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Biochimica et Biophysica Acta 1719 (2005) 69 – 81
BIOCHIMICA ET BIOPHYSICA ACTA
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Review

Gap junctions in skeletal development and function

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Received 1 September 2005; received in revised form 26 October 2005; accepted 28 October 2005
Available online 14 November 2005

Abstract

Gap junctions play a critical role in the coordinated function and activity of nearly all of the skeletal cells. This is not surprising, given the elaborate orchestration of skeletal patterning, bone modeling and subsequent remodeling, as well as the mechanical stresses, strains and adaptive responses that the skeleton must accommodate. Much remains to be learned regarding the role of gap junctions and hemichannels in these processes. A common theme is that without connexins none of the cells of bone function properly. Thus, connexins play an important role in skeletal form and function.

Keywords: Connexin; Bone; Cartilage; Osteoblast; Chondrocyte

Contents

1. Introduction

Bone is a dynamic tissue that is constantly modeled and remodeled in response to a large number of stimuli, including calcitropic hormones, growth factors and mechanical load. The precise and coordinated control of bone remodeling requires a tightly orchestrated interplay among, osteoblasts [the bone forming cells], osteocytes [the bone embedded cells that are the putative mechanosensory cells of bone] and osteoclasts [the bone macrophage-like bone resorbing cells]. Gap junctional communication has been hypothesized to play a critical role in the coordination of bone remodeling. Osteoblasts and osteocytes have been shown to express three major gap junction proteins, connexin43 (Cx43), connexin45 (Cx45) and connexin46 (Cx46); and form functional gap junctions. Chondrocytes, the cells that form cartilage, have also been shown to express Cx43; as do the bone resorbing osteoclasts. In this review, we will summarize recent findings which have elucidated some of the roles of gap junctions in bone development and maintenance.

2. Connexins, gap junctions and hemichannels

Gap junctions are aqueous conduits that are formed by the docking of two hemichannels on juxtaposed cells. The gap junctional hemichannel, or connexon, is composed of a

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doi:10.1016/j.bbamem.2005.10.012

hexameric array of connexin monomers. Each connexin monomer is a single polypeptide, composed of four transmembrane domains, two small extracellular loops, an intracellular loop and intracellular amino- and carboxyl-ends (Fig. 1). More than 17 connexin genes have been identified in the mouse genome and 20 in the human genome [1]. Additionally, a family of gap junction-like proteins, termed pannexins, have been identified in the brain [2]. Orthologs of mammalian connexins, termed innexins, have even been identified and characterized in insect cells [3]. Connexins are almost ubiquitously expressed in cells, implicating that they serve a critical role in the function of multicellular organisms. But what is the precise function of gap junctions? Why are there so many connexin genes?

Gap junctions permit diffusion of ions, metabolites and small signaling molecules (e.g., cyclic nucleotides and inositol derivatives). Depending upon the expressed connexin genes, the resultant 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. Inositol derivatives [4–9] and cADP-ribose [5,10] are capable of diffusion through gap junctions and can elicit a Ca^{2+} response in coupled cells. In fact, Cx43-transfected HeLa cells microinjected with inositol 1,4,5-triphosphate (IP_3) have been shown to propagate Ca^{2+} waves to neighboring cells 2.5-fold more efficiently than untransfected cells [4]. In contrast, Cx45 forms a smaller pore, permitting diffusion of molecules <0.3 kDa, with a preference for positively charged molecules. Further, connexins can assemble as a homomeric or heteromeric hemichannel, and the connexin isotypes that forms the gap junction hemichannels dictate the molecular size and permeability of the resulting gap junction channel [11–15]. Cx43 and Cx45 are two such connexins that can assemble into a single hemichannel composed of both monomeric units. In the resultant Cx43/Cx45 heteromeric channel, the biochemical properties of Cx45 dominate and chemical and electrical coupling among cells is markedly reduced [16–18]. In addition, some connexon (hemichannel) pairs can form heterotypic interactions dependent upon the compatibility of the extracellular loops of the opposing hemichannels (e.g., one cell expressing monomeric Cx43 hemichannels may dock with an adjacent cell expressing monomeric Cx45 hemichannels). These properties provide the gap junction great plasticity in dictating the size permeability and selectivity of the resultant communicative channel, restricting or allowing signaling only to coupled cells. Further, gap junction channels are regulated in a similar fashion as other membrane channel, with open/closed states sensitive to transmembrane voltage and post-translational modification of the connexin subunits. Activation of extracellular signal regulated kinase (ERK), src and protein kinase C have been shown to dynamically regulate Cx43 channel open/closed state by phosphorylation of the C-terminal tail of the connexin monomers [19–21].

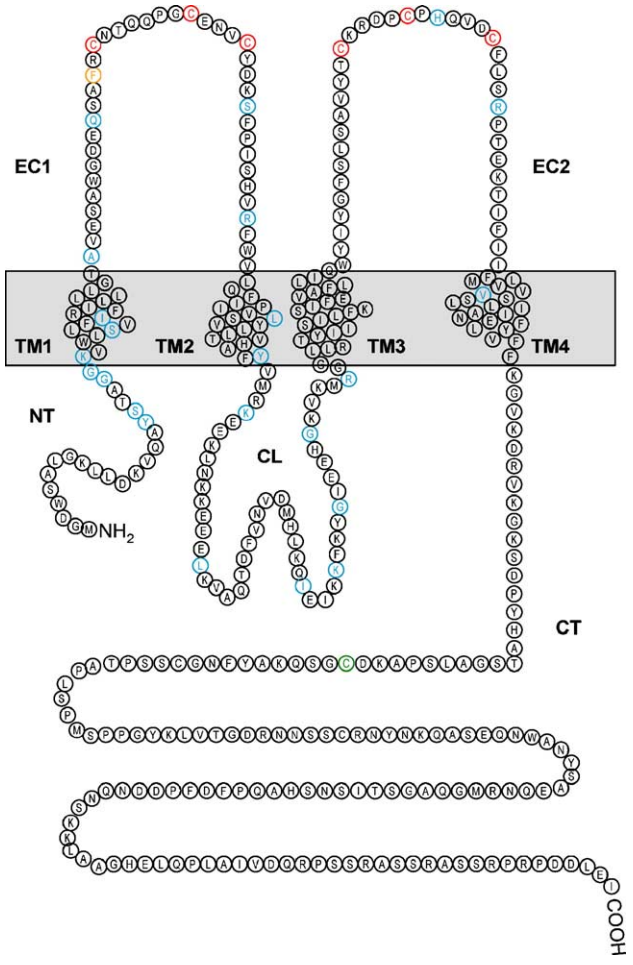

Fig. 1. Structure of the human Cx43 monomer. Connexin43 is a transmembrane protein composed of an amino- (NT) and carboxyl- (CT) terminal cytoplasmic tail, a large cytoplasmic loop (CL), two extracellular loops (EC1–2) and 4 transmembrane domains (TM1–4). The three cysteine residues in each extracellular loop (indicated in red) are required for docking of two hemichannels or connexons to form a gap junction channel. The residues indicated in blue are mutated in the autosomal dominant disorder ODDD. The residue indicated in green indicates a frame shift mutation that produces ODDD as well as an additional skin phenotype. The residue indicated in orange indicates an amino acid duplication that leads to ODDD [44–47,135].

Accumulating evidence from many model systems consistently suggests that the unique profile of connexins expressed by a particular cell type can dictate the types of signals, second messengers, and metabolites that are propagated among cells. In this way, the cells can form a "functional syncytium" within which the cells communicate, with the advantage that the type of signals that can be diffused can be regulated. Thus, not all cells in the network share every signal; while some signals that diffuse through the gap junctions are rapidly distributed, propagation of others may be limited to serve specific functions.

Connexins have also been shown to serve as docking platforms for signaling complexes. Cx43 has been found to co-localize and co-immunoprecipitate with β-catenin [22,23], c-src [24–26], protein kinase C (PKC) α and ε [27,28]. Co-localization has been reported between Cx43 and p38 MAPK [29]; and Cx43 is a target for extracellular signal regulated kinases (ERK), PKC, phosphatidylinositol-3-kinase (PI3K) and src phosphorylation, indicating at least a transient physical association [20,30,31]. Accumulated data support a model in

which connexins are capable of recruiting a unique profile of signal proteins to the resultant channel that is specific for the biologically relevant signal molecule transmitted among cells. Thus, it is not only the permeability of the gap junction, but also the repertoire of signal molecules at the gap junctional plaque that determines the functional consequence of signals passed among coupled cells.

Much recent work has also elucidated the function of Cx43 hemichannel activity [32,33]. Unlike the classic paradigm of the formation of a gap junctional channel between two cells, unpaired hemichannels can open to the extracellular milieu, thus providing an alternative mechanism for connexin function. Hemichannels have been shown to regulate the release of NAD^+ and ATP [32,33], and recently, prostaglandin E2 (PGE_2) [34]. The role of hemichannels in bone cell function will be discussed below.

3. Gap junctions and skeletal development

Gap junctions are involved in many phases of embryonic development and patterning, including heart morphogenesis, left–right asymmetry and limb patterning [35–37]. For the purposes of this review, we will discuss the role of gap junctions in skeletal and limb development.

Bone is formed by two mechanisms, endochondral ossification and intramembranous ossification. The majority of the bones in the body are formed by endochondral ossification, in which a cartilaginous anlage is formed by chondrocytes. Subsequently the anlage becomes vascularized, then infiltrated and mineralized by osteoblastic cells. Conversely, during intramembranous ossification, osteoblastic cells condense and mineralize in the absence of a cartilaginous scaffold. Osteoblastic mineralization occurs through the synthesis and deposition of a complex extracellular matrix, which includes type I collagen, and other non-collagenous proteins, such as osteopontin, bone sialoprotein, and osteocalcin, among others. During the deposition of extracellular matrix, some osteoblasts become embedded in the non-mineralized matrix (osteoid) and eventually become encased within the mineralized bone. These cells, osteocytes, form long cellular processes, which run through canaliculi within the bone tissue, thus coming in contact among themselves and with cells on the bone surface. At the interface between cytoplasmic processes and at contact sites at the bone surface, gap junctions form, thus permitting efficient signal exchanges among distally located cells, embedded in the ossified matrix. Importantly, osteoblasts on the bone surface are also abundantly connected via gap junctions. Indeed, all of the cells of the skeleton express gap junction proteins, including chondrocytes (Cx43), osteoblasts (Cx43, Cx45, and Cx46), osteocytes (Cx43) and even osteoclasts (Cx43) (Fig. 2).

In the developing limb bud, signals from the apical ectodermal ridge, including fibroblast growth factor 4, affect Cx43 expression in the underlying mesenchymal limb bud [38,39]. In fact, in chick limb buds gap junctional communication exhibits a gradient, being most intense in the zone of polarizing activity and almost absent in the opposing side of the limb bud [40,41]. In the developing mouse limb bud, Cx43 gene expression was similarly detected in the zone of polarizing activity, and upon limb outgrowth, Cx43 was detected in the presumptive progress zone. Cx43 message was observed in the condensing limb mesenchyme, but in the more proximal regions where differentiation has been initiated Cx43 expression was more restricted to the presumptive perichondrium [38]. These data suggest that Cx43 expression is regulated by a morphogen gradient that may contribute to limb patterning. Demonstrating the importance of Cx43 for limb development, antisense oligonucleotides inhibition of Cx43 expression in the chick embryo resulted in limb malformations, including truncation of the limb bud, fragmentation into two or more domains, or complete splitting of the limb bud into two or three branches [42,43].

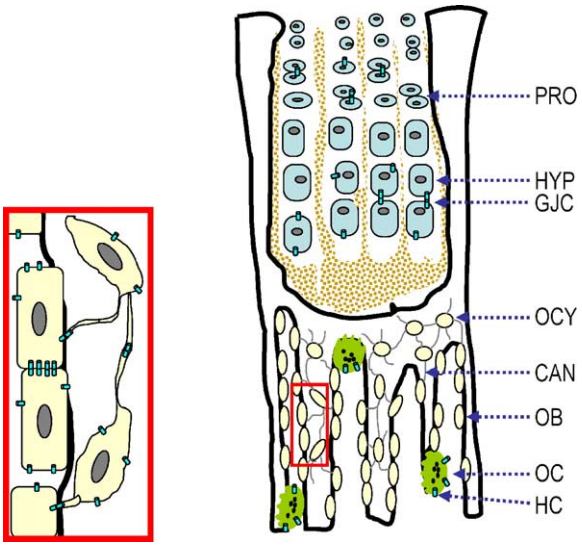

Fig. 2. Gap Junctions in the Skeleton. Chondrocytes of the growth plate form a columnar structure of progressive differentiation from resting chondrocytes > proliferative chondrocytes (PRO) > pre-hypertrophic chondrocytes > hypertrophic chondrocytes (HYP) > terminal differentiated chondrocytes followed by the osteo-chondral border. These cells are known to express Cx43, however whether they function as gap junction channels (GJC) or hemichannels (HC) has yet to be determined. Bone is covered by surface osteoblasts (OB) and osteoclasts (OC). Embedded in the bony matrix is the osteocytes (OCY). Osteocytes form long caniliculae, which interconnect the osteocytes and surface osteoblasts via gap junctions. The interaction among osteoblasts and osteocytes is magnified in the red box to the right to highlight the presence of gap junctions at the intersection of caniliculae, and among adjacent osteoblasts. Note the presence of connexin hemichannels on the surface of osteoblasts and osteocytes, which have been shown to play an important gap junction-independent role in bone. Osteocytes are pictured with putative connexin hemichannels, though the precise distribution and function of these connexins is not clear at this time.

Interestingly, skeletal malformations have been documented in humans with an autosomal dominant disorder termed oculodentodigital dysplasia, or ODDD. Human genetic studies have reported at least 24 separate mutations in the Cx43 gene in association with oculodentodigital dysplasia [44–46] (Fig. 1). The limb phenotype of the affected patients includes syndactyly of the hands and foot, hypoplasia or aplasia of the middle phalanges, and abnormalities in craniofacial elements [44–46]. Affected patients frequently have cranial hyperosto-

sis, a widened alveolar ridge of the mandible, and broad tubular bones. There are also dentition abnormalities including microdontia, adontia, and enamel hypoplasia [44–46]. The Cx43 point mutations found in ODDD patients span much of the Cx43 gene, including the N-terminal tail, the transmembrane domains, and the cytoplasmic loop. Recently, a frameshift mutation in the C-terminal tail of the Cx43 gene has been associated with ODDD as well as an additional skin phenotype (Fig. 1) [47]. The phenotype of these patients suggests that the Cx43 mutants might be hypomorphs of the wild type gene. Indeed, a recent report functionally characterized eight of the Cx43 mutations found in patients with ODDD by overexpressing them in HeLa cells [48]. Two of the mutants, F52dup and R202H, failed to generate gap junctional plaques with the majority of protein found in the intracellular space, mostly associated with the endoplasmic reticulum. However, when co-expressed with the wild type Cx43, the plaque forming defect was rescued, but gap junctional communication was impaired. The other six mutants generated in this study (K134E, I130T, L90V, Y17S, G21R and A40V) all formed gap junctional plaques, yet had markedly reduced or no electrical coupling. Similarly, two additional ODDD Cx43 mutants (G21R and G138R) were screened by Roscoe et al. [49], and found to act in a dominant negative fashion with respect to wild type Cx43. More recently, a new mouse model originated from a mutagenesis screen was identified with a phenotype closely resembling that of ODDD, including syndactyly, enamel hypoplasia, craniofacial abnormalities and cardiac dysfunction [50]. These mice ($Gja1^{Jrt/+}$) carry a new point mutation in the Cx43 gene causing a G60S substitution, and the resulting mutant protein has dominant negative properties, just like most of the other ODDD mutants. Interestingly, these mice also have severely decreased bone mass and mechanical strength, and exhibited bone marrow abnormalities indicative of defects in hematopoietic stem cells, feature not present or not reported in patients with ODDD.

The developmental defects observed in human ODDD, and partly reproduced by this new mouse model are remindful of skeletal abnormalities observed in a model of Cx43 "knockdown" obtained using antisense oligonucleotides in chicken [42,51]. When the antisense Cx43 oligonucleotides were applied to the developing chick face primordia, significant facial defects were observed, including aberrant maxillary and mandibular primordium development and nasal pit defects. Intriguingly, "knockdown" of Cx43 in the chick face, resulted in downregulation of the homeobox transcription factor Msx1, particularly in the affected areas of the developing chick primordia [51]. Msx1 and Msx2 serve critical roles in skeletal development and patterning [52,53]. Double knockouts of Msx1 and Msx2 lack anterior skeletal elements in the developing limb, and single gene deletion results in craniofacial developmental abnormalities, which include cleft palate (Msx1) and craniosynostosis (Msx2) [53,54].

A role for Cx43 in osteogenesis emerges also from other animal models. Recent data demonstrate that development of the *short fin* (*sof*) phenotype in zebrafish is caused by a mutation in the Cx43 gene [55]. The first identified allele, sof^{b126}, expressed markedly reduced amounts of Cx43, while three ENU induced alleles causing *sof* encoded missense mutations of Cx43 (F30V0, F209I, and P191S). The developing zebrafish fin is composed of bony segments, formed by intramembranous ossifications that determine the length of the fin skeleton [56]. Zebrafish homozygous for the mutant *sof* alleles have tail segments that are approximately 1/3 the length of wild type bony segments. Though the molecular details are still unclear, there is an apparent defect in cell proliferation and possibly osteogenic differentiation caused by reduced expression or hypomorphic Cx43 function [55].

Given all of the previous data regarding the role of Cx43 in skeletal growth and development, it is quite surprising that genetic ablation of Cx43 in mice did not overtly affect patterning of the skeleton. Further, only a few of the defects observed in ODDD were recapitulated in Cx43 null mice. These mice die perinatally due to a severe defect in the heart, leading to swelling and blockage of the right ventricle outflow tract [57,58]. Despite no overt impact on skeletal morphogenesis, the Cx43 null mice exhibit profound mineralization defects in the shape and mineralization of skeletal elements derived from both intramembranous and endochondral ossification (Fig. 3). While most skeletal elements originate from the mesoderm; many of the skeletal elements in the head are derived from migratory neural crest cells [59]. Previous studies had revealed a major role for Cx43 in the migration of neural crest cells that likely account for the cardiac defect observed in Cx43 null mice [60]. Not surprisingly, many of the bony elements of the skull derived from the neural crest are affected by loss of Cx43, including a lack of ossification centers in the bones of the cranial vault and delayed ossification of the premaxilla, maxilla and mandibula at E16.5 [61]. Furthermore, the developmental delay in the parietal and frontal bones resulted in a smaller calvarium, a flattened skull and open

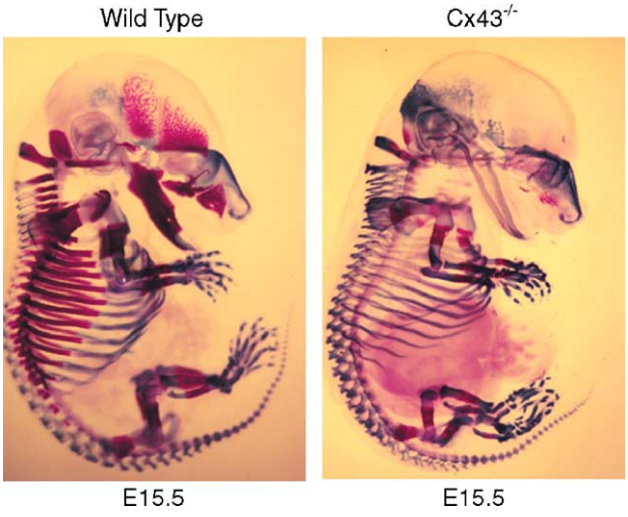

Fig. 3. Delayed Ossification of the Cx43 null mice. Alcian Blue/Alizarin red staining of E15.5 mouse embryos shows that Cx43 deficient mice have severe delay in ossification of many of the craniofacial elements, including the cranial vault and bones of the jaw, as well as the axial and appendicular skeleton. Blue staining indicates cartilaginous scaffold. Red staining is indicative of mineralized tissue.

parietal foramen. This is likely caused by defective migration of the neural crest cells to these skeletal elements. Yet, the hypoplastic and hypomineralized cranial phenotype of Cx43 null and *Gja1*$^{Jrt/+}$ mice is in striking contrast to human subjects with ODDD whose cranial vault is thickened and hyperminer-alized [46]. Clearly, other factors are at play in the development of the adult human phenotype that modify the effect of the mutant Cx43 in ODDD.

In addition to the defects observed in the neural crest derived skeleton, there is also considerable delay in the ossification of the mesoderm derived skeleton in the Cx43 null mice. Affected skeletal elements in mesoderm derived skull elements included basiocciptal and exoccipital bones, which were hypomineralized even at birth. The axial skeleton also was affected by loss of Cx43. The ribs of Cx43 null mice are dysmorphic and hypomineralized at E15.5. Similarly, the axial skeleton was hypomineralized at E15.5 (Fig. 3). At birth, however, many of the skeletal elements appeared ossified, indicating approximately a 2-day delay in skeletal ossification.

Given the data demonstrating a role in bone growth and patterning, it was quite surprising that ablation of Cx43 would not yield a more severe skeletal phenotype, with respect to size and morphology. Also as noted, surprisingly, the Cx43 null mouse is not a phenocopy of human ODDD. One possible explanation is compensation by other connexins, and the best candidate may be Cx45, also expressed by bone cells. Recent findings on another genetic mutation may offer an additional, potential compensatory mechanism for lack of Cx43 attributed to this lack of a massive morphological defect. A recent paper by Pizard et al. [62], using a mouse model of Holt–Oram syndrome, a human dominants disorder characterized by limb malformations and heart disease caused by haploinsufficiency of T-box transcription factor, Tbx5 [63], found that Tbx5 regulates expression of connexin40 (Cx40) [62]. Indeed, many of the skeletal abnormalities present in Tbx5$^{+/-}$ mice are shared by Cx40$^{+/-}$ and Cx40$^{-/-}$ mice. In situ hybridization revealed co-expression of Tbx5 and Cx40 in the developing forelimbs and carpal bones as well as the sternal perichondri-um. These sites of co-expression are consistent with the phenotype of Holt–Oram syndrome, which includes dys-morphic or amorphic thumbs, shortened arms, misshapen and fused bones of the wrist and an abnormal sternum morphology [64]. Mice with only a single allele of Tbx5 exhibited reduced expression of Cx40 at these sites, as well as the heart. The occurrence of skeletal fusions in the wrist bones was identical in Cx40$^{+/-}$ and Cx40$^{-/-}$ mice though the frequency of fusion was markedly increased in Cx40$^{-/-}$ animals. Similar observa-tions of skeletal dysmorphogenesis were observed in the phalanges, carpal bones and sternum of both Tbx5$^{+/-}$ and Cx40 mutant mice, implicating Cx40 as the downstream target of Tbx5 that contributes to limb patterning in the upper limbs and sternum. Interestingly, additional skeletal defects were observed in the Cx40$^{-/-}$ mice in tissues where Cx40, but not Tbx5, is expressed (e.g., the ribs and hindlimbs), suggesting that Cx40 plays a large scale role in skeletal patterning. Compound Tbx5$^{+/-}$ and Cx40$^{-/-}$ mutants were unable to generate live pups. Accordingly, the analyses of Tbx5$^{+/-}$; Cx40$^{+/-}$ mice revealed that the mice die shortly after birth, and demonstrate nearly identical features of the individual mutants or in some cases a mild exacerbation of the phenotype. These data indicate that Cx40 plays a critical role in the formation of endochondral derived elements of the axial and appendicular skeleton.

In summary, data accumulated thus far clearly demonstrate that connexins play a critical role in endochondral (Cx43 and Cx40) and intramembranous (Cx43) skeletal development. The role of Cx45 in ossification has yet to be studied as these mice die very early during embryogenesis (~E10) before ossification has begun and will thus require conditional deletion in the developing skeleton [65]. However, the functional redundancy and distinct control of varying skeletal elements by gap junctions certainly indicates a role for gap junctions in skeletal develop-ment, patterning, growth and, as will be discussed below, cellular function. Indeed, the striking phenotypic differences between humans with ODDD, chickens with Cx43 knockdown, Cx43 null mice or zebrafish with Cx43 tailfin growth mutation may indicate that the degree of redundancy and the connexin isoforms that provide overlapping functions may vary in different species, thus, explaining the different phenotypic expression of Cx43 deficiency so far observed in different models. If the field is to progress in the understanding of the role of gap junctions in skeletal growth and patterning, these differences must be systematically addressed.

4. Gap junctions and skeletal cells

We will now review the current literature on the role of gap junctions and connexin hemichannels on the function of skeletal cells: the chondrocytes, the osteoblast, the osteocyte, the osteoclast and the “other cells” of bone.

4.1. The chondrocytes

Chondrocytes are the cells of bone that form the initial cartilaginous scaffold upon which bone will eventually form. These cells build two critical skeletal structures: the growth plate and the articular cartilage. The growth plate is comprised of chondrocytic cells arranged in columnar fashion in stages of progressive differentiation. The most distal portion of the growth plate with respect to the metaphysis contains the resting chondrocytes, a precursor cell that will enter the next stage of differentiation, the proliferative chondrocyte. As the cells divide and differentiate, they extend the length of the bone. Moving towards the proximal side of the growth plate, the cells undergo hypertrophy, eventually forming a mineralized carti-lage that is eventually replaced by bone (Fig. 2). Conversely, the articular cartilage is essentially a biomechanical shock absorber at the epiphysis of long bones that is produced by articular chondrocytes. These cells produce massive amounts of collagens, proteoglycans and absorbed water, cushioning the load placed upon the bones.

Chondrocytes express Cx43 by immunohistochemistry in vivo in mice and rats [66]. In this study, Cx43 was detected in both the outer layer of the knee joint articular chondrocytes and

in growth plate chondrocytes. However, the functionality of Cx43 in growth plate chondrocytes has not yet been tested, and it is unclear whether gap junctional communication is established between the columnar cells of the growth plate or whether Cx43 is functioning as a hemichannel to release paracrine signals, such as ATP or NAD^+. In contrast, in vitro assays have revealed that articular chondrocytes form functional gap junctions in culture by dye transfer experiments [66–68]. Recently, functional Cx43 gap junctions in the superficial zone of articular chondrocytes have been documented in vivo [69]. The role of Cx43 in chondrocytes has not been extensively studied in vivo, although it is fair to say that Cx43 is not absolutely required in the growth plate for bone growth during embryogenesis, as the long bones of Cx43 null mice are indistinguishable in size from their wild-type littermates [61]. In vitro micromass cultures of chondrocytes from the chick limb bud have demonstrated that gap junctions are required for the differentiation of chondrocytes, as inhibition of gap junctional communication with 18α-glycyrrhetinic acid reduces production of proteoglycans and type II collagen. Furthermore, inhibition of gap junctional communication was demonstrated to be important for the full chondro-anabolic affects of BMP2 on these micromass cultures [70]. A likely explanation for the discrepancy between Cx43 null mice and in vitro studies of inhibition of gap junctional communication may be compensation by other connexins. The effects of 18α-glycyrrhetinic acid on gap junctional communication are not limited to inhibition of only Cx43. As discussed above, the high levels of expression of Cx40 in the perichondrium (and particular abundance in the periarticular perichondrium) coupled to the defects in bone length in the Cx40 mutant mice suggests that other connexins certainly contribute to chondrocyte function and long bone growth [62]. Again these are open questions that, with the sequencing of the mouse genome and identification of all the putative members of the connexin gene family, are ripe to be addressed.

In vitro studies have elaborated on the role of connexins in articular cartilage. It has been shown that gap junctions propagate intercellular Ca^{2+} waves when articular chondrocytes are stimulated by perturbation of a single cell with a micropipette [67,71,72]. Interestingly, the generated Ca^{2+} wave propagation to adjacent cells was attenuated when cells were treated with an inhibitor of gap junctions. Data have suggested that articular chondrocytes accumulate intracellular inositol 1,4,5-trisphosphate (IP_3) following mechanical stimulation and that IP_3 diffuses through gap junctions into adjacent cells to amplify the response [67]. Furthermore, it has been shown that gap junctions form between the articular chondrocytes and synovial fibroblasts in co-culture and that these cells elicit a coordinated Ca^{2+} response to mechanical perturbation [71]. Whether gap junctions form between articular chondrocytes and synovial fibroblasts in vivo remains unclear.

The gap junctional communication between and/or among articular chondrocytes and synovial fibroblasts may have some interesting extrapolations into the field of arthritis. Cx43 has been implicated in the etiology of osteoarthritis. Osteoarthritis is mediated by a dynamic interplay between the articular chondrocytes and the synovial cells. Articular chondrocytes are the single cell type present in articular cartilage. These cells are responsible for producing and maintaining the articular cartilage extracellular matrix. Osteoarthritis changes are accompanied by extracellular matrix degradation and production of a non-functional extracellular matrix, which eventually results in cartilage destruction [73,74]. Synovial fibroblasts are mesenchymal-derived cells, which along with synovial macrophages, form a thin lining of synovial tissue surrounding the fibrous capsule of the joint. The physiologic role of synovial tissue is to produce a synovial fluid that lubricates the joints and supplies nutrients to the articular chondrocytes. However, the pathological changes that occur in the synovium during osteoarthritis shift the balance from an anabolic to a catabolic role, leading to slow progressive destruction of articular cartilage. Despite the fact that osteoarthritis is considered a non-inflammatory form of arthritis, there are changes within the joint that are associated with chronic low-grade inflammation. It has been speculated that this inflammation is a result of the release of cartilage breakdown products into the synovium [75]. These products induce the production of the inflammatory cytokine, interleukin 1β (IL-1β), by the synovial fibroblasts. Indeed, IL-1 is considered one of the most prevalent catabolic factors in osteoarthritic joints, and it has been suggested to be driving cartilage destruction during OA [76,77]. Three important findings suggest that Cx43 plays a role in both articular chondrocytes and synovial fibroblasts during the onset and progression of osteoarthritis: (1) IL-1β upregulates Cx43 expression in cultured chondrocytes [78,79]. (2) In osteoarthritis, there is a pathologic increase of the expression of the gap junction protein, Cx43 (Cx43), in both synovial fibroblasts and articular chondrocytes caused by IL-1β [78,80,81]. (3) Transmission electron microscopic analysis of synovial tissues from healthy and osteoarthritis affected human patients demonstrated an increase in the size and number of Cx43 gap junctional plaques in osteoarthritic synovium relative to non-osteoarthritic synovium [80]. Critically important, in vitro experiments revealed that blocking gap junctional communication with the inhibitors, 18α-glycyrrhetinic acid or octanol, attenuates the production of matrix metalloproteinases, which degrade the cartilaginous extracellular matrix, by synovial fibroblasts following stimulation with IL-1β [80,82]. Thus, it seems apparent that gap junctional communication may amplify catabolic signals among synovial fibroblasts affecting articular cartilage (Fig. 4). As mentioned previously, gap junction-dependent intercellular calcium signaling occurs between articular chondrocytes and synovial cells in co-culture, and these gap junction-dependent calcium signals are amplified by two factors, IL-1β treatment and mechanical perturbation; two major influences on the onset and progression of OA [71,78,79]. Changes in cytosolic Ca^{2+} modulate the articular chondrocyte phenotype, pushing them towards hypertrophy and terminal differentiation, a fate not normally ascribed to articular chondrocytes.

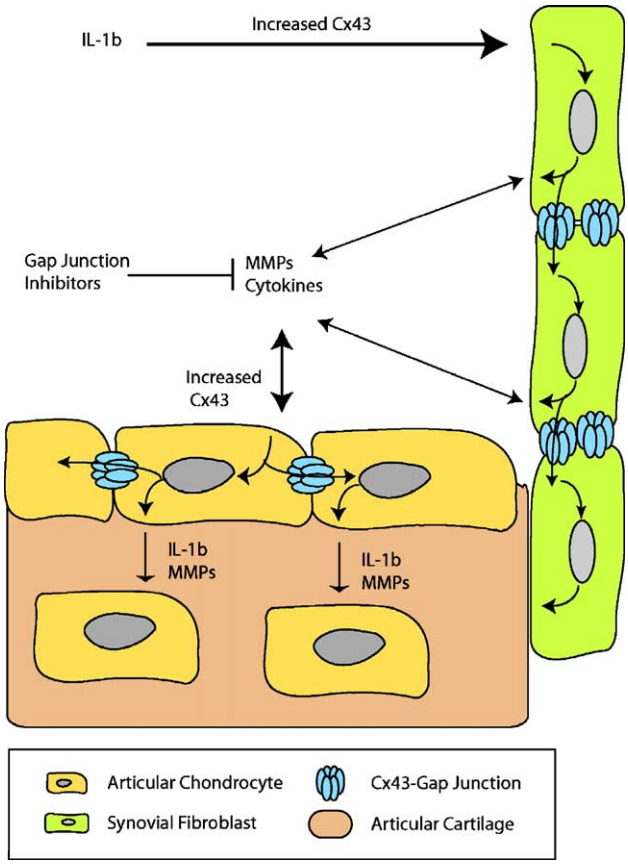

Fig. 4. A putative model of the role of connexin43 in osteoarthritis. Wear-and-tear on the articular cartilage has been shown to induce an inflammatory response by the synovial tissue. The resultant production of IL-1 by the synovium feeds back to upregulate Cx43 expression in both synovial fibroblasts and articular chondrocytes. In turn, Cx43 potentiates signaling further enhancing the production of catabolic factors such as IL-1β, MMPs and additional cytokines. Subsequently, the surface articular chondrocytes produce a response that triggers further degradation of the articular cartilage.

4.2. Osteoblasts and osteocytes

The most studied skeletal cells with regard to gap junctions and connexins are the bone forming osteoblast and the osteocyte. Numerous in vitro experiments have defined the role of gap junctions in regulating osteoblast and osteocyte function. Early work demonstrated the existence of gap junctions among osteoblasts and osteocytes by electron microscopy [83–85]. The data were later confirmed by ultrastructural analyses of histological bone sections [86,87], and subsequently the presence of functional gap junctional communication was established in murine [88] and human [89,90] osteoblasts. Osteoblasts and osteocytes express primarily Cx43 and to a lesser extent Cx45 [90,91]. Cx46 is also expressed, however this protein does not traffic correctly to the plasma membrane and remains in the trans Golgi [92]. The functional consequences or role for Cx46 are thus unknown. Osteoblasts on the bone surface and the bone embedded osteocytes have long been postulated to regulate bone anabolic function via coordinated signaling among the cells via long cellular processes originating from the osteocyte that interconnect osteocytes and osteoblasts by gap junctions.

During osteoblast differentiation in vitro, the expression of Cx43 increases, as does gap junctional communication [93,94]. In contrast, the expression level of Cx45 is unaltered during osteogenic differentiation [93]. In seminal work on the role of Cx43 on osteoblast function, it was shown that inhibition of gap junctional communication retarded the differentiation of these cells, resulting in a reduced ability to form a mineralized extracellular matrix and an attendant reduction in the expression of osteoblastic genes associated with differentiation [93–95]. In fact, it was shown that treatment of osteoblastic cells with pharmacologic inhibitors of gap junctions, 18α-glycyrrhetinic acid and oleamide, not only prevented differentiation into mature osteoblasts, but caused transdifferentiation into adipocyte-like cells [96]. The defective osteogenic differentiation was recapitulated in primary osteoblasts isolated from the calvaria of $Cx43^{-/-}$ mice [61]. These cells failed to express markers of terminal differentiation, including alkaline phosphatase, bone sialoprotein and osteocalcin, but also exhibited a delay in the ability to form mineralized nodules in vitro. These data implicate that Cx43 plays a very important role in osteogenic function.

Interestingly, numerous bone anabolic factors have been shown to upregulate Cx43 protein and gap junctional communication, including bone morphogenetic protein 2 (BMP2), PGE_2 and parathyroid hormone (PTH) [97–102]. Signaling activated by PTH results in a feed forward progression that upregulates Cx43 expression, which in turn amplifies the ability of the cell to respond to PTH. The cAMP response in osteoblast-like cell lines treated with PTH was shown to be attenuated when Cx43 expression was disrupted using antisense RNA [103]. Similarly, the ability of PTH to induce matrix mineralization in mature osteoblasts is markedly reduced when gap junctions are inhibited [104]. A similar observation has been made for PGE_2, which is produced in a gap junction-dependent manner during fluid flow shear stress by osteocyte-like cells in vitro [34,105–107]. The increased PGE_2 production feeds forward to further upregulate Cx43 expression [99,102,108].

A role of gap junctions in mechano-sensing by bone has longed been hypothesized. The gap junctional plaques formed at cell–cell borders of osteocytic processes have been postulated to play a critical role in signaling among osteoblasts and osteocytes in response to mechanical strain. In addition to the studies on PGE_2 and mechanical strain or fluid flow mentioned above, both osteoblasts (MC3T3-E1 cells) and osteocytes (MLO-Y4 cells) have been shown to remodel their gap junctions (Cx43 and Cx45) in response to fluid shear stress using immunofluorescence detection of Cx43 and Cx45 in these cells [109]. This remodeling of gap junctional plaques is dependent upon the mechanical shear stress applied; but in both cell types the fluid shear at 5 dyn/cm^2 or 20 dyn/cm^2 resulted in diminished staining of Cx43 and Cx45 at appositional membranes, and reduced gap junctional communication as assessed by dye coupling. Interestingly, despite the loss in gap junctional coupling, both osteoblasts and osteocytes were shown to increase Cx43 transcription at low fluid shear (5 dyn/cm^2), but not at high shear (20 dyn/cm^2). Cx45 mRNA

showed reciprocal regulation, being increased only at high shear stress [109].

In contrast, recent work in the same osteocyte like cell line has shown that fluid shear stress at 16 dyn/cm^2 increases the amount of Cx43 on the plasma membrane [34]. Importantly, this discrepancy was shown by detection of Cx43 that was capable of being biotinylated in intact cells, thus labeling only surface bound Cx43. Further, the accessibility of biotin to the Cx43 is limited to connexins that have not yet formed a coupled gap junction, and thus represent hemichannels. The authors also show that the levels of PGE_2 released into the extracellular fluid is inversely proportional to the density of plated cells when normalized to cell number, i.e., low density cultures produce more PGE_2 per cell than higher density cultures [34]. In contrast, intracellular levels of PGE_2 remain unchanged. Further, treatment with gap junction inhibitors, 18β-glycyrrhetinic acid or carbenoxolone, or antisense Cx43 RNA ablates the fluid flow induced release of PGE_2 in these cells. The authors argue that low-density cultures implicate the activity of Cx43 hemichannels rather than Cx43 gap junction channels in the resultant effect on PGE_2 release. Indeed, it has been shown that fluid flow or mechanical perturbation can induce the opening of Cx43 hemichannels in osteoblast and osteocytes [34,110].

Similar to osteocytes and chondrocytes, osteoblasts have been shown to respond to mechanical perturbation. Osteoblasts, like chondrocytes, produce synchronized Ca^{2+} waves among cells following mechanical manipulation. These Ca^{2+} waves occur via both gap junction dependent and gap junction independent mechanisms. The gap junction-independent Ca^{2+} waves are a result of the autocrine activity of secreted extracellular ATP on P2X purinergic receptors, and the gap junction-dependent propagation of Ca^{2+}-waves are caused by influx of Ca^{2+} though L-type voltage operated calcium channels [111–113]. These signals converge upon cell function in a connexin43 dependent manner.

Several groups have undertaken detailed molecular analyses to investigate the mechanisms of how alteration in gap junctions and Cx43 in bone cells affects cell phenotype. Work from our group and others have focused on examining how modulating Cx43 function at the plasma membrane can influence osteogenic gene expression. In primary osteoblasts isolated from Cx43$^{-/-}$ mice, transcription of many markers of osteogenic differentiation are reduced, including bone sialoprotein, osteocalcin, and type I collagen [61]. It was subsequently shown that inhibition of Cx43 function in osteoblast-like cell lines could reproduce the attenuation of transcription [94,96,114,115]. Alternately, overexpression of Cx43 in moderately coupled cells could increase transcription of osteoblast genes [95,116]. Gap junction dependent gene expression regulation has also been reported in other cell contexts [117–122], but the molecular mechanisms attending to this novel function of gap junctions remain unclear. Recently, we have shown that disruption of Cx43 function in osteoblasts results in an attenuation in the activation of the extracellular signal regulated kinase (ERK) response to serum stimulation [123]. Further, we have shown that the Cx43-dependent alteration of ERK signaling modulates gene transcription from the promoters of several osteoblast gene promoters [124]. In fact, the transcriptional deficiency observed in cells with disrupted Cx43 gap junctions can be mimicked by inhibition of ERK signaling and rescued by over expression of constitutively active members of the ERK signaling cascade [123]. The consequence of modulation of gap junction dependent ERK signal transduction converges upon an Sp1/Sp3 binding element in the promoters of the osteocalcin and collagen Iα1 genes, two genes downregulated by loss of Cx43. By chromatin immunoprecipitation, we observed that under conditions of robust Cx43-mediated gap junctional communication, the activator Sp1 and the repressor Sp3 both can occupy the promoter at a nearly 1:1 ratio, slightly favoring occupancy by Sp1. As a result transcription from these promoters is high. In contrast, when Cx43-mediated gap junctional communication is inhibited, the occupancy of this promoter element is maintained almost exclusively by the repressor, Sp3, markedly reducing transcription [123,124]. Accordingly, we have shown that ERK cascade dependent phosphorylation of Sp1 mediates the preferential recruitment of Sp1 over Sp3 in well-coupled cells, and loss of Sp1 phosphorylation results in the preferential recruitment of Sp3 [123]. Thus, we termed this Sp1/Sp3 binding element a connexin response element, or CxRE, due to its exquisite responsiveness to the degree of gap junctional coupling in osteoblastic cells. These data led us to propose a model in which cells facilitate a “primary response” to an extracellular cue by ligand–receptor mediated activation of cellular signals. The magnitude of this “primary” response is regulated in part by the bioavailability of ligand and the surface abundance of the receptor in a cell, leading to modulation of signal transduction cascades. In contrast, gap junctional communication permits a “secondary” response, which potentiates the “primary” response of the cells. Signals, in the form of second messengers like IP_3 or cADP ribose, generated by the “primary” response are propagated to adjacent cells via gap junctions initiating this “secondary” response in coupled cells that potentiates and coordinates signaling among a local population of cells (Fig. 5).

Another interesting role for Cx43 and downstream signaling in osteoblasts and osteocytes has been demonstrated. A series of papers have documented the role of Cx43 hemichannels in the anti-apoptotic response of osteoblasts and osteocytes caused by the administration of the bisphosphonate, alendronate. In the first paper, Plotkin et al., show that the bone anabolic therapeutic drug, alendronate, can act on osteoblasts to prevent etoposide and dexamethasone-induced apoptosis, and that this effect required Cx43 [125]. They further demonstrated that this effect was mediated by the src-ERK signaling cascade. Importantly, the authors demonstrated that treatment of MLO-Y4 osteocyte-like cells with alendronate could open Cx43 hemichannels, as assessed by dye uptake. The argument for Cx43 hemichannel function in preventing induced apoptosis of cells was enhanced by showing that cells cultured at low density or in suspension, thus minimizing the likelihood of gap junctional communication, still maintained the activation of ERK and anti-apoptotic effects initiated by

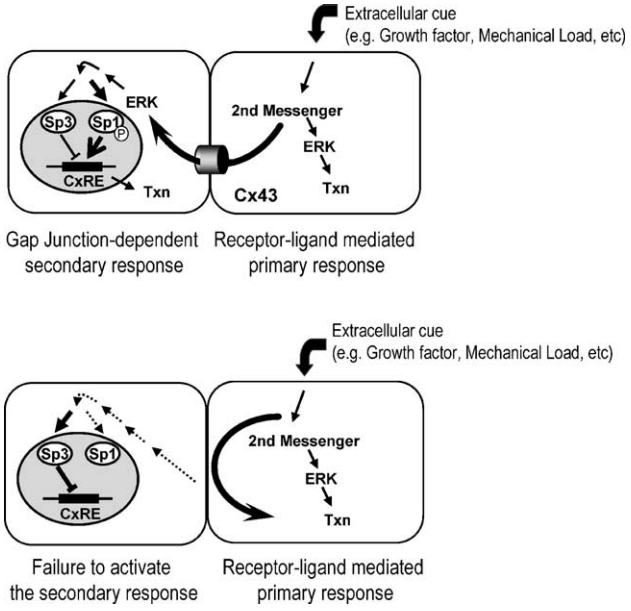

Fig. 5. Model of the role of gap junctions in potentiation of signals from an extracellular cue. For example, we show our model for transcription from the osteocalcin CxRE. Extracellular cues such as growth factors and mechanical loading induce second messengers (e.g., IP_3, cAMP, Ca^{2+} waves), leading to what we have termed a primary cellular response. This response is independent of gap junction. In the presence of gap junctional communication the intercellular propagation of second messengers that activate the ERK cascade, leads to a secondary response in the adjacent, gap junctionally coupled cell. In the example of the osteocalcin Sp1/Sp3-binding CxRE shown here, these signals converge on nuclear function by regulating the recruitment of the transactivator Sp1 to the CxRE in the promoter, leading to robust transcription (Txn). When gap junctional communication is disrupted the cellular response is attenuated, due to the failure to propagate signals among cells (i.e., only the primary response occurs).

alendronate treatment. These data suggested that Cx43 hemichannels may serve as a "receptor" for bisphosphonates, and that the resultant Cx43-dependent ERK activation is essential for the anti-apoptotic effects of alendronate on osteoblasts. More recently, this same group elaborated upon their earlier findings, reporting that the anti-apoptotic effects of alendronate on osteocytes was mediated by cytoplasmic ERK action, rather than the canonical nuclear translocation pathway [126]. Further, they demonstrated that a permeability impaired Cx43 mutant lacking 7 amino acids in the cytoplasmic loop (amino acids, 130–136), failed to protect osteocytes from etoposide induced apoptosis by alendronate. Similarly, they revealed that the C-terminal tail of Cx43 is likewise required for the hemichannel mediated anti-apoptotic effects of alendronate.

In summary, the role of gap junctions in osteogenic function is critically involved in lineage progression and gene transcription. Signals propagated through Cx43 gap junction channels (or hemichannels) regulate osteoblastic and osteocytic phenotype. In the absence of Cx43, osteoblast function is impaired as the responsiveness to hormonal and physical cues are attenuated.

4.3. Osteoclasts

The least studied bone cell type with respect to gap junctions is the hematopoietic derived osteoclast. Osteoclasts have been shown to express Cx43 [87,127]. These bone-resorbing cells are generated from the fusion of monocyte like precursor cells to form multinucleated cells. A role for Cx43 has been implicated in the fusion of these precursor cells into osteoclasts. Treatment of osteoclasts with the gap junction inhibitor heptanol markedly reduced the number of osteoclast like cells [127]. The number of unfused, mononuclear precursor cells was increased, suggesting a defect in fusion. Further, the activity of the osteoclasts that were formed had reduced ability to resorb bone slices. Fewer multinucleated osteoclasts were present and fewer of them were active as determined by bone pit assays. Surprisingly, it was found that the minority of osteoclasts that were active in the heptanol-treated cultures produced larger resorption pits in the bone slices than vehicle-treated samples. Similar results were obtained using a peptide inhibitor of Cx43, known as Gap27 [128]. However, one notable difference is that not only did Gap27 prevent osteoclast fusion and activity, there appeared to be a marked effect on apoptosis in these cells, though the effect was not rigorously analyzed. Analogous results for the resorption pit data were also obtained by another group using different chemical inhibitors of gap junctions [129]. In this study, they found that not only did inhibition of gap junctions results in fewer resorption pits by osteoclasts, but that the ability of PTH and vitamin D_3 to stimulate osteoclast activity was markedly inhibited by blockage of gap junctions.

4.4. Additional cells present in bone

In addition to the "primary" cells of bone listed above, numerous additional cell types are present within the skeletal network. The marrow cavity serves as a critical domain for progenitor cells, and interactions between bone and stem cell populations has been shown to serve a critical niche [130,131]. In addition, vascular invasion of the cartilaginous anlage is critical for endochondral ossification; and the mineralized bone is a highly vascularized tissue. Gap junctions have been shown to form among bone lining cells and marrow stromal cells [84]. In vitro work has demonstrated the formation of gap junctional communication among human bone marrow stromal cells and endothelial cells [132]. Further, in this study, co-culture of human umbilical vein endothelial cells with human bone marrow stromal cells increased the expression of alkaline phosphatase and type I collagen, two markers of osteoblast differentiation, by the stromal cells when placed in direct contact with the endothelial cells [132]. These data suggest that the physical interaction between endothelial cells and stromal cells supports osteoblastogenesis. The authors of this study revealed heterotypic gap junctional communication among the co-cultured cells, and demonstrated that inhibition of gap junctional communication could attenuate the osteogenic effects of co-culture. In subsequent work, it was shown that the contribution of endothelial cell-stromal cell interactions to osteoblast commitment and/or differentiation could be mediated by several additional endothelial cell populations, including human primary vascular endothelial cells, endothelial cells

isolated from cord blood and endothelial cells isolated from the saphen vein [133]. As was observed for the human umbilical vein endothelial cells, these effects were mediated by direct contact and gap junctional communication. The authors of these two studies implicate Cx43 in the heterotypic gap junctional communication among endothelial cells and stromal cells [132,133]; however, in light of the data from the Cx40 mutant mice, it may be interesting to examine whether heterotypic interactions between endothelial cells, which express Cx40 as well as Cx43 [134], and stromal cells contribute to the skeletal phenotype observed in these animals.

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