterozygous G60S point mutation of *Gja1* (*Gja1*^{Jrt/+}). Even though the G60S mutation has never been found in humans, these animals exhibit a phenotype closely resembling that of ODDD, including syndactyly, enamel hypoplasia, craniofacial abnormalities and cardiac dysfunction [39]. A second model was recently reported based on conditional replacement of one wild type allele with a *Gja1*^{G138R} mutant allele [40], frequently found in ODDD patients [34;35]. These animals exhibit craniofacial abnormalities, decreased bone mass and cortical thinning, just as *Gja1*^{Jrt/+} mice do [39;40]. However, neither the *Gja1*^{Jrt/+} nor the *Gja1*^{G138R/+} mutants fully phenocopy human ODDD, the most notable difference being the absence of thickened bones of the cranial vault, frequently observed in ODDD patients [36]. Such discrepancies might be related to species differences or to the effect of aging. As noted later, some common structural features of long bones are beginning to emerge from the different Cx43 mutant mouse models, suggesting a similarity with the aging process. Nonetheless, the fact that skeletal abnormalities represent the major phenotypic feature of ODDD in humans and mice provides genetic proof that the skeleton is one of the major sites of action of Cx43.

Connexins in Adult Skeletal Homeostasis

The development of mouse models of conditional, osteoblast specific *Gja1* deletion has allowed to overcome the perinatal lethality of the germline null mutation [41]. As noted above, ablation of *Gja1* using the $\alpha_1(I)$ collagen promoter – active in differentiated osteoblasts – does not cause skeletal malformations, but results in reduced bone mass at skeletal maturity [30]. A similar degree of osteopenia is present in *Gja1*^{Jrt/+} mice [39]; and it has also been confirmed in the conditional *Gja1*^{G138R/+} mutant [40]. Despite these similarities, the cellular bases of the low bone mass in ODDD mouse models are still not totally clear. In contrast to *Gja1* deletion, which severely impairs osteoblast differentiation and mineralization potential [30], the presence of ODDD mutant does not lead to major functional abnormalities of committed osteoblastic cells [42], suggesting that the dominant negative effect of the mutant Cx43 may not be sufficient to disrupt full osteoblast differentiation. However, earlier steps of osteogenesis could be affected by a dominant negative Cx43 mutant that may also interfere with the function of other connexins. Preliminary results from a new mouse model in which *Gja1* is ablated in

cells that give rise to chondro-osteoprogenitors suggest that such an early deletion leads to much more severe skeletal defects than osteoblast-specific ablated mice [43]. These intriguing and somewhat unexpected findings are opening new directions of research on GJIC in the bone marrow microenvironment, and in particular on direct cell-cell interactions between skeletal (mesenchymal) and hematopoietic cell systems.

On the other hand, *Gjal* is deleted in osteocytes as well as osteoblasts when ablation is obtained using either the $\alpha_1(I)$ collagen or other more broadly expressed promoters [30]. Whereas GJIC and hemichannels are important for both osteocytes and osteoblasts, the contribution of the former cells to the phenotype of Cx43-deficient mice remains to be established. Osteocyte-specific gene deletion models are currently being developed, but preliminary data on *Gjal* ablation using the osteocalcin promoter, which is active at late stages of osteoblast differentiation, are somewhat contradictory. While one group has reported only modest, if any osteopenia [44], other investigators found an osteopenic phenotype quite similar to that seen in $\alpha_1(I)$ collagen-driven *Gjal* deletion [45]. Strain background differences may explain the discrepancies, although given the preliminary nature of these reports, such results should be taken with caution. Nonetheless, the accumulated data on mouse genetics clearly point to the idea that Cx43 is at least as important at earlier than it is at later stages of osteoblast differentiation. They also suggest that compensatory mechanisms, perhaps other connexins, may be operative in differentiated osteoblasts and osteocytes.

An important phenotypic pattern emerging from the in vivo studies just mentioned is the abnormal structure of long bones of *Gjal* deficient or mutant mice. Larger cross-sectional areas and cortical thinning have been consistently observed in both conditional *Gjal* null [45;46] and ODDD mutants [39], suggesting that these phenotypic features may represent a common denominator of osteoblast Cx43 deficiency or dysfunction. Such novel findings support the idea that Cx43 is critically involved in bone modeling and perhaps in modulating adaptive responses to external stimuli in adult life. In fact, larger but thinner long bones are reminiscent of the changes in bone structure that occur with aging. Such changes also imply increased endocortical bone resorption with unbalanced periosteal bone apposition, leading to the conclusion that Cx43 might be important for osteoclast function as well. Although such role of Cx43 was postulated by earlier in vitro studies [25;47], a clear osteoclast phenotype has not been reported in *Gjal* null or ODDD mouse models. Therefore, the potential role of Cx43 for osteoclast biology and its link to the structural abnormalities seen in Cx43 mutant animals require a more thorough reassessment.

Connexins in Skeletal Response to Pharmacologic and Physical Stimuli

An emerging body of data indicates that Cx43 is involved in conditioning bone cell responses to hormonal stimulation. One proof of his hypothesis is provided by the attenuated anabolic effect of parathyroid hormone (PTH) in *Gjal* deficient mice. Based upon earlier in vitro data demonstrating that interference with *Gjal* expression diminishes PTH stimulation of cAMP production [48] and matrix mineralization by osteoblasts [49], an in vivo study was conducted in conditional *Gjal* deleted mice. Treatment with daily doses of teriparatide® (PTH fragment 1-34) resulted in severely attenuated increments in bone mass and reduced activation of bone formation rates relative to wild type mice, the consequence of an inability of Cx43 deficient osteoblasts to mount a full response to the hormone [30]. These results have important ramifications for translational research, as PTH analogs are currently used as anabolic therapy for osteoporosis [50].

Recent studies show that Cx43 involvement in bone anabolic responses is not limited to PTH, but it extends to other anabolic stimuli, in particular mechanical load. As in the case of PTH, stimulation of mineral apposition rate at the endocortical surface by application of a 3-point

bending regime to tibiae in vivo was significantly reduced in conditionally *Gja1* deleted mice relative to wild type animals [46]. In addition, these mutant mice required approximately 40% more force to generate the same endocortical strain as did wild type mice [46], solidifying the concept that Cx43 plays an instrumental role in adaptive response to osteogenic stimuli of different nature.

Cx43 has also been proposed as part of a mechanism of skeletal response to pharmacologic inhibitors of bone resorption. Aside its potent inhibitory action on osteoclasts, the bisphosphonate, alendronate can prevent pharmacologically-induced apoptosis in osteoblasts and osteocyte-like cells, an effect that may require Cx43 [21]. Intriguingly, the anti-apoptotic action of bisphosphonates seems to be mediated not by effects on GJIC, but by alendronate-induced, src-ERK-dependent opening of Cx43 hemichannels [11]. The potential role of Cx43 in the mechanism of action of bisphosphonates can now be tested in vivo.

Mechanisms of Connexin Action in Bone

As discussed earlier, Cx43 deficiency alters osteoblast differentiation and expression of osteoblast genes, in particular $\alpha_1(I)$ collagen and osteocalcin [20;30]. To garner insights on the molecular mechanisms of Cx43 regulation of osteoblast function, a series of studies were conducted by taking advantage of the dominant function of Cx45 on Cx43 in determining permeability of heteromeric Cx43/Cx45 channels [5;6;51]. Thus, expression of chick *Gjc1* (Cx45 gene) in osteoblastic cells, which express abundant Cx43, down-regulates transcription of $\alpha_1(I)$ collagen and osteocalcin [19]; whereas over-expression of *Gja1* in cells with low abundance of Cx43 up-regulates osteoblast gene transcription [19;52]. These observations have been reproduced in other osteoblast-like cell lines [53;54]. Further studies using the same approach to modulate Cx43 channel function led to the identification of a specific DNA promoter element that confers connexin sensitivity to the osteocalcin and $\alpha_1(I)$ collagen promoters, via binding of Sp1/Sp3 transcription factors [55]. Thus, Cx43 via either gap junctions or hemichannels modulates osteoblast gene expression by interfering with specific signaling mechanisms that may be activated by extracellular stimuli. Accordingly, *Gja1* deficiency or Cx45-mediated interference with Cx43 function alters ERK signaling, and this in turn modulates gene transcription from osteoblast gene promoters via decrease of ERK-dependent phosphorylation of Sp1 with preferential recruitment of Sp3 to connexin response elements [56]. Therefore, by modulation of connexin-sensitive signaling pathways, different modes of gap junctional communication may allow (or restrict) propagation of signals generated in one cell to other cells by permitting (or restricting) diffusion of second messengers, such as cAMP, IP₃ or cADP ribose, which can permeate gap junction channels formed by Cx43 but not those formed by Cx45 (Fig. 2). Hence, depending upon the connexin expressed within a cell network, signal propagation may allow a larger number of cells than those expressing the receptor to contribute to the response. Alternatively, signaling may be restricted to a smaller number of cells to generate a localized response [56;57].

Transduction of mechanical signals represents another aspect of bone cell regulation where connexins play a critical role. Deformation of the bone matrix by physical load generates an array of biophysical signals that affects bone cell activity and differentiation, thereby contributing to the anabolic effect of mechanical load. Fluid flow is thought to represent the primary biophysical signal involved in mechanotransduction, and several biologic effects have been described after application of fluid flow to osteoblasts of osteocytes [58]. Interestingly, work on fluid flow has disclosed a prominent role of gap junction hemichannels in bone cells. Cx43 hemichannels are active in osteocytic cells, where they mediate fluid flow-induced PGE₂ [10] and ATP [59] release, although dependence of fluid flow stimulated release of ATP from osteoblastic cells has not been universally linked to Cx43 hemichannels [60]. One of the major challenges in this area is represented by the inherent difficulties in rigorously

distinguishing hemichannel-mediated diffusion from exocytosis or other related events. Obviously, this area will require further investigation.

Considering their unique location within mineralized bone, osteocytes are the best candidates for detecting and coordinating responsiveness to mechanical signals. Since osteocyte dendritic processes are in contact with osteoblasts on the bone surface and with adjacent osteocytes via adherens junctions and gap junctions [13;14], it is commonly believed that osteocyte communicate mechanical signals to osteoblasts and perhaps other skeletal cells to affect their function. Indeed, in vitro studies demonstrated that GJIC contributes to mechanically induced Ca^{2+} wave propagation from osteocytic to osteoblastic cells [61]. However, surprisingly few studies have examined the physiological consequence of mechanical signals detected by osteocytes and communicated to osteoblasts. This hypothesis was recently put to test in a novel in vitro system whereby osteocytic cells could communicate via gap junctions with osteoblastic cells, but only one cell type could be subjected to fluid flow. Intriguingly, only when osteocytes were exposed to fluid flow did alkaline phosphatase activity increase in osteoblasts; when fluid flow was directly applied to the osteoblastic cells no such effect was observed [62]. Furthermore, the ability of osteocytic cells to communicate the mechanical signal to osteoblastic cells was sensitive to pharmacologic inhibition of GJIC, or to removal of physical contact between osteocytic and osteoblastic cell [62]. These in vitro data provide the first evidence to the notion that direct communication via gap junctions (or hemichannels) between osteocytes and osteoblasts is critical for osteoblast response to physical stimuli. The availability of new, conditional or inducible gene recombination approaches should allow testing of this hypothesis in vivo.

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Fig. 1.

Gap junctions and hemichannels in the osteoblast-osteocyte network. Gap junction transcellular channels, formed by the apposition of two connexons (hexameric arrays of 6 connexin subunits), are abundantly present between osteoblasts on the bone surface, as well as at the interface between osteocytic processes and between these processes and osteoblasts on the surface. Connexons also exist as membrane channels, without docking to an apposing connexin, thus representing gap junction “hemichannels”. Both gap junctions and hemichannels contribute to connexin role in bone biology.

Fig. 2.

Schematic model integrating the main putative mechanisms of connexin function in bone cells. By allowing cell-to-cell diffusion of second messengers or small metabolites, gap junctions may allow propagation of signals generated by ligand binding to receptors (for example PTH) in a responsive cell to less responsive cells, thus recruiting a larger number of cells to the hormonal response. Connexins, via yet unknown mechanisms, modulate specific cellular signaling systems, in particular the MAPK/ERK and PLC pathways, so that when Cx43 is abundant and cell are well coupled, ERK phosphorylates transcription factor Sp1, which then binds to specific connexin response elements (CxRE) present in osteoblast promoters, for example the $\alpha_1(I)$ collagen and osteocalcin, resulting in activation of gene transcription. In the absence of MAPK/ERK signaling, Sp3 binding to CxRE prevails leading to gene repression. ERK signaling can also be activated by hemichannel opening by bisphosphonates, potent bone resorption inhibitors, leading to inhibition to apoptosis. Finally, both gap junctions and hemichannels can be activated by mechanical stimulation. In the latter case, ATP and/or PGE₂ may be released and feedback on bone cells as autocrine factors, thus amplifying the response.