The Unstoppable Connexin43 Carboxyl-Terminus

New Roles in Gap Junction Organization and Wound Healing

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ABSTRACT: **Intercellular connectivity mediated by gap junctions (GJs) composed of connexin43 (C×43) is critical to the function of excitable tissues such as the heart and brain. Disruptions to C×43 GJ organization are thought to be a factor in cardiac arrhythmias and are also implicated in epilepsy. This article is based on a presentation to the 4th Larry and Horti Fairberg Workshop on Interactive and Integrative Cardiology and summarizes the work of Gourdie and his lab on C×43 GJs in the heart. Background and perspective of recently published studies on the function of C×43-interacting protein zonula occludens-(ZO)-1 in determining the organization of GJ plaques are provided. In addition how a peptide containing a PDZ-binding sequence of C×43, developed as part of the work on cardiac GJ organization is also described, which has led to evidence for novel and unexpected roles for C×43 in modulating healing following tissue injury.**

KEYWORDS: **gap junctions; connexin; electrical coupling; conduction; wound healing**

INTRODUCTION

The gap junction (GJ) plaque is an aggregate of intercellular channels that provides for exchange of nutrients, messengers, and ions between the cytoplasms of neighboring cells.[1–3] The channels comprising the GJ are made up

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of proteins encoded by the connexin gene family.[4] Assembly of GJs from connexins is understood to proceed in multiple steps.[5] Following translation, six connexins oligomerize into a connexin hemichannel, which is then trafficked to the cell membrane. The hemichannel docks with a second hemichannel from the apposed membrane of an adjacent cell to form an intercellular channel. In a process that may occur in association with the docking step, intercellular channels aggregate to form the GJ plaque. Phosphorylation is a posttranslational modification that appears to be of particular significance to the function and life cycle of connexins.[6,7]

In the heart and other excitable tissues, electrotonic couplings mediated by GJ aggregates contribute to uniform propagation of electrical activation.[8–13] Disruptions to GJ organization are thought to be a factor in cardiac arrhythmias[8–16] and have been implicated in a form of epilepsy.[17] As cellular circuits defined by GJs are recognized as important in health and disease, the mechanisms determining the size and positioning of GJs between cells are attracting increasing attention by researchers. Moreover, the prospect that the connexin subunits of GJs may have functions that are independent of intercellular communication is arousing interest and debate.[18–21]

Here, we review our work on the main GJ protein in the mammalian heart α1 connexin43 (C$\times$43), with particular focus on its interaction with zonula occludens (ZO)-1, a protein directly interacting with the carboxyl terminus of C$\times$43. Among other information, a perspective is given on our studies of how ZO-1 interaction with C$\times$43 may determine GJ organizational patterns and we also outline evidence for a novel and unexpected role for peptide fragments derived from the C$\times$43 carboxyl terminal (Ct) in wound healing.

CARDIAC GJs SHOW HIGHLY REGULAR PATTERNS OF SPATIAL ORGANIZATION

The main GJ proteins in the mammalian heart are Cx40, C$\times$43, and Cx45.[8–16,20–31] C$\times$43 is expressed in atrial myocardium and parts of the conduction system, but is found most prominently in the ventricle where numerous GJs composed of C$\times$43 couple working ventricular myocytes together. Cx40 and Cx45 are also expressed in atrial myocardium and comprise GJs within tissues of the pacemaking and conduction system. There is evidence for conservation of Cx40 and Cx45 in the hearts of nonmammalian vertebrates.[28,32,33] However, mammals appear to be unique among the chordates in expressing C$\times$43 in cardiac muscle.[33] This phylogenetic oddity is made even more curious by the high abundance at which C$\times$43 is expressed in hearts of all mammalian species relative to the near undetectable levels of connexin of any type present in the working myocardium of adult nonmammals (reviewed in Ref. 9)

The large numbers of C$\times$43 GJs in the working ventricular myocardium of the adult mammal display characteristic and highly regular patterns of spatial

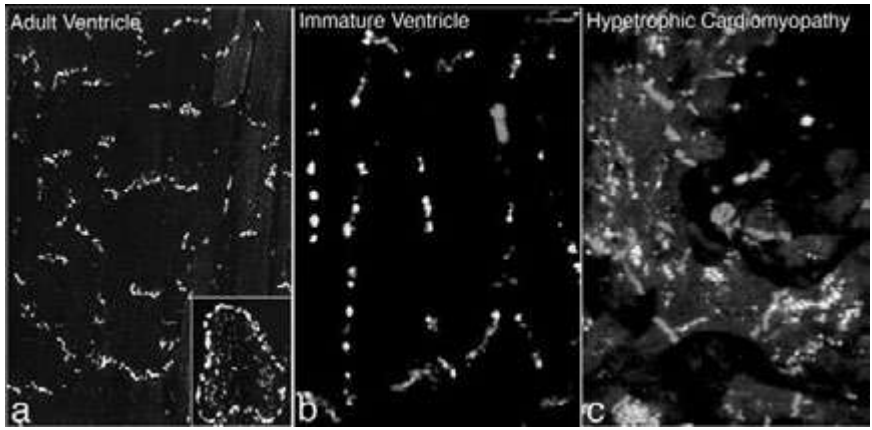

FIGURE 1. C×43 GJ organization in the adult, immature, and diseased mammalian ventricular myocardium. (**A**) Immunolabeled C×43 GJs are largely polarized at myocyte intercalated disks in the normal adult mammalian ventricle.[25] Inset (A) Large C×43 GJs form prominent rings encircling the intercalated disk. (**B**) C×43 GJs (green) are distributed along lateral membranes and dissociated from the heaviest concentrations of intercellular adhesion junctions (as delineated by red desmoplakin-labeled desmosomes) at intercalated disks in the immature mammalian ventricle.[40] (**C**) C×43 GJs (green) are dissociated from desmoplakin-labeled (red) intercalated disks in a zone of myofiber disarray in a human patient with hypertrophic cardiomyopathy (HCM).[44] The dissociation in electrical and mechanical intercellular junction distribution in HCM is reminiscent of the pattern seen during developmental remodeling of C×43 GJs from lateral distributions to intercalated disks (i.e., as in FIG. 1 B). Shown in color in the online version.

organization.[9,10,15,16,25,26,33–40] The polarized localization of GJs at intercalated disks is perhaps the most widely appreciated of these characteristics (FIG. 1 A). Further evidence of the regulated order of GJs in the ventricle can be recognized at the scale of the intercalated disk. Confocal imaging reveals ring-like arrangements of large GJs encircling the perimeter of disks[25,35] (inset FIG. 1 A). An even finer substructure has been discriminated within the plaque of individual GJs. Ultrarapid freezing has shown GJs at the intercalated disk to be irregularly packed structures comprising small rafts of uniformly aggregated channels separated by narrow particle-free aisles.[36]

POSTNATAL REMODELING OF LATERAL *Cx43* GJS INTO INTERCALATED DISKS

Characterization of the developmental processes accounting for the spatial order of C×43 GJs in ventricular myocardium has been a long-term focus of the lab. The first descriptions of how C×43 GJs change from lateral distributions at side-by-side appositions between myocytes to become polarized at

intercalated disks over postnatal growth of the rat heart were provided by Gourdie and co-workers[26,37]—a phenomenon later confirmed by others in mice[38] and humans.[39] Subsequently, we showed that postnatal remodeling of GJs was preceded by striking increases in intercellular adhesion junctions at intercalated disks and proposed that this transient dissociation in the distribution of electrical and mechanical junctions (e.g., FIG. 1 B) was key to understanding how GJs progressively assume mature organizational patterns in ventricular myocardium following birth.[40] Our proposal was that GJs are maintained within the intercalated disk (and conversely lost from lateral membranes over postnatal development) owing to a stabilizing proximity of disk-localized GJs to adherens junctions and desmosomes in the mechanically active tissue.

The concept that intercellular mechanical junctions are critical for GJ stability has received support from studies by other workers. First, it has been demonstrated that the components of the adherens junction and C×43 GJ form a multiprotein complex in NIH-3T3 cells.[41] Second, a hierarchical interdependence between maintenance of GJs and calcium-dependent adhesion junctions has been shown in Drosophila embryos.[42] Third, elegant transgenic work from Radice and co-workers have demonstrated that cardiac-specific knockout of N-cadherin in postnatal mice results in dissolution of intercalated disk structure, loss of membrane localization of C×43 GJs, and progressive lethality from ventricular arrhythmia.[43] Finally, evidence for interdependence of the stability of electrical and desmosomal intercellular junctions have come from studies of human patients with hypertrophic cardiomyopathy and inherited diseases of the myocardium (FIG. 1 C).[44,45]

INTERACTION OF THE ACTIN-BINDING MAGUK PROTEIN ZO-1 WITH Cx43

In addition to describing processes giving rise to the characteristic organizational patterns of GJs in the mammalian heart, we are also interested in the intracellular machinery governing the generation of this spatial order. The actin-binding protein ZO-1 has been proposed as a candidate for regulating cardiac GJ organization at the molecular level[46,47] and has been a major focus of our research of the last 5 years. Originally discovered in association with the tight junction, ZO-1 is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins that function in targeting, signal transduction, and determination of cell polarity.[48] In immunoprecipitation studies in cultured cells, and in yeast-two hybrid analyses, it was shown that C×43 interacts with the second PDZ domain of ZO-1 via a short PDZ-binding motif at extreme carboxyl terminus of C×43.[46,47]

Initially, it was assumed that the function of ZO-1 interaction with C×43 was analogous to the presumed role of ZO-1 as a scaffolding protein at the tight junction. Namely, ZO-1 was thought to stabilize GJs at the plasma membrane

by linkage to the actin cytoskeleton. However, subsequent reports have indicated that protein–protein interactions between connexins and ZO-1 encompass other, more dynamic functions. In particular, changes in ZO-1–C×43 interactions have been noted during remodeling of the organization and subcellular distribution of C×43 GJs in various cell types.[49–54]

Interaction between C×43 and ZO-1 during Remodeling of GJ Organization

Based on reports that ZO-1 interacts with C×43,[46,47] we investigated ZO-1 and C×43 association in the rat ventricular myocardium *in vivo*.[52] Our initial goal was to probe whether ZO-1 served within an actin scaffold, stabilizing GJs at the intercalated disk by direct interaction with C×43. However, results from immunofluorescence, immunoelectron microscopic, and immunoprecipitation studies did not seem to fit well with this proposal. First, we found that levels of co-localization between C×43 and ZO-1 in the ventricle *in vivo* were relatively modest. Second, it was determined that enzymatic dissociation of myocytes, a treatment causing loss of GJs from the sarcolemmal membrane,[55] increased C×43-ZO-1 association levels. Subsequently, other workers have confirmed that GJ-localized connexins and ZO-1 show modest levels of co-localization *in vivo*.[56,57] Moreover, others have confirmed increased association between C×43 and ZO-1 following induction of GJ re-distribution from the membrane to the cytoplasm.[54]

Fusion of GFP to the C×43 Carboxyl Terminus Yields a Connexin Molecule That Is Incompetent to Interact with ZO-1 and a Loss of GJ Size Control

We next attempted to observe the dynamics of C×43 and ZO-1 interaction directly in living cells.[58,59] To achieve this goal, we generated HeLa cell lines stably expressing C×43-GFP (FIG. 2). However, it was found that GJ plaques formed by C×43-GFP excluded ZO-1.[59] We later realized that this loss of ZO-1 co-localization was consistent with previous results from Giepmans and co-workers.[60] In pull-down studies, this group had shown that Ct tagging of C×43 with GFP, as well as other sequences, resulted in loss of function of the C×43 PDZ-binding domain. Although a setback, as outlined in Hunter *et al.*,[58] we soon appreciated that the loss of competence of the C×43-GFP mutant protein in interacting with ZO-1 provided a useful opportunity.

As is illustrated in FIGURE 2, the GJs formed by C×43-GFP molecules are abnormally large, relative to GJs formed by wild-type C×43 in HeLa cells (e.g., FIG. 2 B) and also in comparison with C×43 GJs observed *in vivo* (e.g., FIG. 1). To determine whether the organization of the large C×43-GFP GJs

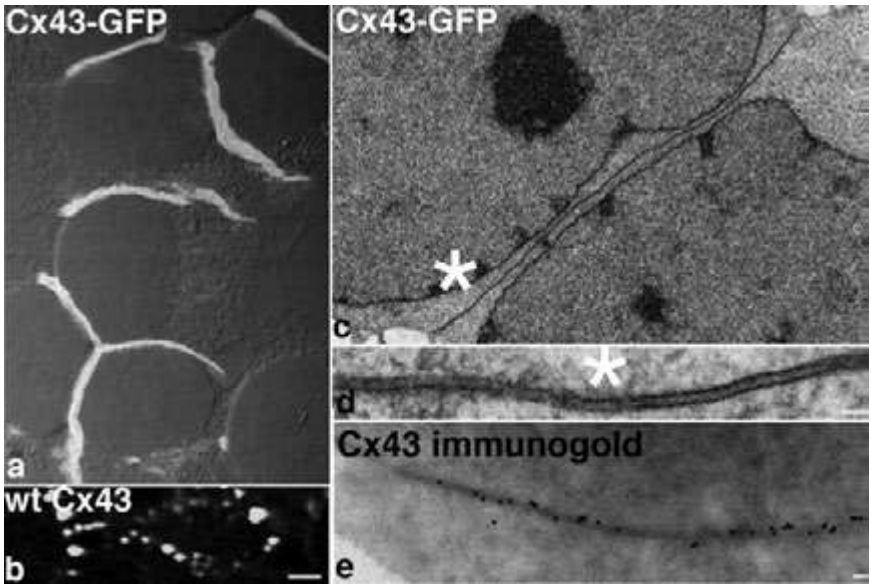

FIGURE 2. A C×43-GFP construct that is incompetent to interact with ZO-1 (see Refs. 58 and 59). (**A**) HeLa cells stably expressing the C×43-GFP construct form large, sheet-like GJs. White-gray signal at cell borders is GFP fluorescence. Darker gray background is a differential interference contrast image of the HeLa cells. (**B**) Normally sized and distributed punctate GJs in HeLa cells stably expressing wild-type (wt) C×43 as immunolabeled by anti-C×43 antibodies. (**C**) Transmission electron micrograph of an ultrathin section showing a large GJ between two C×43-GFP expressing HeLa cells. Note that the GJ spans the entire width of the interface between the two cells. Asterisk marks the region of GJ shown at higher resolution in (**D**). (**E**) anti-C×43 immunogold labeling electron micrograph of a GJ between two HeLa-C×43-GFP cells. Scale: A, B = 1 μm; D, F = 40 nm.

could be altered or rescued to be more *in vivo*-like, we expressed wild-type C×43 at varying levels in the HeLa cell line.[58] As relative amounts of wild-type C×43 increased, GJs in the C×43-GFP expressing cell line assumed regular size distributions and organizations. Pulse chase labeling studies with [^{35}S]-methionine established that there was little difference in half-life between C×43-GFP and wild-type C×43 when expressed singly or together, suggesting that C×43-GFP GJs did not get larger as a result of slowed turn-over of the mutant protein.[58] In experiments using immunoprecipitation of metabolically labeled connexins, we determined that an average stoichiometry of 1 C×43-GFP to 5.4 wild-type C×43 molecules was required for normalization of GJ organization.[58] This result suggested that it was necessary for each six-subunit hemichannel aggregated within a GJ needed to be mostly composed of wild-type C×43 for size control to be recovered for the GJ as a whole.

Binding of ZO-1 Regulates the Rate of Accretion of C×43 Hemichannels to the GJ

In Zhu *et al.*,[61] we reported high-resolution analyses of ZO-1 localization patterns at C×43 GJs in cultured monolayers of neonatal myocytes. These studies revealed a preferential association of ZO-1 with the perimeter of the C×43 GJ plaque. Subsequently, in Hunter *et al.*,[58] we determined that the rescue of size control in C×43-GFP expressing HeLa cells by expression of wild-type C×43 was associated with the re-establishment of localization of ZO-1 to the GJ periphery. In earlier work, Ellisman and co-workers[62] identified the GJ periphery as a site of assembly for new junctional membrane. Based on our data, we hypothesized that ZO-1 may be regulating GJ size via governing the rate of recruitment of C×43 hemichannels at the plaque periphery.

Our next step was to develop a strategy for inhibiting ZO-1 binding in situ at already established GJs between C×43-expressing cells. To achieve this goal, we synthesized a short peptide comprised of an antennapedia internalization sequence linked to the PDZ-binding domain of C×43[58] (FIG. 3 A). This rationally designed inhibitor of ZO-1 interaction with C×43 was shown to specifically interact with the PDZ2 domain of ZO-1 and inhibited PDZ2 interaction with normal full-length C×43 *in vivo* (FIG. 3 B). Importantly, we demonstrated that the inhibitor measurably reduced levels of ZO-1 localization at the GJ plaque edge *in vivo* (FIG. 3 C) and increased GJ size in neonatal myocytes and in HeLa cells expressing wild-type C×43 (FIG. 3 C).

Consistent with the concept that ZO-1-C×43 binding influences GJ size via affecting recruitment of C×43, changes in GJ size and ZO-1 co-localization induced by the peptide were similar, albeit less pronounced, to those observed in the C×43-GFP expressing HeLa cells. Western blotting indicated that this increase in GJ size was not associated with an increase in C×43 abundance.[58] These results were interpreted as indicating that the peptide caused redistribution between different parts of the cellular pool C×43—i.e., as opposed to increasing bulk levels of the protein. To probe this hypothesis, detergent fractionation was used to segregate the cellular pool of C×43 into junctional and nonjunctional components.[58] Consistent with the increase in GJ size observed in response to the peptide, fractionation of C×43 in this assay indicated a shift in C×43 from nonjunctional to junctional pools following peptide treatment.

Based on the microscopic and biochemical evidence, we concluded that ZO-1 limits C×43 GJ size via influencing rate of accretion of channels to the GJ. This rate-affecting mechanism is probably most active at the periphery of the GJ, where assembly of new junctional membrane proceeds. As such, ZO-1 may be conceived as somewhat like a "gate keeper" regulating, though not inhibiting, admission of hemichannels to the GJ channel aggregate. An interesting possibility is that regulation of ZO-1 interaction level provides a mechanism for altering C×43 hemichannel density in the plasma membrane. In such a scenario, dynamic adjustment to ZO-1 levels at the plaque edge might

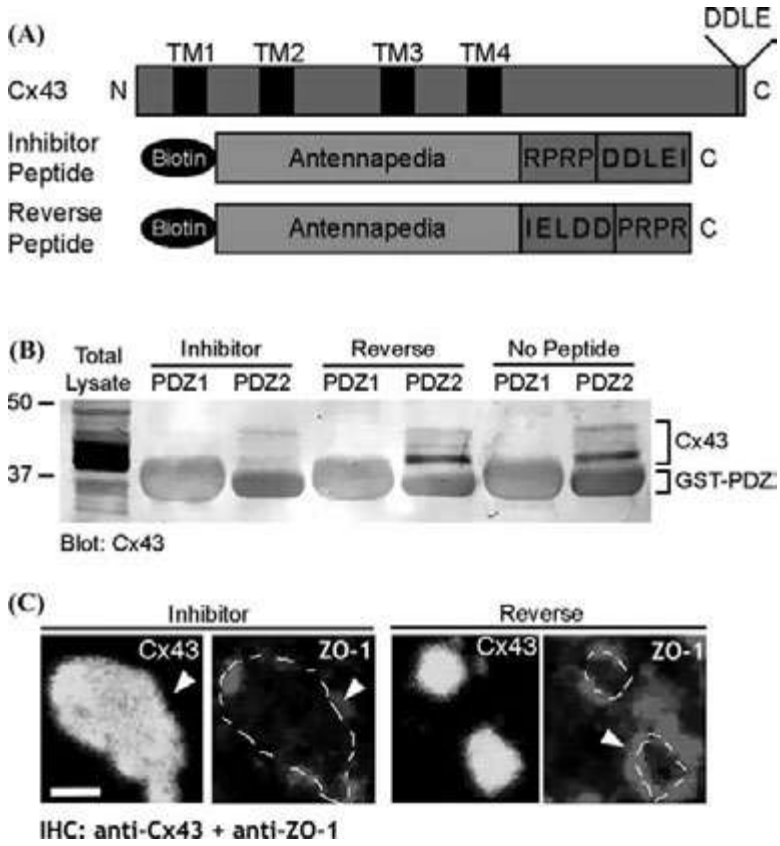

FIGURE 3. A peptide that specifically binds ZO-1 and blocks interaction with C×43.[60] (**A**) C×43, inhibitory peptide and reverse control peptide. (**B**) Blot showing that C×43 pulled down from HeLa lysates by GST-PDZ2 beads was reduced by inhibitor peptide. (**C**) In HeLa cells the peptide reduces C×43 GJ size and ZO-1 localization at the GJ perimeter as compared to reverse peptide treated cells. Two channel views of C×43 and ZO-1 signals are shown from a double immunolabeling. The perimeter of GJ plaques is indicated on the ZO-1 channel by dotted lines. Scale: C = 1 μm.

prompt conversion of dispersed connexons into GJ aggregates, reducing the numbers of hemichannel available for opening in response to an appropriate prompt.

ROLE OF PEPTIDE CONTAINING Cx43 PDZ DOMAIN IN WOUND HEALING

Intercellular communication between cells is a key aspect of tissue repair following injury.[63] While specific mechanisms remain to be characterized,

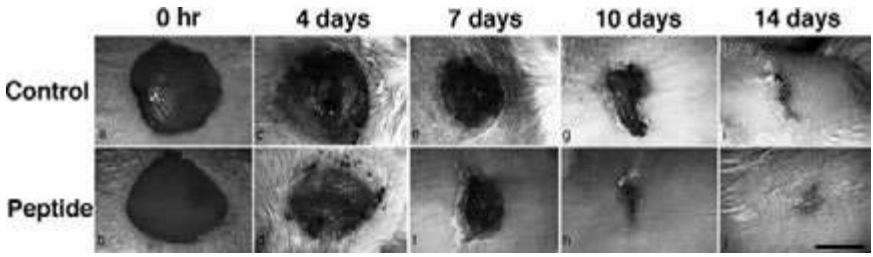

FIGURE 4. The inhibitory peptide improves healing of a excisional skin injury in adult mice. Anesthetized adult mice had 8-mm wide excisional skin injuries made by scalpel down to the underlying muscle in the dorsal mid line between the shoulder blades [i.e., as shown in (**A**) and (**B**)]. Hundred microliters of a solution of 30% pluronic gel containing either no (control) or dissolved peptide (FIG. 3 A) at a concentration of 100 μM was then applied to the injuries. Control or peptide containing gel was applied again subsequently 24 h. The peptide-treated excisional injury (B, D, F, H, I) was less inflamed, healed faster, and had better appearance than the control injury (A, C, E, G, J) over the 14-day time course. Note that images of the same injury on the same animal are shown at the different time points during the healing time course. Scale = 4 mm.

connexins appear to have functions in coordinating inflammatory response, wound repair, and scar tissue formation following injury.[63–66] Of particular note, downregulation of C×43 mRNA by antisense targeting has been shown to accelerate healing and reduce scar differentiation in a wound model of mouse skin.[64] Moreover, ZO-1 localization patterns in migrating fibroblasts in corneal injury have recently led Taliana and co-workers to propose roles for ZO-1 in coordination of cell migration and adhesion during wound healing.[67]

Based on the literature and experiments on cell migration from "scratch wounds" of NIH-3T3 fibroblast monolayers, we undertook studies of the effects of the PDZ-targeting peptide (as shown in FIG. 3 A) on skin wound healing in a mouse model *in vivo*.[68] The data indicated that relative to controls, the peptide increased the healing rate of excisional wounds and promoted the generation of skin with more normal-looking histology following closure of the injury (FIG. 4). Additionally, strength testing of 90-day-old healed wounds indicated peptide treatment resulted in significantly improved mechanical properties compared to controls. The effects of the peptide on wound healing were in some respects not similar to those reported for C×43 antisense treatment,[64] and it is unclear whether the C×43 antisense or the peptide designed to target ZO-1 act via related mechanisms.

CONCLUDING REMARKS

Can results obtained from skin be applied to a cryo-injury model of the heart? A further interesting question is whether our synthetic peptide is enhancing

an endogenous response of mammalian tissues to injury involving the C×43 carboxyl terminus. This issue brings us to a point raised at the outset of this review. It is widely accepted that orderly C×43-based coupling between cardiac muscle cells is required for the stability of the heartbeat. However, it is remains unclear why mammals express C×43 in the heart at such abundance, or indeed at all. Efficient electrical coupling is achieved between heart muscle cells in nonmammalian chordates from tunicates to birds in the absence of C×43, and with only a tiny fraction of the intercellular channels that are apparently required in mammals.[9] Cardiac-specific gene knockout of C×43 in mice, while eventually causing lethal arrhythmia during postnatal life, is in fact consistent with regular propagation of cardiac activation up to the point that these transgenics succumb to conduction instability.[69] Could it be that C×43 is required for both the stability of the heartbeat and cardiac injury response in mammals? C×43 is also expressed at high levels in mammalian skin,[63–66] as well as other tissues, especially in embryonic development.[70] Apart from perfunctory assumptions about the importance of communication to cell and tissue homeostasis, the actual requirement for abundant C×43 expression in tissues like the skin, as in the heart, is not that well understood. In our ongoing work, we seek to determine whether C×43 has a generic role in mediating response to injury in mammals. Also, it will be of great interest to determine whether the peptide based on the PDZ-binding domain of C×43 shifts the balance during healing from fibrotic scar tissue to regeneration of cardiac muscle in the injured heart.

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