CONNEXINS AND CELL SIGNALING IN DEVELOPMENT AND DISEASE

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Abstract  Gap junctions contain hydrophilic membrane channels that allow direct communication between neighboring cells through the diffusion of ions, metabolites, and small cell signaling molecules. They are made up of a hexameric array of polypeptides encoded by the connexin multi-gene family. Cell-cell communication mediated by connexins is crucial to various cellular functions, including the regulation of cell growth, differentiation, and development. Mutations in connexin genes have been linked to a variety of human diseases, including cardiovascular anomalies, peripheral neuropathy, deafness, skin disorders, and cataracts. In addition to their coupling function, recent studies suggest that connexin proteins may also mediate signaling. This could involve interactions with other protein partners that may play a role not only in connexin assembly, trafficking, gating and turnover, but also in the coordinate regulation of cell-cell communication with cell adhesion and cell motility. The integration of these cell functions is likely to be important in the role of gap junctions in development and disease.

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INTRODUCTION

Gap junctions are intercellular membrane channels found in a diverse array of organisms spanning from nematodes, echinoderms, ascidians, molluscs, and arthropods to vertebrates such as frog, chick, rodents, and humans. In vertebrates, gap junctions are made up of a multi-gene family called connexins (Willecke et al. 2002), whereas invertebrate gap junctions are encoded by a separate gene family known as the innexins (Phelan & Starich 2001). Connexins and innexins share an identical membrane topology, but little sequence homology. Given space constraints, this review is focused exclusively on the vertebrate connexins.

We begin with a brief overview of the vertebrate gap junction channel structure and physiology, followed by a discussion of connexin protein interactions with other membrane and cytoplasmic proteins. We then provide a brief survey of the role of connexins in development and disease, with emphasis on studies of transgenic and knockout mouse models and the recent discoveries of connexin mutations associated with human diseases. We consider in more detail the specific role of connexins in cardiovascular development, and within this context, propose a role for connexins in mediating cell signaling that may not require the gap junction channel. We hope to stimulate debate and discussion, and no doubt these provocative ideas will require revision and reevaluation as new data emerge. Ultimately, understanding the functional role of connexins in development and disease will likely require paradigm shifts that promise to keep us engaged. We apologize for the inevitable gaps in our review and refer readers to other excellent gap junction reviews (Bruzzone et al. 1996, Evans & Martin 2002, Goodenough & Paul 2003, Harris 2001, Kumar & Gilula 1996, Saez et al. 2003, White & Paul 1999, Willecke et al. 2002).
GAP JUNCTIONS

Gap Junction Structure and Permeability Properties

Gap junctions are specialized intercellular junctions characterized by the apposition of the plasma membrane of apposing cells such that there is only a narrow 2–3 nm gap. They contain hydrophilic membrane channels that mediate the passage of ions, metabolites, and small cell signaling molecules less than 1 kDa in size (Kumar & Gilula 1996). Electron microscopy and X-ray crystallographic studies showed gap junctions are tightly packed in a hexagonal array termed connexons, with each connexon having a hemi-channel made up of six protein subunits known as the connexin (Kumar & Gilula 1996, Yeager 1998) (Figure 1). Thus a gap junction is formed when two connexons are joined end to end in the extracellular space. Hydrophy plots of connexins revealed the presence of two extracellular domains (E1 and E2), four hydrophobic membrane-spanning domains (M1–M4), and three cytoplasmic domains including an intracellular loop, and amino and carboxyl termini (NT and CT, respectively) (Figure 1A). The four transmembrane domains of each connexin subunit exhibit an α-helical structure, whereas the extracellular domains from apposing connexons form a tight seal that prohibits the exchange of substances between the channel lumen and the extracellular milieu (Unger et al. 1999). Gating of the gap junction channel is controlled by multiple factors such as calcium concentration, pH, transjunctional membrane potential and protein phosphorylation (Harris 2001).

Connexin Multi-Gene Family and Heteromeric/Heterotypic Gap Junctions

To date, 20 connexin genes have been identified in the mouse genome and 21 in the human genome (Sohl & Willecke 2003). These genes show 40% sequence identity. Most of the connexin genes share a common structure: a first exon containing the 5′ untranslated region (5′-UTR) followed by an intron of varying length, and a second exon containing the remaining 5′-UTR, the coding sequence, and the 3′-UTR (Willecke et al. 2002). However, several exceptions have been reported, including differential splicing of 5′-UTR and interruption of the coding sequence by introns (Condorelli et al. 1998; Neuhaus et al. 1995; Sohl et al. 1996, 1998, 2001).

Connexin proteins have been described using two different nomenclature systems, either by molecular mass or based on sequence homology. Thus a 43 kDa connexin protein is referred to as either connexin43 or α1 connexin. Using the latter nomenclature system, connexin proteins have been subdivided into at least three subgroups referred to as connexin α, β, or γ. Thus Cx43 is referred to as α1 connexin, Cx32 is β1 connexin, etc. To minimize confusion, we use a hybrid nomenclature, such that Cx43 or α1 connexin is referred to as Cx43α1. Most cells and tissues express more than one connexin isotype. Connexons may be composed of six identical connexin subunits (homomeric) or more than one connexin
Figure 1  Schematic diagram showing connexin membrane topology and heteromeric/heterotypic arrangements of gap junction contacts. (a) Model showing membrane topology for the connexin polypeptide. M1–M4 represent the four transmembrane domains, E1 and E2 the two intracellular loops; the amino (N) and carboxy (C) termini are intracellular (from Kumar & Gilula 1996). (b) Schematic drawing showing possible arrangements of connexons to form heterotypic and heteromeric gap junction channels. Connexons consisting of six connexin subunits (red and blue) may be homomeric (identical subunits) or heteromeric (more than one connexin isotype), and when associated end to end, form membrane channels that may be homotypic or heterotypic (adapted from Kumar & Gilula 1996).

isotype (heteromeric), and two identical connexons can form a homotypic channel, or a heterotypic channel can be generated by two connexons having different connexin isotypes (Figure 1B). The ability to form homomeric/heteromeric and homotypic/heterotypic channels provides greater complexity in the regulation of gap junctional communication.

Differential Gating of Gap Junction Channels

The gap junction channel can be differentially gated by different mechanisms. Closure of the gap junction channel has been observed in the presence of high
concentration of calcium ion (micromolar range), which suggests that the gap junction channel can be modulated by a variety of calcium-dependent cellular events. It has been proposed that calmodulin serves as a mediator for this calcium effect because it can bind Cx32β1 (Hertzberg & Van Eldik 1987). The depletion of calmodulin has been shown to reduce the sensitivity of gap junctional communication to elevated calcium levels in *Xenopus* oocytes (Peracchia et al. 1996).

Intracellular pH (pH$_i$) is another factor that can modulate gap junction gating behavior. The regulatory sites for pH gating are believed to be located at the intracellular loop and CT domains of the connexin proteins, a region showing little sequence homology across the connexin multi-gene family. Hence, not surprisingly, different homotypic and heterotypic gap junction channels exhibit a variable degree of sensitivity to intracellular acidification (Ek-Vitorin et al. 1996; Francis et al. 1999; Homma et al. 1998; Morley et al. 1996, 1997; White et al. 1994; Yahuaca et al. 2000). On the basis of a series of studies involving the use of mutagenesis and heterologous expression of connexin CT domains, Delmar and colleagues have proposed a particle-receptor model to describe the mechanism of connexin pH gating. According to this model, intracellular acidification leads to the binding of Cx43α1 CT domain (acting as particle) to a region including histidine 95 (acting as receptor), and this action is proposed to close the channel (Ek-Vitorin et al. 1996, Homma et al. 1998, Morley et al. 1996). Several recent studies suggest that this model may not universally apply to all connexins (Eckert 2002, Stergiopoulos et al. 1999).

Gap junction conductance can also be regulated by transjunctional or transmembrane voltage. Most vertebrate connexins are sensitive to transjunctional voltage and form closed channels when large transjunctional voltages are applied (Dahl 1996). Cx26β2, Cx43α1, and Cx45α7 also display transmembrane voltage dependence (Barrio et al. 1991, 1997; White et al. 1994). Different homotypic and heterotypic gap junction channels exhibit voltage gating to different degrees and the voltage-gating properties of a connexin may also be species-specific (Barrio et al. 1991, 1997; White et al. 1995). Mutagenesis studies have identified four separate domains where mutations can affect connexin voltage gating: NT (Verselis et al. 1994), E1 (Rubin et al. 1992), M2 (Suchyna et al. 1993), and CT (Moreno et al. 1992, 2002).

**Connexin43 Protein-Protein Interactions**

Connexin proteins have been shown to interact with a diverse array of proteins to form multi-protein complexes (Duffy et al. 2002, Herve et al. 2004). Such interactions are likely to regulate connexin functions at several levels, including connexin assembly, trafficking, turnover, and channel gating. In addition, recent studies suggest connexin protein interactions may modulate connexin function in response to physiological stimulation and pathological conditions (Thomas et al. 2002). Below we summarize the current evidence for interaction between Cx43α1 and other membrane and cytoplasmic proteins and consider whether such interaction may indicate a novel role for connexins in cell signaling. Elucidating connexin
protein interactions may also provide mechanistic insights into the role of connexin mutations in human disease.

Interactions with Adherens Junction–Associated Proteins

A number of studies have indicated a close association between gap junctions and cadherin-based adherens junctions. Cadherins are comprised of a major family of transmembrane glycoproteins known to play an important role in the regulation of cell adhesion and cell motility (Juliano 2002; Wheelock & Johnson 2003a,b). Inhibition of cadherin function can disrupt gap junction formation and inhibit cell-cell coupling, suggesting that localization of cadherin to cell-cell contact sites may be a prerequisite for gap junction formation (Frenzel & Johnson 1996; Hertig et al. 1996a,b; Kanno et al. 1984; Keane et al. 1988; Kostin et al. 1999; Meyer et al. 1992; Zuppinger et al. 2000). Conversely, inhibition of Cx43α1 can block adherens junction formation (Meyer et al. 1992).

Cadherin-mediated cell adhesion can be regulated by signaling through the Rho GTPases, the Wnt pathway, and receptor tyrosine kinases (Wheelock & Johnson 2003a), as well as through a variety of extracellular signals that include signals from gap junctions (Paul et al. 1995). The cytoplasmic domain of cadherins binds α/β-catenins and other F-actin binding proteins including α-actinin, vinculin, and zonula occludens-1 (ZO-1) and thus provide linkage to the actin cytoskeleton (Nagafuchi 2001). In rat cardiac myocytes, β-catenin interacts with Cx43α1 (Ai et al. 2000), and the formation of Cx43α1/ZO-1/β-catenin complex is required for targeting of Cx43α1 to the plasma membrane (Wu et al. 2003). Another recent study also suggests that α-catenin is important for Cx43α1 trafficking and assembly (Govindarajan et al. 2002). Given that N-cadherin and catenins are coassembled in the endoplasmic reticulum/Golgi compartments (Wahl et al. 2003), this raises the possibility that Cx43α1 is assembled as part of a multi-protein complex that may coordinate the regulation of adherens and gap junction assembly. Interestingly, studies in mouse neural crest cells, as well as in NIH3T3 cells, have shown that Cx43α1 is colocalized with N-cadherin and p120ctn at regions of cell-cell contact (Wei et al. 2003, Xu et al. 2001). In Cx43α1 and N-cadherin-deficient neural crest cells, the subcellular distribution of p120ctn was altered in conjunction with the perturbation of cell motility, suggesting the possibility that Cx43α1 and N-cadherin may modulate cell motility by engaging in a dynamic cross-talk with the cell’s locomotory apparatus through p120ctn signaling (Xu et al. 2001).

Cytoskeletal Protein Interactions

A number of studies indicate that Cx43α1 gap junctions may be closely associated with cytoskeletal proteins. One study showed that formation of functional Cx43α1 gap junctions required elevated cAMP and intact microfilaments, which suggests the possibility that clustering of Cx43α1 gap junction may involve protein kinase A (PKA) and actin filaments (Wang & Rose 1995). The association of Cx43α1 with actin filaments and perhaps other actin-binding proteins was also implicated
in studies with cultured astrocytes, where microinjection of anti-actin antibody impaired Cx43α1 membrane trafficking and inhibited gap junctional communication (Theiss & Meller 2002). Direct interaction of Cx43α1 with microtubules also has been demonstrated (Giepmans et al. 2001a, Guo et al. 2003). Although the nature of this interaction is still unclear, it is possible that Cx43α1 acts as an anchor to stabilize microtubules, or it may serve to regulate Cx43α1 expression and distribution via the integrin-mediated cell signaling pathway (Giepmans et al. 2001a, Guo et al. 2003).

Tight Junction–Associated Proteins

The CT domain of Cx43α1 has been shown to bind ZO-1 in several cell types, and this interaction is regulated by phosphorylation of Cx43α1 by Src tyrosine kinases (Giepmans et al. 2001a,b; Toyofuku et al. 1998). ZO-1 is a peripheral membrane scaffolding protein that is specifically enriched at the tight junctions of epithelial and endothelial cells (Stevenson et al. 1986). It functions to tether transmembrane proteins to the actin cytoskeleton (Denker & Nigam 1998) and is also part of adherens junctions (Itoh et al. 1993). Although the role of ZO-1 in Cx43α1 function is not clear, it is possible that ZO-1 serves as a scaffold to recruit signaling molecules and/or actin filaments to Cx43α1, which may help in coordinately regulating gap junction formation with modulation of the actin cytoskeleton or with intracellular signaling.

Caveolin and Membrane Microdomains

Cx43α1 has been shown to interact with caveolin-1, a structural protein that resides in a specialized lipid raft domain known as a caveola (Schubert et al. 2002). Lipid rafts are membrane microdomains enriched in cholesterol and glycosphingolipids. These domains serve as a platform for a number of diverse cellular processes such as signal transduction, endocytosis, and cholesterol trafficking (Pike 2004). The functional significance of Cx43α1 interaction with caveolin-1 is still unclear. Perhaps it recruits Cx43α1 to membrane domains enriched in signaling proteins; for example, a recent study showed that Cx43α1 gap junction regulated by PKCγ occurs through caveolin-1-containing lipid rafts (Lin et al. 2003).

Protein Kinases and Cx43α1 Phosphorylation

All connexins characterized so far are phosphoproteins with the exception of Cx26β2, whose CT domain is relatively short (Herve & Sarrouilhe 2002, Lampe & Lau 2000). Phosphorylation of the connexin CT domains is important in gap junction assembly, trafficking, channel gating, and turnover. Because of the large sequence variation among connexin CT domains, the mechanisms of connexin phosphorylation appear to be complex. For instance, Cx32β1 gap junctional communication is increased by the activated cAMP-dependent PKA (Saenz et al. 1990), whereas intercellular communication is reduced by protein kinase C (PKC) phosphorylation of chick lens Cx56α3 (Berthoud et al. 1997). Connexins can also be differentially phosphorylated in a tissue-specific manner (Kadle et al. 1991), and
the communication competence of several cell lines has been correlated with the pattern of protein phosphorylation (Musil et al. 1990).

Cx43α1 may be subject to modification by as many as 10 different protein kinases. Among the kinases that phosphorylate Cx43α1, interactions with Src and PKC have been studied most extensively (Herve & Sarrouilhe 2002, Lampe & Lau 2000, Warn-Cramer & Lau 2004). It has been proposed that Src phosphorylation regulates the interaction between Cx43α1 and ZO-1, and disruption of Cx43α1/ZO-1 interaction is suggested to underlie the inhibitory effect of c-Src on gap junctional communication (Toyofuku et al. 2001). Cx43α1 phosphorylation by PKC was found to inhibit cell-cell coupling via a reduction in the channel unitary conductance (Lampe et al. 2000) and also altered cell-cycle events (Solan et al. 2003). PKC phosphorylation of Cx43α1 has also been reported with fibroblast growth factor-2 (FGF2) stimulation and was associated with the inhibition of DNA synthesis and cell growth. Interestingly, these changes occurred independently of channel permeability and/or the subcellular distribution of Cx43α1 (Doble et al. 2004).

**Connexins in Development and Disease**

Gap junctions have long been speculated as playing a role in development. An attractive hypothesis is that gap junction communication may mediate the formation of morphogen gradients. Consistent with this notion, gap junction communication in the developing embryo shows functional subdivision into communication compartments that coincide with developmentally significant domains (Bagnall et al. 1992; Kalimi & Lo 1988, 1989; Lo & Gilula 1979a,b; Martinez et al. 1992; Warner et al. 1984; Warner & Lawrence 1982; Weir & Lo 1982, 1984, 1985). Such communication compartments may provide the context for generating morphogen gradients that can regulate growth, patterning, and differentiation (Lo & Gilula 1979b, 2000). Although morphogen gradients in development are well described, there is little evidence for gap junctions playing a role in the formation of such gradients. Nevertheless, it is worth noting that recent work by Levin and colleagues indicates that gap junction coupling may modulate left-right patterning (Levin 2002). This was demonstrated using several different methods to alter or inhibit gap junctional communication in *Xenopus* and chick embryos (Levin & Mercola 1998, 1999). These animal model experiments are in agreement with an earlier report that Cx43α1 mutations can cause viscerocriral heterotaxia (VAH) in humans (Britz-Cunningham et al. 1995), although other groups examining different patient populations did not confirm the latter findings (Casey & Ballabio 1995, Debrus et al. 1997, Gebbia et al. 1996).

Recent studies using reverse genetic approaches with the analysis of transgenic and knockout mouse models have provided more conclusive evidence of the requirement for gap junctions in regulating growth and development, and in modulating tissue and organ physiology (Willecke et al. 2002). Thus knockout mouse models have indicated an indispensable role for connexins in the development and function of a variety of tissues and organs (Willecke et al. 2002). Furthermore,
clinical studies have demonstrated a role for connexin mutations in a wide spectra of human diseases, such as in demyelinating neuropathies, various skin disorders, cataracts, sensorineural deafness, and oculodentodigital dysplasia (ODDD) (Willecke et al. 2002). The challenge now is to unravel the mechanism(s) by which connexins contribute to development and disease. Together with transgenic mouse models, human connexin mutations are nature’s genetic experiments that can help elucidate connexin protein structure-function relationships. However, it is that the collection of connexin mutations associated with human diseases shows no correlation with protein domains known to be required for the formation or regulation of the gap junction channel itself, the only exception being connexin mutations associated with cataracts.

**Connexin Mutations Associated with Peripheral Neuropathy**

Mutations in the connexin protein, Cx32β1, are associated with X-linked form of Charcot-Marie-Tooth (CMTX) disease (Bergoffen et al. 1993a,b). CMTX is a demyelinating syndrome with progressive degeneration of peripheral nerves brought on by a defect in Schwann cells. Over 200 Cx32β1 mutations have been identified in CMTX patients (Nelis et al. 1999). Most of these mutations are situated within the coding region and, surprisingly, span the entire length of the protein. Compared with CMTX patients, Cx32β1 knockout mice develop relatively mild peripheral neuropathy (Nelles et al. 1996, Scherer et al. 1998) that is associated with a distinct pattern of gene dysregulation in Schwann cells (Nicholson et al. 2001). Other defects include reduced nerve stimulation and hormone-induced hepatic glucose release, as well as reduced bile flow following sympathetic stimulation (Nelles et al. 1996, Scherer et al. 1998). In addition, Cx32β1 knockout mice appeared to be more susceptible to chemically induced liver carcinogenesis (Nelles et al. 1996).

**Connexin Mutations Associated with Deafness**

At least 5 connexin proteins are reported to be involved in deafness (syndromic and nonsyndromic), including Cx26β2, Cx30β6, Cx31β3, Cx32β1, and Cx43α1 (Kelsell et al. 2001, Richard 2003). Autosomal-recessive (DFNB1) and autosomal-dominant (DFNA3) forms of hearing impairment have been associated with more than 50 mutations in the coding region of Cx26β2 (Kelsell et al. 2001, White 2000). The most common mutation is a recessive frame shift mutation (35delG) that causes premature translation termination (Zelante et al. 1997). Recent data indicate that DFNB1 in some patients may be associated with a 342 kb deletion involving Cx30β6 (Del Castillo et al. 2003). Interestingly, as with CMTX, connexin mutations causing deafness are not restricted to any functional domain but are distributed throughout the length of the protein.

Knockout mouse studies have provided some insights into the role of Cx26β2 and Cx30β6 in auditory function. Recent work using targeted ablation demonstrated that Cx26β2 is essential for cochlear function and cell survival in the
sensory epithelium of the inner ear (Cohen-Salmon et al. 2002), whereas a Cx30β6 knockout mouse model exhibited severe constitutive hearing impairment, suggesting that Cx30β6 plays a role in generating endocochlear potential and mediating survival of the auditory hair cells after the onset of hearing (Teubner et al. 2003). However, it should be noted that connexin mutations associated with hearing loss are not restricted to connexin protein domains required for channel function.

Connexin Mutations Associated with Skin Disorders

Some of the mutations in Cx26β2, Cx30β6, and Cx31β3 are linked not only with deafness, but also with skin disorders (Richard 2003). In the epidermis, gap junctions appear to play a critical role in regulating keratinocyte growth and differentiation (Choudhry et al. 1997), and studies in mice revealed that at least nine different connexin genes are coexpressed in the epidermis (Richard 2000). Mutations in Cx26β2 have been shown to be linked to Vohwinkel syndrome, an autosomal-dominant condition with mutilating keratoderma accompanied by deafness (Maestrini et al. 1999). Another type of skin disease, erythorokeratoderma variabilis (EKV) was linked to mutations in Cx31β3 (Richard et al. 1998). EKV was recently also reported to be associated with mutations in Cx30.3β4 (Richard et al. 2003). However, Cx31β3-deficient mice exhibit no morphological or functional skin or inner ear defects, which were possibly compensated by other redundantly expressed connexins (Plum et al. 2001). As with mutations associated with CMTX and sensorineural deafness, connexin mutations causing skin disorders are not restricted to any particular connexin protein domain.

A recent transgenic mouse study with epidermal expression of a mutant Cx26β2 (D66H) is reported to mimic true Vohwinkel syndrome (Bakirtzis et al. 2003). Such transgenic mice showed keratoderma similar to humans with the same mutation, and they also exhibited premature keratinocyte cell death and marked thickening of the epidermal cornified layers. These findings suggest the possibility that connexin function plays a role in regulating formation of the cornified envelope. It is interesting to note that in this animal model, transgene expression was associated with the cytoplasmic accumulation of both wild-type Cx26β2 and Cx30β6 connexins, indicating a dominant-negative effect of the mutant connexin protein on trafficking of wild-type connexins.

Connexin Mutations Associated with Cataracts

In the lens, three connexins are expressed: Cx43α1, Cx46α3, and Cx50α8. Fiber cells of the lens are interconnected by an extensive network of gap junctions containing Cx46α3 and Cx50α8, both serving to maintain homeostasis and support cell growth (White 2002). Point mutations in the Cx50α8 and Cx46α3 connexins have been identified in patients with inherited zonular pulverulent cataracts. Perhaps indicative of the unique requirement for gap junction communication in maintaining lens tissue homeostasis, human lens connexin mutations are largely restricted to the extracellular loop or transmembrane domains (Berry et al. 1999,

Studies using knockout mouse models revealed that loss of Cx46α3 function caused severe cataracts, but otherwise had no effect on lens growth and development (Gong et al. 1997, 1998). In contrast, the deletion of Cx50α8 caused a significant reduction in lens growth together with mild cataracts (Rong et al. 2002, White et al. 1998). Interestingly, knockin replacement of Cx50α8 with Cx46α3 rescued lens clarity but was unable to restore normal growth (Martinez-Wittinghan et al. 2003). In contrast, double Cx43α1/Cx50α8 knockout mice exhibited normal lens development, suggesting that neither Cx43α1 nor Cx50α8 is required for prenatal lens development (White et al. 2001). Overall, these observations indicate that the intrinsic properties of Cx50α8 are likely required for cellular growth, whereas Cx46α3 is essential for normal lens differentiation (White 2002).

Connexin Regulation of Germ Cell Development

Knockout mouse studies have identified an essential role for Cx37α4 and Cx43α1 in germ cell development. Cx37α4 is expressed in oocytes and presumably generates heterotypic gap junctions linking oocytes with granulosa cells (Kidder & Mhawi 2002), whereas Cx43α1 is expressed abundantly in granulosa/granulosa gap junctions (Saez et al. 2003, White & Paul 1999). Cx37α4-deficient mice show defects in oocyte development before competence is reached, together with the perturbation of antral follicle development (Kidder & Mhawi 2002, Simon et al. 1997). In Cx43α1-deficient mice, neonatal mice show germ cell deficiency that arises during fetal life. Grafting experiments show that Cx43α1 deficiency in the testis resulted in a “Sertoli cell only” phenotype (Roscoe et al. 2001), whereas in the ovary, follicles are arrested early in development with incompetent oocytes (Juneja et al. 1999). Interestingly, the replacement of Cx43α1 with either Cx40α5 or Cx32β1 in Cx40α5 and Cx32β1 knockin mice, respectively, could not rescue the germ cell defect (Plum et al. 2000). Because Cx43α1 is expressed in mouse embryonic ovaries and testes from the earliest stages of development, perhaps it is required for prenatal expansion of germ cells during fetal gonadal development (Perez-Armendariz et al. 2003). Another possibility to consider is whether Cx43α1 may be required for germ cell migration and targeting to the gonads, a possibility worth considering in light of the known role of Cx43α1 modulating the migration of neural crest and proepiardielly derived cells (Huang et al. 1998a, Li et al. 2002).

Cx43α1 Mutations and Oculodentodigital Dysplasia

Another human disease recently shown to be associated with connexin mutations is oculodentodigital dysplasia (ODDD). ODDD is a congenital disorder characterized by developmental abnormalities of the face, eyes, limbs, and dentition and is linked to dominant mutations in Cx43α1 (Paznekas et al. 2003). Cx43α1 mutations associated with ODDD are broadly distributed except for the singular absence of
any mutation in the CT (Paznekas et al. 2003). In contrast, human patients with VAH have point mutations in the cytoplasmic tail of the Cx43α1 gap junction protein (Britz-Cunningham et al. 1995).

CONNEXINS AND CARDIOVASCULAR DEVELOPMENT AND FUNCTION

Gap junctions play an essential role in cardiac function, as they mediate the spread of electrical impulses that allows synchronous contraction of the cardiac chambers (Severs 2001). In the adult mammalian working myocardium, Cx43α1 is the major connexin protein expressed and is principally responsible for electrical synchrony. In addition, there are two other connexin isotypes found in the adult heart, Cx45α7 and Cx40α5. Cx45α7 is expressed in the atrioventricular node and adjoining His bundles, whereas Cx40α5 is expressed in the fast conducting tissues of the His-Purkinje system, nested within the Cx45α7 expression domain (Gourdie et al. 1993). Given that Cx40α5 generates channels with high conductance and Cx45α7 forms voltage-sensitive channels with very low conductance (Veenstra et al. 1992), this compartmentalized connexin expression pattern is expected to provide for the orderly sequential spread of activation from the atrial to ventricular chambers (Gourdie & Lo 1999).

Each of these three connexin genes has now been deleted by embryonic stem cell targeting, and analysis of the phenotypes in the respective knockout mouse models shows that all three genes are required for heart conduction. Thus conditional deletion of Cx43α1 restricted to the adult working myocardium showed that Cx43α1 is essential for adult heart conduction, further indicating that Cx43α1 deficiency may provide an arrhythmogenic substrate that could contribute to heart dysfunction (Gutstein et al. 2001). Knockout mice deficient in Cx45α7 died early in gestation, exhibiting conduction block as well as endocardial cushion defects (Kumai et al. 2000), whereas Cx40α5 knockout mice were viable but showed slowed conduction and a partial atrioventricular block (Bevilacqua et al. 2000; Kirchhoff et al. 1998, 2000; Simon et al. 1998). Recent knockin gene replacement studies in which Cx40α5 (Cx43KI40 mice) or Cx32β1 (Cx43KI32) were substituted for Cx43α1 showed no conduction abnormalities, indicating that heart conduction is independent of the unitary conductances of the gap junction channel (Plum et al. 2000).

In addition to their roles in heart conduction, analysis of the Cx43α1, Cx40α5, and Cx45α7 knockout mouse models showed that these connexin genes also play an essential role in heart morphogenesis. In recent studies reexamining the Cx40α5 knockout mouse phenotype, 16% of newborn homozygous Cx40α5 knockout mice were reported to die at birth with atrioventricular septation defects (Kirchhoff et al. 2000); in a second study, 33% of homozygous Cx40α5 knockout mice were reported to have outflow tract malformations consisting of double outlet right ventricle or Tetralogy of Fallot (Gu et al. 2003). Analysis of the Cx45α7 knockout
mice shows that this connexin gene is required for the development of the endocardial cushions, whereas Cx43α1 plays an essential role in heart outflow tract morphogenesis and development of the coronary arteries (see below).

**Cx45α7 and Development of the Endocardial Cushions**

Knockout mice deficient in Cx45α7 die of heart failure at embryonic day 10.5 (E10.5), showing a looping defect, reduced trabeculation, disrupted formation of the endocardial cushions, and a conduction block (Kumai et al. 2000). Significantly, expression of the calcium-dependent transcription factor, NF-ATc1, was inactivated in the endocardium. We note that NF-ATc1-deficient mice have defects associated with formation of endocardially derived valve tissues (de la Pompa et al. 1998, Kruger et al. 2000, Kumai et al. 2000, Ranger et al. 1998), thereby suggesting the possibility that Cx45α7 has a role in modulating calcineurin signaling required for NF-ATc activation. Although another study suggests that Cx45α7 knockout mice die from abnormal vascular development (Kruger et al. 2000), recent experiments using a floxed allele of Cx45α7, together with a myocardial specific Cre, showed that the requirement for Cx45α7 resides in the myocardium (Nishii et al. 2003). It should be noted that these conditional knockout mice did not have endocardial cushion defects, confirming that the cushion defects likely arise from a requirement for Cx45α7 in the endocardium (Nishii et al. 2003).

**Cx43α1 and Heart Outflow Tract Morphogenesis**

The gene for Cx43α1 was the first connexin gene to be knocked out (Reaume et al. 1995) and also the first to be linked to a human disease; mutations in the CT of Cx43α1 were reported to be associated with pulmonary atresia and VAH (Britz-Cunningham et al. 1995). Cx43α1 knockout mice survived to term, but expired at birth from pulmonary outflow obstruction. This result was surprising at the time because Cx43α1 was known to be expressed from the 4–8 cell stage and was expected to cause preimplantation lethality (Nishi et al. 1991). However, subsequent studies showed that at least half a dozen connexin genes (and perhaps more) are expressed in the preimplantation mouse embryo (De Sousa et al. 1997).

The cardiac phenotype of the Cx43α1 knockout mouse consisted of pulmonary outflow obstruction associated with conotruncal malformations and defects in the patterning of the coronary arteries (Reaume et al. 1995, Ya et al. 1998). The finding of outflow anomalies was unexpected because this particular connexin gene is not expressed in abundance in the outflow tract myocardium (Gourdie et al. 1992, van Kempen et al. 1991). However, as tissue remodeling in this region of the heart is known to require the activities of neural crest cells (Kirby & Waldo 1995), we generated several transgenic mouse models to examine the effects of manipulating Cx43α1 function in the cardiac neural crest cells.

Neural crest cells are migratory cells derived via an epithelial-mesenchymal cell transformation from the dorsal neural fold. They migrate in streams and are dispersed throughout the embryo, generating all the cells of the peripheral
nervous system, as well as providing melanocytes, cranial mesenchyme, and cells that contribute to the aortic arches and outflow tract septation complex. Cx43α1 is expressed in abundance in migrating neural crest cells and, moreover, migrating neural crest cells are functionally coupled (Lo et al. 1997). Significantly, transgenic mice with gain (CMV43 transgenic mice) or loss (FC transgenic mice) of Cx43α1 function targeted to neural crest cells displayed conotruncal heart defects and outflow obstruction (Ewart et al. 1997, Huang et al. 1998b, Sullivan et al. 1998). These findings suggest that the outflow anomalies in the Cx43α1 knockout mice do arise from the perturbation of cardiac neural crest cells. However, one puzzling fact was that heart defects in the CMV43 or FC transgenic mice were not identical to that of the Cx43α1 knockout mouse. Thus conotruncal pouches typically found at the base of the pulmonary outflow tract in the knockout mice were never observed in either transgenic mouse model (Lo et al. 1999). Additional studies have since shown that the formation of conotruncal pouches likely arises from defects involving not only the cardiac neural crest cells, but also another migratory cell population, the proepicardially derived cells (Li et al. 2002). The proepicardial cells are a mesothelial cell population found near the septum transversum and liver primordia. They delaminate over the surface of the heart to form the epicardium and also invade the heart, providing all of the vascular smooth muscle and endothelial cells of the coronary arteries. Our studies suggest that alterations in the migratory behavior of both cell populations underlie the

Figure 2 Altered neural crest cell migration in transgenic mice exhibiting deficiency or overexpression of Cx43α1. (a–f) The distribution of cardiac neural crest cells in E14.5 mouse fetal hearts was examined using a lacZ reporter transgene driven by the Cx43α1 promoter (Lo et al. 1997). Shown are the front (a–c) and side views (d–f) of X-gal stained hearts from wild-type (a, d), CMV43 (b, e) and Cx43α1 knockout mice (c, f). The white asterisk denotes presumptive neural crest cells in the conus in the closing seam of the aorticopulmonary septum. Note the increased abundance of lacZ-labeled neural crest cells in the CMV43 heart (b, white asterisk); lacZ-labeled neural crest cells were reduced in the Cx43α1 knockout heart (c, white asterisk). Black arrows denote bulging of the conotruncus in both CMV43 (e) and Cx43α1 knockout (c, f) hearts. Abbreviations: p, pulmonary trunk; a, aorta; rv, right ventricle. (g–i). Altered neural crest cell motility was indicated by time lapse videomicroscopy of neural crest explant cultures derived from E8.5 wild-type (g), CMV43 (h) and Cx43α1 knockout (i) mouse embryos. A ring of neural crest outgrowth can be observed surrounding the central dense mass of neuroepithelial tissue in the 24-h explant culture (g–i). The color lines represent the migration paths of individual neural crest cells observed over a 20-h interval; the black circles denote the position at time zero. Note that the migratory paths of Cx43α1 knockout neural crest cells were more tortuous than those of the wild-type (g) and CMV43 embryos (h). In addition, the migratory paths of the CMV43 neural crest cells (h) were longer, reflecting an enhanced rate of cell migration compared with that of the wild-type or knockout neural crest cells.
elaboration of the conotruncal heart malformations and coronary artery defects in the Cx43α1 knockout mouse (Huang et al. 1998a, Li et al. 2002).

**Cx43α1 and the Modulation of Cell Motility**

Development at the base of the heart, the region most severely affected in the Cx43α1 knockout mouse, requires the precise temporospatial regulation of
cardiac neural crest and proepicardial cells deployment to the heart. In the Cx43α1 knockout mice and our transgenic mouse models, the migratory behavior of the cardiac neural crest and proepicardial cells is altered. Neural crest explant experiments showed that CMV43 neural crest cells have an enhanced rate of migration, whereas the FC transgenic and Cx43α1-deficient neural crest cells have a reduced migration rate (Huang et al. 1998a) (Figure 2). These in vitro migration studies were confirmed with in vivo studies using a lacZ reporter transgene to track the deployment of neural crest cells (Huang et al. 1998b, Lo et al. 1997). Thus lacZ-labeled crest cells were increased in abundance in the outflow tract in CMV43 transgenic embryos; in Cx43α1 knockout mouse embryos there was a marked reduction (Huang et al. 1998a) (Figure 2). Overall, these studies indicate that the apparent rate of neural crest cell migration increased or decreased in parallel with the up- or down-regulation of dye coupling in the CMV43 versus Cx43α1-deficient or FC neural crest cells (Huang et al. 1998a). Given this, we previously hypothesized that gap junctions may help coordinate the deployment of neural crest cells to the heart by mediating the cell-to-cell movement of second messengers and other cell signaling molecules involved in regulating cell locomotion (Huang et al. 1998a, Lo & Wessels 1998). However, the tenability of this hypothesis has been called into question given the results of more recent studies using time lapse videomicroscopy, together with motion analysis, to examine more closely the changes in neural crest cell motility in these and other transgenic and knockout mouse models (Xu et al. 2001). These studies showed no consistent correlation between changes in the level of dye coupling and alterations in the directionality versus speed of cell locomotion in neural crest cells derived from the CMV43, FC transgenic, and Cx43α1 knockout mice (Huang et al. 1998a) (Table 1). Such discrepancies were also found in dye coupling levels and cell motile behavior in neural crest cells derived from the N-cadherin and Wnt1 knockout mice (Xu et al. 2001) (Table 1). Particularly striking is the finding that Wnt1-deficient neural crest cells with very low levels of dye coupling nevertheless exhibited cell migration behavior indistinguishable from wild-type neural crest cells (Xu et al. 2001) (Table 1). Significantly, such Wnt1-deficient neural crest cells continued to express Cx43α1 abundantly at the cell surface (Xu et al. 2001). This divergence between changes in neural crest cell motility and alterations in the level of dye coupling suggests that the modulation of cell motility by Cx43α1 may involve a novel function that is independent of gap junction channel activity. We note that several other studies have also shown no consistent correlation between changes in the level of gap junctional communication and alterations in cell proliferation or motile cell behavior (Huang et al. 1998a, Moorby & Patel 2001, Qin et al. 2002). In addition, the ectopic expression of the CT portion of Cx43α1 was observed to be as effective as wild-type Cx43α1 in growth suppression (Dang et al. 2003, Moorby & Patel 2001, Zhang et al. 2003). In yet another study, a mutation in the second extracellular loop of Cx43α1 was shown to prevent cell surface trafficking, yet this mutant Cx43α1 protein also retained its ability to suppress growth (Olbina & Eckhart 2003). Below we outline a model in which Cx43α1 may mediate cell signaling independently of its channel
function. It should be noted that a dual functional role for a cell junction protein is not unprecedented, as this is well described for a number of cell junction and junction-associated proteins such as E-cadherin and β-catenin (Ben-Ze’ev 1999, Chunthapong et al. 2004, Hagen et al. 2004).

### A SPECULATIVE MODEL

Gap junctions, given their hydrophilic membrane channels, are generally assumed to modulate various biological processes through mediating the passive movement of ions and second messengers. Although the gap junction channel undoubtedly is important and essential for some biological processes, a separable signaling function is suggested by the lack of a consistent correlation between changes in the level of gap junctional communication and alterations in cell proliferation or motile cell behavior.

We propose that Cx43αl, through interactions with a diverse array of protein binding partners, including signaling proteins (α/β-catenin, p120ctn), structural proteins (ZO-1, caveolin-1), membrane proteins (cadherins), and proteins that interact with or are part of the cell cytoskeleton (α-actinin, microtubule), may cross

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**TABLE 1** Alterations in neural crest cell motility not correlated with dye coupling levels

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Dye coupling</th>
<th>Directionality</th>
<th>Speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx43α1 KO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>+/−</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>−/−</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>CMV43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontransgenic</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>transgenic</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>FC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontransgenic</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Wnt-1 KO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
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<tr>
<td>+/−</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>−/−</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
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<tr>
<td>N-cadherin KO</td>
<td></td>
<td></td>
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<tr>
<td>+/+</td>
<td>↓</td>
<td>↓</td>
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<tr>
<td>+/−</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>−/−</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

*Huang et al. 1998a, Xu et al. 2001; C.W. Lo, unpublished data. ↓, decrease; ↑, increase; ↔, no change.
talk with cell signaling pathways that regulate cell adhesion, cell motility, and the actin cytoskeleton (Figure 3). Several protein kinases, including Src, PKC, and MAPK (PKs) can phosphorylate the CT of Cx43α1, potentially altering not only gating of the channel but also protein interactions that may be important in cell signaling. In addition, transcriptional effects may be elicited via p120ctn/Kaiso, or β-catenin-TCF/LEF, resulting in additional long-term effects through gene expression changes.
In short, our model places Cx43α1 as an integral member of a large signaling complex that includes β-catenin, p120ctn, N-cadherin, and a host of other proteins, and together, they facilitate cross talk to affect the coordinate regulation of cell-cell communication with cell adhesion, cell motility, and possibly other cell processes such as cell growth and proliferation. The fact that connexin mutations linked with human diseases are generally not restricted to protein domains required for channel function is consistent with a separable nonchannel activity attributable to connexin proteins.

One of the important challenges in the future is to examine the functional properties of the various human connexin mutations, and, using tissue culture and animal models, determine how they may contribute to the various disease processes. In the context of this model, it is possible to envision that connexin mutations perturb the assembly or function of this signaling complex, thereby providing a unified mechanism that could account for the wide spectrum of human diseases associated with connexin mutations.

ACKNOWLEDGMENTS

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Figure 3  Cx43α1 protein interactions and cell-cell signaling. Cx43α1 has a diverse array of potential protein-binding partners, including signaling proteins (α/β-catenin, p120ctn), structural proteins (ZO-1, caveolin-1), membrane proteins (cadherins) and proteins that interact with or are part of the cell cytoskeleton (α-actinin, microtubule). Illustrated is the putative arrangement of a Cx43α1/cadherin complex anchored to the actin cytoskeleton through a multi-protein complex containing α-catenin/β-catenin and various other actin-binding proteins such as α-actinin, vinculin, and ZO-1. ZO-1, which can directly bind Cx43α1, may also help anchor Cx43α1 to the actin cytoskeleton, whereas interactions with caveolin-1 may target Cx43α1 to lipid rafts and recruit Cx43α1 to membrane domains enriched in other signaling proteins. Rac1/cdc42/IQGAP1, as well as the binding of p120ctn to cadherin’s juxtamembrane region may regulate the adhesive strength of adherens junction, and at the same time, these signaling pathways together with p120ctn/Rho GTPases may cross talk with Cx43α1 to affect changes in cell motility. Several protein kinases, including Src, PKC, and MAPK (PKs) can phosphorylate the CT of Cx43α1, potentially altering not only gating of the channel but also protein interactions that may be important in cell signaling. It should be noted that p120ctn binding with the transcriptional factor Kaiso or β-catenin interactions with TCF/LEF may elicit downstream transcriptional effects that can result in additional long-term effects through gene expression changes.
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