Published in final edited form as:

Genesis. 2013 February ; 51(2): 75–82. doi:10.1002/dvg.22349.

Determining how defects in Connexin43 cause skeletal disease

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Abstract

Gap junction channels mediate direct cell-cell communication via the exchange of second messengers, ions, and metabolites from one cell to another. Mutations in several human *connexin* (*cx*) genes, the subunits of gap junction channels, disturb the development and function of multiple tissues/organs. In particular, appropriate function of Cx43 is required for skeletal development in all vertebrate model organisms. Importantly, it remains largely unclear *how* disruption of gap junctional intercellular communication causes developmental defects. Two groups have taken distinct approaches towards defining the tangible molecular changes occurring downstream of Cx43-based gap junctional communication. Here, these strategies for determining how Cx43 modulates downstream events relevant to skeletal morphogenesis are reviewed.

Keywords

gap junction; Cx43; bone growth; skeletal development

Introduction

Gap junctions are proteinaceous channels that lie in the plasma membranes between adjacent cells, permitting the exchange of small molecules including ions and second messengers (Goodenough et al., 1996; Kumar and Gilula 1996) and playing critical roles in development and homeostasis. Connexins (*cx*), the subunits of gap junctions, consist of four transmembrane-spanning domains, two extracellular loops, one intracellular loop, and intracellular amino and carboxy ends. Six connexins comprise a connexon, or hemichannel, and a functional gap junction channel is composed of two connexons (one from each cell, see Figure 1). Connexons may be homomeric (same connexin isotype) or heteromeric (two or more connexin isotypes), increasing the potential for specificity differences in the types of molecules shared between cells. For example, mouse Cx43 and Cx45 can assemble into heteromeric gap junctions that exhibit coupling properties distinct from either homomeric type (Martinez et al., 2002). Several studies show that Cx43 is important for the growth and development of the vertebrate skeleton. However, little is known mechanistically regarding how Cx43 regulates events such as cell proliferation and cell differentiation. Thus, the challenge is to identify the tangible events regulated by gap junctional intercellular communication (GJIC). In this review, we will first summarize the important roles of Cx43 in skeletal development. Second, we will discuss two independent experimental strategies

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aimed at revealing how Cx43 mediates downstream molecular signaling events, in turn altering cell function.

Cx43 exhibits conserved functions in the vertebrate skeleton

Defective Cx43 function causes skeletal defects in human, mouse, chick, and zebrafish. Missense mutations in human *CX43* result in an autosomal dominant disorder called oculodentodigital dysplasia (ODDD, Paznekas et al., 2003). Patients with ODDD exhibit major skeletal malformations and craniofacial abnormalities such as cranial hyperostosis and broad tubular bones. This disease has additional pleiotropic phenotypes including eye abnormalities leading to vision loss and dental anomalies (reviewed in Pfenniger et al., 2010). The range of phenotypes affecting multiple tissues is likely due to the fact that *CX43* is expressed in most cell types. The *Gja1^{tr/+}* mouse carries a mutation coding for a dominant missense mutation in the *CX43* gene, and exhibits skeletal and pleiotropic phenotypes similar to those observed in human ODDD (Flenniken et al., 2005). Indeed, recently generated knock-in alleles, where human Cx43-missense mutations replace the endogenous wild-type Cx43 allele in the mouse, also mimic human ODDD (Dobrowolski et al., 2008; Watkins et al., 2011). In contrast, the *CX43* knockout mouse (*CX43^{-/-}*) dies perinatally due to defects in the heart outflow tract and reduced blood flow to tissues (Reaume et al., 1995). Continued investigation revealed that these mice exhibit delayed ossification of both the intramembranous and endochondral skeletons (Lecanda et al., 2000). To further overcome the lethality of *CX43^{-/-}*, conditional *CX43* knockout mouse lines have been generated. While mice lacking Cx43 activity in osteoblasts are viable, they exhibit reduced bone mineral density throughout their skeletons, consistent with phenotypes observed in the complete knockout (Chung et al., 2006; Watkins et al., 2011; Zhang et al., 2012; Bivi et al., 2012). Targeted gene knockdown of *CX43* in adult chicks and in chick embryos results in facial defects and limb malformation, respectively (Makarenkova et al., 1999; McGonnell et al., 2003). Finally, in the zebrafish, homozygous mutations in the *cx43* gene cause the *short fin* (*sof^{b123}*) phenotype, which primarily affects the fin skeleton. The *sof^{b123}* mutant exhibits short bony fin rays due to short bony segments and reduced levels of cell proliferation (Iovine et al., 2005). Targeted gene knockdown of *cx43* causes embryonic heart defects, suggesting *cx43* is essential (Iovine et al., 2005). Collectively, these data demonstrate that Cx43 function is conserved from fish to man and is required for typical skeletal morphogenesis in vertebrates.

There is some evidence that connexins, including Cx43, may exhibit activities independent of, or in addition to, GJIC. For example, since Cx43 in particular has a long carboxy tail exposed to the cytoplasm, it may serve as a signaling platform that functions independent of GJIC. Indeed the carboxy tail of Cx43 is known to bind to the components of signaling complexes including β -catenin and src (Saez et al., 2003; Giepmans, 2004; Herve et al., 2004). Alternatively, the signaling complexes may be activated by GJIC, such that channel function regulates signaling events. There is also evidence that Cx43 hemichannels play important functions in skeletal growth and development (reviewed in Plotkin, 2011). Connexin hemichannels are unpaired connexons that may permit the exchange of small molecules between the cytoplasm and the extracellular space. The focus of this review is on current strategies used to define the cellular events occurring downstream of Cx43 function,

whether as a gap junction channel, a hemichannel, or in a channel-independent manner. Thus, it should be noted that while GJIC is often the presumed function for Cx43 in the described studies, its precise role(s) has not been exclusively determined.

Cx43 regulates gene expression in osteoblast cell lines

It is well established that Cx43 enhances the expression of osteoblast genes. Recently, considerable effort has gone into defining the molecular mechanisms underlying Cx43-based regulation of gene expression. This work has been completed using a reporter assay in osteoblast cell lines (i.e. ROS17/2.8 cells and/or MC3T3 cells). The reporter construct contains the promoter of the osteoblast-specific gene osteocalcin upstream of the luciferase coding sequence (Figure 2). Luciferase activity can be precisely calculated and is directly proportional to the level of gene transcription from the osteocalcin promoter. When Cx43 function is high, luciferase activity is high. When Cx43 function is abrogated either by over-expression of Cx45 (which has been shown to reduce Cx43-dependent GJIC by modifying the size and specificity of the heteromeric gap junction channel, Lecanda et al., 1998) or by the addition of pharmacological inhibitors of GJIC (Stains and Civitelli, 2005), luciferase activity is reduced. Using this system, two distinct Cx43-dependent response elements have been identified in the osteocalcin promoter, and a mechanism for Cx43-dependent transcriptional activation has been suggested (described below and shown in Figure 3).

Using the system described above, a minimal Cx43 response element (CxRE) was identified in the osteocalcin promoter (Stains et al., 2003). It was determined that Cx43 function regulated the level of phosphorylation of the transcription factor Sp1, which in turn bound to the CxRE. Thus, increased phosphorylation of Sp1 favored its recruitment to the CxRE and caused increased gene transcription. In contrast, reduced phosphorylation of Sp1 favored the recruitment of an alternate transcription factor, Sp3, and lead to reduced gene transcription. Continued studies revealed that the phosphorylation of Sp1 occurred through Cx43-dependent activation of the ERK signal transduction cascade (Stains and Civitelli, 2005). Moreover, this Cx43-dependent recruitment of Sp1 was also correlated with recruitment of the osteoblast transcription factor Osx/Sp7 to the osteocalcin promoter (Niger et al., 2011). A second pathway for Cx43-dependent gene transcription occurs via activation of the transcription factor Runx2. Phosphorylated Runx2 interacts with the osteoblast-specific element OSE2, also found in the osteocalcin promoter. Cx43 function leads to activation of PKC δ , which is an intermediate in the FGF2 signaling pathway. Activation of PKC δ leads to the phosphorylation of Runx2 and increased gene transcription (Lima et al., 2009). Alternatively, FGF2 signaling can activate ERK independent of Cx43, also leading to Runx2-phosphorylation (Niger et al., 2012). These data provide evidence that FGF2 and Cx43 synergize to influence Runx2-phosphorylation and therefore increased gene expression of at least a subset of osteoblast-specific genes.

How are the ERK and FGF2 growth factor dependent signaling cascades related to Cx43 function? The Stains group suggests that both primary and secondary responses to growth factor-mediated signal transduction pathways are responsible for coordinated regulation of gene expression among a population of osteoblasts (Figure 3). The primary response occurs in cells expressing appropriate receptors for externally provided cues, such as growth

factors. Growth factor binding to its receptor leads to the intracellular production of second messengers, which in turn can be shared with adjacent cells via gap junctions, thereby promoting the same response in neighboring cells (i.e. even when those cells lack the appropriate receptor). The identification of signal transduction cascades working synergistically with Cx43-based gap junctions suggests that one role of gap junctions may be to amplify typical growth-factor induced responses. Moreover, since the carboxy-tail of connexins may act as a signaling center by binding to components of various signaling complexes (Bivi et al., 2011), the mediators of the second messengers may be closely associated with gap junctions. For example, Ras, a mediator of the ERK pathway, is known to associate on the inner leaflet of the plasma membrane and could associate with gap junctions (Stains and Civitelli, 2005). Moreover, PKC δ , a known mediator of FGF2 signaling, was recently shown to physically associate with the carboxy-tail of Cx43 (Niger et al., 2010).

These studies suggested that the Cx43 gap junctions reinforce typical signal transduction cascades by propagating second messengers to neighboring cells that may not have received the primary signal. Such a mechanism allows a signal that is initiated in a small number of cells to be coordinated within a tissue. Furthermore, these studies provide opportunities to begin to identify the relevant second messengers that may permeate Cx43-based gap junction channels (Niger et al., 2012).

Cx43 regulates gene expression in vivo

A second strategy to explore how Cx43 controls gene regulation in a mechanistic pathway is to evaluate Cx43 effects in vivo. Our lab evaluates the fin phenotypes of the *sof* mutant (Figure 4) to identify Cx43-dependent functions in the skeleton. The fin is composed of multiple bony fin rays and each fin ray consists of multiple bony segments separated by joints. Fin growth occurs at the distal end of the fin ray and can be monitored during typical ontogenetic growth or during regenerative growth following amputation (reviewed in Poss et al., 2003; Akimenko et al., 2003). The fin ray includes several tissues. The bone matrix of opposed hemirays surrounds the central mesenchymal compartment, while the hemirays are surrounded by a multi-layered epithelium. The population of dividing cells contributing to new fin growth is located in the distal mesenchyme, and is referred to as the blastema in the regenerating fin. Skeletal precursor cells (the pre-osteoblasts and pre-joint forming cells) are located laterally in the mesenchymal compartment, adjacent to the blastema. During regeneration, the *cx43* mRNA is up-regulated in blastemal cells. We have suggested that communication between the blastema and the skeletal precursor cells could be mediated by Cx43 function (Brown et al., 2009).

The Iovine lab has taken a molecular genetics approach to identify *cx43*-dependent genes contributing to skeletal morphogenesis. The original allele, *sof*^{b123}, exhibits reduced levels of *cx43* mRNA, short fins, short bony fin ray segments, and reduced levels of cell proliferation (Iovine et al., 2005). In addition to the *sof*^{b123} allele, three alleles coding missense mutations in the *cx43* gene were isolated using a non-complementation screen. All four *sof* alleles are hypomorphic and exhibit reduced fin length, segment length and cell proliferation. Further, morpholino-mediated knockdown of *cx43* recapitulates all of the *sof*

phenotypes (Hoptak-Solga et al., 2008). In contrast to the *sof* mutants, the *another long fin* (*alf^{dy86}*, van Eeden et al., 1996, Figure 4) mutant exhibits increased levels of *cx43* mRNA, long fins, and stochastic joint failure (i.e. long segments on average). Remarkably, the *alf^{dy86}* phenotypes are rescued by *cx43* knockdown (Sims et al., 2009). Since the *alf^{dy86}* mutant exhibits phenotypes opposite to that of *sof*, and since *cx43* knockdown rescues those phenotypes, we consider *alf^{dy86}* to be a mimic of *cx43* over-expression. Based on these and other data we further hypothesize that the role of Cx43 in the regenerating fin is to coordinate fin growth/cell proliferation with skeletal patterning/joint formation. In other words, Cx43 mediates *two* activities: promoting cell proliferation and suppressing joint formation.

In order to identify genes whose expression is regulated by Cx43, we completed a microarray analysis. We generated a list of genes that are both down-regulated in *sof^{b123}* (when *cx43* is down-regulated) and up-regulated in *alf^{dy86}* (when *cx43* is up-regulated). Notably, we found the *cx43* gene among the top 50 candidates, suggesting that our approach was successful. From this list, we decided to focus first on the *semaphorin3d* (*sema3d*) gene (Ton and Iovine, 2012). Class 3 semaphorins, such as *Sema3d*, are secreted signaling molecules known to be involved in activating signaling pathways that influence diverse cellular functions (reviewed in Yazdani and Terman, 2006). Expression of *sema3d* is located in the lateral skeletal precursor cells. Since *sema3d* is not expressed in the *cx43*-positive cells, it appears not to be a direct target for Cx43 function. However, since *sema3d* knockdown recapitulates all of the *cx43* knockdown phenotypes (including rescue of the *alf^{dy86}* phenotypes), *sema3d* is a likely candidate for mediating Cx43-dependent functions (Figure 5a).

Semaphorins utilize Neuropilins (Nrps) and Plexins (Plxns) as typical cell-surface receptors. Based on gene expression and gene function of candidate genes encoding the Nrps and Plxns, we found that independent receptors mediate either cell proliferation or joint formation (Ton and Iovine, 2012). For example, the *Nrp2a* receptor appears to mediate *Sema3d*-dependent cell proliferation while the *PlxnA3* receptor appears to mediate *Sema3d*-dependent joint formation. Interestingly, knockdown of *nrp2a* causes an increase in cell proliferation, while knockdown of *sema3d* causes reduced cell proliferation. We interpret these and other data to suggest that *Sema3d* inhibits *Nrp2a* function, thereby promoting cell proliferation by inhibiting a negative signal (Ton and Iovine, 2012). Based on these analyses of a single gene identified by our novel microarray strategy, we established the beginning of a molecular pathway dependent on Cx43 function (Figure 5). Continued insight into Cx43-dependent events may be obtained by placing additional genes identified by the microarray in this pathway. Indeed, of the nine newly validated genes from the microarray, four have established connections to mammalian skeletal diseases (Table 1). We anticipate that continued evaluation of these and other microarray genes will reveal a network of Cx43-dependent events that coordinate tangible cellular functions (such as cell proliferation and joint formation) and thereby control skeletal morphogenesis.

The case for Cx43-dependent GJIC during skeletal morphogenesis

Work from both the Stains lab and the Iovine lab provides evidence that Cx43-based GJIC contributes to skeletal development. For example, recall that Cx43 and FGF2 signals synergize to activate the ERK2-mediated phosphorylation of Runx2 and concomitant increased transcription of the luciferase reporter (Niger et al., 2012). When treated cells were examined using flow cytometry it was possible to demonstrate that the increased luciferase activity was due to an increase in the *number* of cells responding to the Cx43 and FGF2 signals, and not due to a more robust response from the same number of cells. This finding is consistent with their proposed model that the primary signal from the FGF2 growth factor is amplified via Cx43-based GJIC. Prior work from our lab demonstrated that the three missense alleles of *cx43* exhibit reduced levels of GJIC in heterologous assays (these alleles also appear to be capable of trafficking to the plasma membrane, Hoptak-Solga et al., 2007). Moreover, the severity of the segment length and cell proliferation phenotypes is correlated with the reduced level of GJIC (Iovine et al., 2005; Hoptak-Solga et al., 2008), strongly suggesting that reduced Cx43-based GJIC is responsible for the observed skeletal defects.

There are two possibilities to explain how Cx43-dependent GJIC in the blastemal cells influences *sema3d* gene expression in the adjacent skeletal precursor cells. First, Cx43-GJIC causes changes in gene expression in the *cx43*-positive compartment that lead to the secretion of an unidentified growth factor. This growth factor will bind to its receptor on the adjacent cells and cause an increase in *sema3d* expression in the lateral compartment (Figure 6a). Such a mechanism may be revealed by continued examination of genes identified from the microarray analysis. Alternatively, it remains possible that heterotypic gap junctions (i.e. when each cell contributes a connexon composed of different connexins) exist between cells of the blastema and the skeletal precursor cells, directly influencing gene expression between the two compartments (Figure 6b). Expression of a *connexin* gene has not been identified in the lateral compartment. However, since the zebrafish genome has at least 37 connexin genes (Eastman et al., 2006), one may still be found. This latter model, if true, may represent an in vivo example of second messengers traveling from one cell population to another in order to coordinate changes in gene expression, as elucidated by work from Stains et al.

Conclusions

The goal in defining the relevant Cx43 functions during skeletal morphogenesis is to reveal the initial Cx43-dependent event that regulates changes in cellular function. How do the different connexin functions lead to tangible changes in cell proliferation or cell differentiation? What are the relevant molecules that mediate these functions? Does the channel activity enhance propagation (as suggested in the work of Stains et al.), or is the channel-function secondary to the carboxy-tail as a scaffold for signaling molecules? How does hemichannel function fit in? The strategies described here have revealed tangible events downstream of Cx43 function and have provided novel insights into the types of cellular changes that are regulated by Connexins. In the future, we and others hope to work from these downstream events towards the initial Cx43-dependent function.

Acknowledgments

The authors wish to thank the members of the Iovine lab for providing constructive comments on the manuscript. Some of the work described in this review was supported by the NIH (HD047737) and the NSF (IOS-1145582) to MKI.

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Figure 1.

Gap junction channels connect the cytoplasm of adjacent cells. Each connexin contains four transmembrane-spanning domains, with both the amino and carboxy ends located in the cytoplasm. Six connexins comprise a connexon, or hemichannel. Two connexons, one from each cell, dock together at the plasma membrane to make a single gap junction channel. IL, intracellular loop; EL, extracellular loop; TM, transmembrane-spanning domain.

Figure 2.

The in vitro system used to evaluate Cx43-dependent regulation of gene expression. The reporter construct contains the osteocalcin (OCN) promoter followed by the coding sequence for the luciferase gene. The level of luciferase transcription is determined by quantitating the amount of luciferase activity. Osteoblast cell lines transfected with this reporter demonstrate that luciferase activity is dependent on the level of Cx43-based function.

Figure 3.

Model showing how gap junctions may amplify signals originating from growth-factor mediated cascades. A primary signal originates from a growth factor-receptor interaction that can initiate a response/change in gene expression in cell one (not drawn). At the same time, second messengers resulting from the primary signal transverse gap junction channels to initiate a similar change in gene expression in cell two. This is the secondary signal. Described Cx43-dependent changes include Ras/ERK (triangle) mediated phosphorylation of Sp1 (Stains and Civitelli, 2005) and PKCδ (diamond) mediated phosphorylation of Runx2 (Lima et al., 2009). PKCδ may also independently activate ERK (Niger et al., 2012). Phosphorylated Sp1 and Runx2 favor transcription of certain osteoblast genes.

Figure 4.
Fin length mutants exhibit defects in skeletal morphogenesis. Top: wild-type zebrafish.
Middle: *sof*^{b123} mutant. Bottom: *alf*^{dy86} mutant.

Figure 5.

Cx43 influences cell proliferation and joint formation by regulating the Sema3d pathway. (a) Expression of the *sema3d* gene is regulated by *cx43*. The Sema3d protein is secreted and interacts with its putative receptors, Nrp2a and PlxnA3, to regulate cell proliferation and joint formation respectively. (b) Compartmental expression of the genes in the Cx43 pathway. The *cx43* mRNA is up-regulated in the proliferating cells of the blastema (red), while *sema3d* is up-regulated in the lateral skeletal precursor cells and basal layer of the epidermis (green). The *nrp2a* gene is expressed in the distal blastema (yellow), where it may regulate cell proliferation in the blastemal cells. The *plxna3* gene is expressed in the skeletal precursor cells (blue), where it may regulate joint formation. This figure was originally published in Ton and Iovine, 2012, and is reprinted here with permission.

Figure 6.

Examples of how Cx43-dependent GJIC may influence gene expression in the regenerating fin. (a) Up-regulation of *cx43* in the blastema (red) may lead to the secretion of a growth factor that can interact with its receptor located on adjacent skeletal precursor cells (green), leading to increased *sema3d* expression. (b) Heterotypic gap junctions may exist between cells of the blastema and skeletal precursor cells, permitting the direct exchange of secondary messengers. These second messengers may influence *sema3d* expression.

Table 1

Validated microarray genes with known skeletal relevance

name	disease associated with mammalian ortholog	OMIM ID
<i>hsp47</i>	osteogenesis imperfecta	600943
<i>hapln1a</i>	dwarfism and craniofacial defects (mouse)	115435
<i>twist1b</i>	craniosynostosis type I	601622
<i>hoxda13</i>	brachydactyly; syndactyly	142989

OMIM: Online Mendelian Inheritance in Man is a database that catalogs disease-causing mutations and phenotypes. Mutations in the mammalian orthologs to the four listed zebrafish genes are known to cause skeletal disease. For complete list of identified genes, please see Ton and Iovine, 2012.