Connexin43 Remodeling Caused by Inhibition of Plakophilin-2 Expression in Cardiac Cells

Eva M. Oxford,* Hassan Musa,* Karen Maass, Wanda Coombs, Steven M. Taffet, Mario Delmar

Abstract—Desmosomes and gap junctions are distinct structural components of the cardiac intercalated disc. Here, we asked whether the presence of plakophilin (PKP)2, a component of the desmosome, is essential for the proper function and distribution of the gap junction protein connexin (Cx)43. We used RNA silencing technology to decrease the expression of PKP2 in cardiac cells (ventricular myocytes, as well as epicardium-derived cells) obtained from neonatal rat hearts. We evaluated the content, distribution, and function of Cx43 gap junctions. Our results show that loss of PKP2 expression led to a decrease in total Cx43 content, a significant redistribution of Cx43 to the intracellular space, and a decrease in dye coupling between cells. Separate experiments showed that Cx43 and PKP2 can coexist in the same macromolecular complex. Our results support the notion of a molecular crosstalk between desmosomal and gap junction proteins. The results are discussed in the context of arrhythmogenic right ventricular cardiomyopathy, an inherited disease involving mutations in desmosomal proteins, including PKP2. (Circ Res. 2007;101:703-711.)

Key Words: adenovirus ■ arrhythmogenic right ventricular cardiomyopathy ■ connexin43 ■ gap junction ■ plakophilin 2

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited disease that presents with sustained monomorphic ventricular tachycardia and sudden cardiac death. The disease is characterized by progressive fibrofatty infiltration of the myocardium, most prominent in the free wall of the right ventricle.1 Recent studies have linked ARVC with mutations in proteins of the cardiac desmosome;2 a component of the intercalated disc essential for mechanical coupling between cardiac cells.3 It is estimated that as many as 70% of the mutations linked to familial ARVC are in the gene coding for plakophilin (PKP)2,4 a 98-kDa desmosomal protein. PKP2 interacts with plakoglobin, desmoplakin, and the desmosomal cadherins via its amino terminal (“head”) domain.5-6 Loss of PKP2 destabilizes the desmosome,7 and its genetic deletion in mice leads to rupture of the myocardial wall during the embryonic stage.7

Loss of desmosomal integrity could lead to disruption of mechanical function in hearts afflicted with ARVC; yet, the latter does not directly explain the highly arrhythmogenic nature of the disease, particularly in cases in which life-threatening arrhythmias occur in the absence of severe displacement of myocardium with fatty or fibrous tissue.8 Recently, Saffitz and colleagues proposed that disruption of mechanical coupling may lead to loss of gap junction-mediated electrical communication between cells.8-10 This hypothesis awaits confirmation in a cellular model in which protein expression can be manipulated and intercellular communication can be assessed directly.

Here, we used small interfering (si)RNA technology to silence PKP2 expression in neonatal cardiac cells, and we explored the effect of loss of PKP2 expression on the distribution and function of gap junctions. Our studies focused primarily on 2 cell populations: cardiac myocytes and epicardium-derived cells (EPDCs). Although the importance of cardiac myocytes in the context of ARVC and arrhythmias seems self-evident, a possible role for EPDCs in ARVC has not been described. Yet, as progenitors of the cardiac fibroblast cell lineage, the function of EPDCs deserves attention. Our results show that loss of PKP2 leads to a redistribution of connexin (Cx)43 inside the cell, loss of gap junction plaques detectable by immunofluorescence, and reduction in lucifer yellow (LY)-permeable gap junctions between cells. Additional studies show that Cx43 and PKP2 can coexist in a common macromolecular complex. This is the first demonstration of a link between PKP2 disruption and loss of Cx43-mediated cell–cell communication. Our data open a new avenue for the understanding of the molecular mechanisms that may be responsible for ARVC.

Materials and Methods
All details for methods are provided in the online data supplement at http://circres.ahajournals.org. Brief descriptions are presented below.
Cell Culture and PKP2 Silencing

Experiments were conducted in neonatal rat primary cell cultures. After dissociation, cells were resuspended in supplemented M199 media and preplated for 2 hours to allow for nonmuscle cells to attach to the plate. These dishes were used for experiments conducted in “preplated” cells (see under Results). After preplating, myocytes were plated to 70% confluence and maintained in supplemented DMEM media at 37°C.

Preparation of epicardial–mesenchymal cells (EPDCs) followed the method of Chen et al. Hearts from 1- to 4-day-old rats were excised, and ventricles were dissected. Each ventricular section was cut into 4 pieces, and each piece was placed epicardium-side down onto a 60-mm dish coated with 0.1% gelatin. Ventricular pieces were covered with supplemented DMEM media. Ventricular sections were removed after 4 days of culture. After 3 additional days, cells were trypsinized and plated to 70% confluence.

We used viral transfer technology to silence the expression of PKP2 in neonatal rat ventricular myocytes (NRVMs). Cells in culture were infected with adenosine containing short hairpin (sh)RNA for PKP2 (shRNA-PKP2). Cells untreated or treated with virus coding for green fluorescent protein (GFP) were used as controls. An alternative construct, predicted as a potential PKP2 silencer but shown not to interfere with PKP2 expression, was used as an additional control (shRNA-PKP2Ø). Unless otherwise indicated, experiments were conducted at 100 multiplicities of infection (mois) for shRNA-PKP2, 25 mois for GFP, and 100 mois for RNA-PKP2Ø and were performed 5 days after infection.

For PKP2 silencing in EPDCs, plates were separated in 3 groups: untreated, treated with Lipofectamine and the silencing construct (stealth RNAi interference [RNAi]; Invitrogen), or treated with Lipofectamine and a scrambled oligonucleotide. The scrambled silencer but shown not to interfere with PKP2 expression, was used as an additional control (shRNA-PKP2Ø). Unless otherwise indicated, experiments were conducted at 100 multiplicities of infection (mois) for shRNA-PKP2, 25 mois for GFP, and 100 mois for RNA-PKP2Ø and were performed 5 days after infection.

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Immunochemical and Functional Assays

All immunochemical protocols, as well as the production of recombinant glutathione S-transferase (GST) fusion proteins and the GST pull-down assays, followed standard techniques. Details are provided in the online data supplement.

Dye transfer through gap junctions in NRVM cell pairs followed the method described by Valiunas et al. Dye transfer in monolayers of EPDCs followed conventional methods, and details are provided in the online data supplement.

Results

Loss of PKP2 Expression in NRVMs After shRNA-PKP2 Treatment

To characterize the effect that loss of PKP2 expression has on function and distribution of Cx43, NRVMs in culture were infected with adenovirus containing a PKP2-silencing sequence (shRNA-PKP2). Figure 1 shows an example. PKP2 was present in control conditions and after transfer of CDNA coding for enhanced GFP (control) but decreased significantly after shRNA exposure in a manner that was dependent on the viral concentration used. Subsequent experiments were conducted using 100 moi for shRNA-PKP2. For quantification, each band density was measured relative to its corresponding actin control. Treatment with 100 moi shRNA-PKP2 caused a decrease in the density of the PKP2 signal recorded 5 days after exposure to virus to a level that was 10.80±2.23% of control (mean±SEM; n=8). In contrast, the actin-calibrated PKP2 signal obtained in cells treated with an enhanced GFP virus was not different from that recorded from untreated cells (101.36±5.23%; n=4). We used this experimental model to assess the effect of PKP2 silencing on the distribution of Cx43.

Effect of PKP2 Silencing on Content and Distribution of Cx43

Figure 2A shows the immunolocalization of Cx43 and PKP2 in NRVMs. Colocalization to the site of cell–cell contact was apparent in untreated conditions (Figure 2A, left images) and when cells were infected with virus coding for a construct that failed to silence PKP2 (ie, shRNA-PKP2Ø; middle images; see also Figure 2B). Yet, loss of PKP2 expression induced by shRNA-PKP2 correlated with a drastic redistribution of Cx43 (Figure 2A, right images); few gap junction plaques were detectable, and, instead, Cx43 was found mostly within the intracellular space. Similar results were obtained in 4 experiments. Consistent with previous observations, loss of PKP2 expression also led to significant remodeling of desmoplakin and of desmin in NRVMs (Figure 1 in the online data supplement). Separately, we measured Cx43 protein content by Western blot. As shown in Figure 2B, the density of the Cx43 signal recorded from cells treated with shRNA-PKP2 was 48.56±6.04% of that recorded from untreated cells (n=4). Additional controls were obtained from cells treated with a virus containing a construct that did not silence PKP2 (lanes labeled RNA-PKP2Ø). In that case, the density of the Cx43 signal was 97.54±5.60% of that recorded from untreated cells (n=4). Although PKP2 silencing led to a decrease in total Cx43 content, we did not observe a shift in the ratio of low versus high-mobility bands. This ratio was 1.91±0.07 in untreated cells, 1.91±0.15 in cells treated with shRNA-PKP2, and 1.89±0.10 in cells treated with shRNA-PKP2Ø (n=4 for all experiments). Overall, these data show that loss of PKP2 leads to a decrease in total Cx43 content and a significant disruption of the structural integrity of Cx43 gap junction plaques. As a next step, we asked whether loss of PKP2 expression affected the function of gap junctions.

Loss of Dye Transfer in PKP2-Silenced NRVMs

Transfer of LY across gap junctions allows for assessment of the extent of functional coupling between cells. Here, we determined whether loss of PKP2 expression correlated with changes in the extent of dye transfer between cell pairs.

Figure 1. Western blot for PKP2 or actin (bottom bands) obtained from NRVMs. Cells were either untreated or infected with a replication-deficient adenovirus containing cDNA coding for GFP or infected with adenovirus containing a construct designed to silence PKP2 expression (shRNA-PKP2). Concentration of viral particles is expressed in multiplicity of infection units. The experiment was conducted 5 days after viral infection (or 5 days after incubation in control [CTRL]). Notice the gradual decrease in PKP2 abundance subsequent to shRNA-PKP2 exposure.
Figure 3A shows fluorescent images of dye transfer in a cell pair maintained in control conditions. An LY-filled patch pipette was used to gain access to the intracellular space of one cell in the pair (cell 1) and the extent, and the time course of diffusion into the partner cell (cell 2) was recorded. Fluorescent images in Figure 3A were obtained 1, 5, and 9 minutes after patch break, respectively. Dye diffused from the pipette into cell 1 and from there into cell 2. Yet, as shown in Figure 3B, dye transfer was significantly decreased in cells treated with shRNA-PKP2. The plot in Figure 3C shows the average fluorescence intensity recorded from both cell 1 and cell 2 (relative to the maximum fluorescence in cell 1 for each individual experiment) as a function of time after patch break. Data for fluorescence intensity in cell 2 are depicted for control (open squares) and cells treated with shRNA-PKP2 (open circles). The time course and extent of diffusion observed in control was similar to that previously reported.14 In contrast, dye diffusion was severely interrupted after PKP2 silencing, and the fluorescence intensity in cell 2 measured 10 minutes after patch break was significantly different from that observed in control ($P<0.005$).

**PKP2 Expression and Cx43 Distribution in Nonmyocyte Cardiac Cells**

Loss of gap junction plaques in cardiac myocytes may result from the mechanical strain imposed on the beating myocytes in the absence of proper mechanical junctions. As a first approach to the study of an alternative nonbeating cardiac cell population, we used cells that were retained in the preplating step during dissociation of neonatal rat hearts. These cultures contained cells of at least 3 different morphologies:

1. Triangular or polygonal shape with clear cytoplasm and a “bulk” volume that allowed the membrane to raise slightly over the bottom of the dish; these cells clustered in monolayers, reminiscent of those seen in cultures of epithelial cells, and they were positive for E-cadherin (see supplemental Figure II).
2. Cells of bigger dimensions and clear cytoplasm that laid flat over the bottom of the dish; consistent with the morphology of fibroblasts.

3. Contractile cells of smaller dimension and darker cytoplasm (cardiac myocytes). The latter category represented not more than 5% of the total cell population. Cells described in no. 1 were intensely positive (by immunofluorescence) for both PKP2 and Cx43 and amenable to Lipofectamine-mediated transfection. Silencing of PKP2 was confirmed by Western blot (supplemental Figure III). Drastic changes were observed on Cx43 distribution after PKP2 silencing. Results are shown in Figure 4. Figure 4A through 4C shows microscopic images of cultured cells in control conditions. Cells were labeled with antibodies detecting PKP2 (red; Figure 4A) and Cx43 (green; Figure 4B). An overlay image is shown in Figure 4C. As expected, both proteins preferentially localized at, or near, the cell membrane, with little or no signal originating from the intracellular space. Similar results were obtained when a scrambled RNAi sequence was transfected (Figure 4D through 4F). This pattern was significantly altered by pretreating cells with RNAi for PKP2. As shown in Figure 4G, PKP2 signal was minor or absent in cells treated with PKP2 RNAi, and loss of PKP2 signal correlated with drastic redistribution of Cx43 (Figure 4H and 4I). In this case, there was a significant increase in the amount of signal localized to the intracellular space, at the expense of the membrane-selective localization seen under control conditions. Overall, these results indicate that loss of PKP2 leads to significant remodeling of Cx43 in this cell population.

Dye Transfer in Nonmyocyte Cardiac Cells

Loss of PKP2 expression (and Cx43 redistribution) correlated with changes in the extent of dye transfer in a cell cluster. A patch pipette filled with LY was used to enter the intracellular space of a cell in the center of a cluster (Figure 5A through 5C). The extent and time course of dye diffusion into surrounding cells was recorded. Figure 5D shows a fluorescent image of dye transfer in a group of cells maintained in untreated conditions (10 minutes after patch break; exposure time, 2 seconds). The dye diffused from the impaled cell (asterisk) into the cells in the outer layers, thus demonstrating the presence of dye-permeable gap junctions. A similar recording was obtained from a cell cluster pretreated with scrambled RNAi (Figure 5E). However, a different picture emerged from cells where PKP2 expression had been abolished. In that case (Figure 5F), the impaled cell filled rapidly with the dye but the number of cells receiving the dye was significantly reduced, and the average intensity of the fluorescent signal decreased significantly with distance. A summary of data is presented in Figure 5G and 5H. Two parameters were measured: mean fluorescence intensity per cell layer (Figure 5G) and percentage of dye-positive cells

Figure 3. Dye coupling between pairs of rat neonatal cardiac myocytes. Cells were either untreated (A) or infected with 100 mois of virus shRNA-PKP2 (B). Fluorescence micrographs were taken at 1, 5, and 9 minutes after patch break. The graph in C shows the rise in dye intensity in control and silenced donor cells (□ and ●) and in recipient cells (□ and ○) as a function of time. Notice the reduction in dye transfer in cells silenced for PKP2. LY diffusion was measured in 10 untreated and 8 shRNA-treated myocyte pairs.
within each successive layer (Figure 5H). A partial effect of the scrambled construct was observed. The reasons for this effect are unclear; the transfection procedure may have caused a certain degree of damage (perhaps intracellular acidification or a rise in intracellular calcium) that reflected in dye coupling. Yet, a much larger effect was observed in the PKP2-silenced cultures, and the effect was significant when compared with either scrambled or control. Overall, the data show that loss of PKP2 expression led to a decrease in the ability of LY to diffuse between cells.

PKP2-Cx43 Crosstalk in EPDCs

The phenotypic characteristics of the cells presented in Figures 4 and 5 were consistent with those of EPDCs. To obtain a less heterogeneous cell population, we cultured EPDCs directly from explants of neonatal rat hearts using the technique of Chen et al. After 8 days in culture, cells were transfected with either siRNA-PKP2 or the scrambled construct. Figure 6A through 6C (untreated) and Figure 6D through 6F (scrambled) show the characteristic colocalization of PKP2 and Cx43. In contrast, cells treated with siRNA-PKP2 revealed the loss of detectable PKP2 and a significant redistribution of Cx43 (Figure 6G through 6I), mostly to compartments within the perinuclear space (similar pattern also observed in Figure 4H and 4I). Consistent with observations in cardiac myocytes, Western blot for Cx43 in EPDCs demonstrated a reduction in total Cx43 protein content and a preservation of the ratio of low versus high-mobility bands (Figure 6H). It is worth noting that loss of PKP2 expression did not cause significant remodeling of the adherens junction protein cadherin (see supplemental Figure IV). Overall, these data confirm that PKP2 silencing leads to Cx43 remodeling, even in the absence of mechanical strain imposed by regular beating. Whether Cx43 and PKP2 are able to interact, directly or indirectly, was assessed by the experiments described below.

PKP2 and Cx43 As Part of a Common Macromolecular Complex

Figure 7, left, shows that a recombinant protein corresponding to the head domain of PKP2 (PKP2H) can pull down Cx43 out of a rat heart lysate. GST-fused PKP2H was bound to glutathione beads and incubated either in the absence (lanes marked “minus”) or presence of a cell lysate obtained from an adult rat heart (lanes marked “plus”). Two controls were used: a GST protein (left 2 lanes) and GST fused to the cytoplasmic loop domain of Cx43 (GST–loop). GST alone failed to pull down Cx43 whereas, as expected, GST–loop brought down cardiac Cx43. More importantly, a Cx43-immunoreactive protein of the appropriate size was recovered from the precipitate of beads coated with GST–PKP2-H, indicating an interaction between the 2 proteins. The reverse pull down is shown at right in Figure 7, where GST-Cx43CT (a recombinant protein corresponding to the CT domain of Cx43) pulled down PKP2 out of both a mouse heart (last lane) and a rat heart extract (second-to-last lane). Additional
studies showed that wild-type PKP2 and Cx43 were coimmunoprecipitated from rat heart lysate (see supplemental Figure V). Overall, the results indicate that Cx43 and PKP2 are part of a common macromolecular complex and that the presence of PKP2 is necessary for the proper distribution and function of Cx43 channels.

**Discussion**

In recent years, investigators have proposed that structures involved in mechanical coupling may crosstalk with those involved in maintaining electrical synchrony.\textsuperscript{18–19} This concept, interesting from the point of view of basic sciences, has gained relevance after the discovery that a number of cases of ARVC may be linked to mutations in desmosomal proteins\textsuperscript{2} and that in those cases studied, diseased hearts showed remodeling of gap junction plaques.\textsuperscript{8–9} Here, we demonstrate that loss of PKP2 leads to redistribution of Cx43 protein inside the cell, loss of gap junction plaques detectable by conventional immunofluorescence, and a reduction in the ability of LY to diffuse from cell to cell. This is the first demonstration of a link between PKP2 disruption and partial loss of Cx43-mediated cell–cell communication. Our studies further show that crosstalk between desmosomes and gap junctions is not necessarily caused by the mechanical strain imposed by the contractile forces in the beating heart. The data suggest that PKP2 may regulate (and/or coordinate) the formation and interaction of mechanical and electrical junctional complexes. The mechanism through which these 2 molecular entities (PKP2 and Cx43) interact is likely indirect and remains to be determined. Yet, our data show that PKP2 and Cx43 can be present within a common macromolecular complex. As such, our data are consistent with the possibility of a molecular crosstalk mediating gap junction remodeling subsequent to disruption of the desmosome.

The possible interaction of gap junctions with other intercalated disc structures is further emphasized by the recent
studies of Shaw et al. These authors showed that Cx43 is targeted for delivery near adherens junctions in the membrane through microtubule plus ends. Their studies further demonstrated that siRNA-mediated silencing of β-catenin (an integral protein of the adherens junction) compromised this interaction and resulted in diminished gap junction plaque size. Interestingly, both PKP2 and Cx43 have been shown to interact with β-catenin. Although further experiments are required, it is tempting to speculate that β-catenin may be an important link in the crosstalk between Cx43 and PKP2 and in the integration of gap junctions, adherens junctions, and desmosomes at the intercalated disc.

We have conducted experiments using not only cardiac myocytes but also EPDCs. This cell system allowed us to study the fate of Cx43 after PKP2 silencing in a nonbeating preparation. Yet, we should note that EPDCs are not completely void of mechanical strain, because loss of desmosomes in epithelia may be accompanied by a certain degree of cell retraction. Although our results indicate that cadherin remained present at (or near) the cell membrane after PKP2 silencing (see the online data supplement), preservation (or not) of adherens junctions after PKP2 silencing remains to be determined. On the other hand, the coexistence of PKP2 and Cx43 in heart precipitates strongly suggests that the fate of Cx43 could be linked to the presence of PKP2 through an intermolecular crosstalk between the 2 molecules, likely mediated through common molecular partners.

In addition to their utility as an experimental cell system, the study of EPDCs may bear relevance to the understanding of ARVC. These cells are progenitors of the cardiac fibroblast cell lineage. Recent studies show that in the presence of appropriate agonists, EPDCs retain their ability to proliferate and differentiate not only during fetal and neonate stages but also during adult life. The hypothesis emerges as to whether ARVC-relevant mutations can alter the balance of epicardial–mesenchymal transformation in EPDCs, and, if so, the cytoplasmic loop domain of Cx43 (GST–loop) was used as positive control for the pull down of Cx43.17
whether this disruption is a factor in the fibrofatty infiltration that characterizes the disease. Previous studies show that a reduction in Cx43 expression significantly affects motility and proliferation of proepicardial cells,25 and other investigators have documented the importance of cell–cell adhesion in the control of epithelial–mesenchymal transformation.26–27 As such, a role for PKP2 and/or Cx43 on the migratory, proliferative, and metaplastic behavior of the epicardium seems plausible.

Our studies show that loss of PKP2 expression leads to Cx43 remodeling. We speculate that in the setting of ARVC, reduced expression of wild-type PKP2 (as it occurs in patients afflicted with dominant mutations) could in itself alter the integrity of the junctional structures. In that regard, it is worth noting that heterozygous deletion of desmoplakin leads to fibrofatty infiltration in mice.28 Whether a similar disruption (and/or arrhythmogenesis) occurs in PKP2 heterozygous mice remains to be determined.

Although the actual intracellular compartment hosting Cx43 after PKP2 silencing remains to be defined, our images suggest that Cx43 is more concentrated in the perinuclear region. Future studies will characterize the specific intracellular compartment, and the coexistence of other intercalated disc proteins in the same compartment, following loss of PKP2. Similarly, it will be interesting to determine whether expression of ARVC-relevant PKP2 mutants, rather than silencing of the wild-type protein, leads to a similar redistribution of Cx43.

Our data indicate that loss of PKP2 expression leads to a decrease in the total content of Cx43. Future studies will address whether the decrease in Cx43 content is attributable to changes in gene transcription, protein synthesis, protein degradation or a complex combination of these factors. Moreover, it is surprising that despite the loss of gap junction plaques, the relative density of low versus high mobility bands remained constant. In fact, previous studies have indicated that the mobility of Cx43 in SDS-PAGE correlates with its phosphorylation state29 and that internalization of Cx43 associates with a decrease in the phosphorylated form of the protein.30 Further studies will be necessary to address this apparent inconsistency. One possibility is that the internalized (presumably dephosphorylated fraction) is also degraded at a faster rate. The latter could explain the combination of a total decrease in Cx43 content but an apparent preservation of the high- versus low-mobility ratio.

Our data show a drastic loss of Cx43 gap junction plaques and yet only a decrease of cell–cell dye coupling. It is important to note that gap junction plaques do not define the location of all gap junctions, because the optical resolution of our system is incapable of discerning for the presence of isolated areas where functional gap junctions may remain. Furthermore, previous studies have shown that Cx43 is not the only connexin expressed in heart cells. Expression of Cx45 has been reported both for ventricular myocytes and for cardiac fibroblasts,31 and changes in Cx45 content in human failing hearts have been reported.32 Additional studies in our laboratory indicated that Cx45 is present in our preparations, and its expression may be slightly increased after PKP2 silencing, in a manner similar to that detected by Yamada et al.32 (supplemental Figure VI). It is indeed possible that Cx45 provides a pathway for dye coupling in our preparations, which may remain available after PKP2 silencing.

The preservation of dye coupling suggests that electrical coupling may be maintained (although to a lesser extent than in control), even after total loss of PKP2. Whether the decrease in functional gap junctions is enough to explain arrhythmogenesis remains unclear. Previous studies show that a significant reduction in Cx43 content is necessary to modify conduction velocity,33–34 although even a 50% reduction in Cx43 content can increase the susceptibility to arrhythmias under conditions such as ischemia.35 Our experiments have only explored the presence and the function of gap junctions. Other ion channel proteins, particularly those with preference for localization at the intercalated disc36 may also be affected by the loss of desmosomal coupling. Cx43 remodeling may be one of a number of factors that provide a substrate for arrhythmias during ARVC. Finally, it is worth noting that gap junctions allow not only for passage of electrical currents but of molecular signals as well.37 Thus, loss of functional gap junctions may play an important role in the coordination of cellular events (such as apoptosis or fibrofatty infiltration) that are fundamental to the natural history of ARVC.

In summary, this is the first demonstration of an intermolecular crosstalk between PKP2 and Cx43. The limitations of our experimental system notwithstanding, our results support the idea that changes in gap junctions may participate in the pathophysiological processes leading to ARVC in a subgroup of the afflicted population. The exact mechanisms mediating this molecular crosstalk and its consequences to the synchronization of cellular events within the heart will be determined in future studies.

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Disclosures
None.

References


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MATERIALS AND METHODS

Primary culture of neonatal rat ventricular cells.
Experiments were conducted in neonatal rat primary cell cultures. Details of the methods have been previously described.\(^1\) Briefly, hearts from 1-2 day old rat pups were excised, ventricles dissected and cut into small pieces with dissecting scissors. The pieces of ventricle were dissociated in calcium and magnesium-free HBSS with pancreatin (60 μg/ml), and 1% trypsin. Cells were subsequently spun down, and pellets resuspended in a solution of M199 media supplemented with 1% penicillin and streptomycin, and 10% FBS. Cells were pre-plated for 2 hours to allow for fibroblasts and other non-muscle cells to attach to the bottom of the plate. Within this time frame, myocytes tended to remain in the solution, thus allowing for separation of myocytes from the other cells upon washout.\(^1\) After preplating, myocytes were plated to 70% confluency in either 60mm (for Western blots) or 35mm (for microinjection) dishes. Alternatively, cells were plated at 70% confluency onto coverslips in a 12-well dish for immunofluorescence. Myocytes were maintained in DMEM media supplemented with 10% FBS and 200 U/ml penicillin and 200μg/ml streptomycin in a 37° C incubator containing 5%CO\(_2\). Separate experiments were conducted in cells retained in the preplating step. These cells were allowed to grow undisturbed for 3-5 days at which point they were trypsinized, plated, and maintained as described above.

Primary culture of neonatal rat epicardium-derived cells (EPDCs)
Preparation of EPDC’s followed the method of Chen et al (2002).\(^2\) Hearts from 1-2 day old rat pups were excised and ventricles were dissected. Each ventricular section was cut into 4 pieces with dissecting scissors, and placed epicardium-side down onto a 60mm dish coated with 0.1% gelatin. The ventricular pieces were then covered with DMEM media including 15% FBS, penicillin/streptomycin, and 15mM HEPES. Dishes were allowed to incubate 4 days, at which time the ventricular sections were removed and media was changed to DMEM with 10% FBS, 100 U/ml penicillin and 100μg/ml streptomycin. After 3 more days, cells were trypsinized using .25% trypsin/EDTA, and plated to 70% confluency in 35mm plates for dye transfer experiments. Cells were also
 plated in 6 well dishes for Western blots, or on glass coverslips in 12 well dishes for immunofluorescence.

PKP2 silencing in NRVMs

We have used viral transfer technology to silence the expression of PKP2 in NRVMs. Cells in culture were infected with adenovirus containing an shRNA for PKP2 (shRNA-PKP2) based on the RNAi construct of sequence GAAACTCTACCAGATTTGGTTTCTA. This sequence was predicted as a potential silencer of PKP2 expression as per Invitrogen’s Block-it™ software. Cells untreated, or treated with the virus coding for GFP were used as controls. An alternative construct (GAGGTAGCTGAACTGAATG) was shown not to interfere with PKP2 expression and was used as an additional control (cont-shRNA). Silencing of PKP2 was confirmed by Western blot or immunofluorescence. NRVMs were plated at 80-90% confluency in M199 culture media (10% FBS, 100 U/ml penicillin and 100μg/ml streptomycin) and the viral particles were added to the media. Unless otherwise indicated, experiments were conducted at 100 MOI for shRNA-PKP2, given that cells were better preserved at this concentration and yet, PKP2 was silenced to less than 15 % from control. Concentrations of viral particles for GFP and cont-shRNA were 25 MOI and 100 MOI, respectively. All experiments were carried out five days after viral infection.

PKP2 silencing in EPDCs

Plates were separated in 3 groups: untreated, treated with lipofectamine and the silencing construct (stealth RNAi; Invitrogen), and treated with lipofectamine and a “scrambled” oligonucleotide. The RNAi construct was designed using Invitrogen’s online BLOCK-iT™ RNAi Designer and consisted of the sequence GAAACUCUACCAGAUUUGGUUUCUA. The “scrambled” construct corresponded to a series of the same bases organized in order to avoid any existing coding sequence (GAACUAUCCGAUAUUGGUUUCACUA). A BLAST search for both the silencing and the scrambled construct resulted in no homology to sequences coding for known proteins other than PKP-2. Transfection was accomplished by diluting RNAi in lipofectamine and then presenting it to culture cells. For each ml of media, 1 μl of
lipofectamine was diluted in 25 µl of serum- and antibiotic-free media. After 15 minutes, this solution was mixed with one containing 5 µl RNAi (40 uM) in 25 µl of serum- and antibiotic-free media per 1 ml of media. After an additional 15-minute incubation period, this solution was added to the cells. After 24 hours, the serum- and antibiotic-free DMEM media was replaced with DMEM media containing 3% FBS and penicillin/streptomycin. Experiments were carried out 72 hours after completion of the PKP2 silencing protocol. Silencing of PKP2 was confirmed on every experiment by Western blot.

**Western blots**

Cells were harvested in ice cold PBS. After a 10 minute centrifugation, the cell pellet was resuspended in 150 µl Triton X-100 lysis buffer, briefly sonicated, and incubated on ice for 30 minutes. After the incubation, 10 µl 6X SSB buffer per 50ul of sample were added. Samples were run on an 8-16% tris-glycine gradient gel, transferred to nitrocellulose membranes and blocked for 1 hour at room temperature (blocking buffer consisted of 1% non-fat milk and 0.05% Tween in PBS). Membranes were then incubated in primary antibodies overnight at 4°C followed by incubation in secondary antibody (anti-mouse HRP, Sigma) for 45 minutes at room temperature. Signal was detected by chemiluminescence (Pierce SuperSignal West Pico Chemiluminescent Substrate).

Primary antibodies used were: a mouse monoclonal anti-PKP2a and 2b that recognizes an epitope on the CT domain of the protein (Biodesign; 1:50 diluted in 0.05% Tween in PBS), and a monoclonal mouse Cx43 (diluted 1:100 in .05% Tween in PBS) that recognizes the amino terminal (NT) domain of Cx43 (Fred Hutchinson Cancer Research Center). Immunodetection of actin was used as a loading control on a stripped and reprobed blot using mouse monoclonal β-actin (Sigma).

The ratio of the densities of low-mobility/high-mobility bands (P/NP) of Cx43 was determined from Cx43 Western blots performed using the NT antibody. The densities of the P and NP bands were determined separately, background subtracted and adjusted for loading utilizing beta-actin as a control. P/NP ratios were calculated by dividing the P
band values by NP band values for each experimental condition. Ratio values are represented as mean +/- S.E.M.

Co-Immunoprecipitation:

Immunoprecipitation was performed based on the protocol developed by Lukas et al. (1998). Whole ventricular tissue of adult Sprague Dawley rats was homogenized for 30 sec at 4°C in 5 ml lysis buffer containing 1% Triton X-100, 0.5% NP-40, 20 mM HEPES pH 7.4, 50 mM NaCl, 1 mM EGTA, 5 mM u-glycerophosphate, 30 mM sodium pyrophosphate, 1 mM NaVO₄, 50 mM NaF, 1mM Phenyl methanesulfonyl fluorid (PMSF) and 2x Complete® proteinase inhibitor (Roche). Samples were centrifuged at 16000g for 20 min and ~ 4ml of supernatant were transferred to a fresh tube. To pre-clear the lysates, 200 ul of protein A-sepharose (Sigma-Aldrich Corporation, St. Louis, MO) were added and the samples were incubated for 3h at 4°C on a Nuator (Fisher). Tubes were spun at 4°C and 16000g for 1 min, the pre-cleared supernatant transferred to a fresh tube and a protein concentration assay (Lowry) was carried out. Immunoprecipitations for Cx43 protein were carried out in a volume of 1000 ul for 300 ug of total protein. A total of 14 ug of rabbit anti-Cx43 antibodies (Chemicon,) were added to the pre-cleared lysates and the samples were incubated overnight at 4°C on a Nuator. The next day, 40 ul of protein A-sepharose were added and the samples were incubated at 4°C for 2h on a Nuator (Fisher). The antibody-protein A-sepharose complex was spun down at 16000g for 10 sec and washed with ice-cold lysis buffer for three times. After the final spin, the precipitate was washed with ice-cold 20 mM HEPES including 1 mM NaVO₄, 50 mM NaF, 1 mM PMSF and 2x Complete® proteinase inhibitor to minimize electrophoresis artifacts due to ionic composition during SDS-PAGE. Twenty ul Laemmli buffer were added to the precipitate, samples were incubated at 65°C for 5 min and spun at 16000g for 5 min to pellet the protein A-sepharose immuno-complex. Fifteen ul of supernatant per sample were subjected to SDS-PAGE and co-immunoprecipitation was tested by immunoblot analysis using monoclonal mouse plakophilin 2 antibodies (#K44262M, Biodesign International, Saco, ME). Immunoprecipitation of Cx43 was tested by re-probing stripped membranes with monoclonal anti-Cx43NT1 (clone P1E11) (Fred Hutchinson Cancer Research Center) antibodies. As negative controls, reactions were
carried out in parallel with the same amount of pre-cleared homogenate omitting anti Cx43 antibodies and subjected to the same SDS-PAGE. As positive controls, the supernatants were precipitated by 20% trichloroacetic acid on ice for 30 min. The precipitates were pelleted by centrifugation at 16000g for 15 min at 4°C, washed with ice-cold acetone, air-dried and resuspended by brief sonification in 150µl of Laemmli buffer. Five µl, corresponding to a 1:30 dilution of the initial amount subjected to the immunoprecipitation reactions, were subjected to the same SDS-PAGE.

Preparation of recombinant GST-fusion proteins
Recombinant GST fusion proteins were generated in BL21 E. coli. Production of a protein fragment corresponding to the carboxyl terminal domain of Cx43 (GST-Cx43CT) followed the methods described elsewhere. Briefly, crude lysates were prepared by sonication of one liter bacterial pellets in 20 mls of PBS protein purification buffer containing 138mM NaCl, 10mM phosphate, 2.7mM KCl pH 7.4,1mM DTT, lmM PMSF and 1% NP-40), followed by centrifugation at 12,000rpm for 25 minutes. One ml of crude bacterial lysate was mixed with 50 µl of Glutathione Sepharose 4B (GE Healthcare) overnight at 4 ºC. Unbound proteins were removed by washing three times with heart lysis buffer (50mM Tris-HCl, 50mM NaCl, 2mM MgCl2, 2mM EDTA, 50mM NaF, 1mM NaVO4, 1 % b-mercaptoethanol, 1% TritonX-100, 1X Complete protease inhibitor (Roche Diagnostics); pH 7.5. Production of a protein fragment corresponding to the head domain of PKP2 (GST-PKP2H; amino acids 1-335 of PKP2a) followed the same protocol.

Preparation of heart lysates
Fresh mouse or rat heart lysates were prepared by homogenizing tissue in heart lysis buffer on ice. The components of the lysate were separated by centrifugation at 12,000 rpm for 25 minutes. One ml of supernatant was exposed to 250 µl of glutathione sepharose beads for 30 minutes at 4 ºC. After centrifugation, the pre-cleared supernatant was used for the pulldown assays described below. Total protein content of heart lysate was determined by DC protein assay (Bio-Rad) with bovine serum albumin as a standard.
**GST-pulldown assays**

Bound GST fusion proteins were incubated with approximately 15 mg of pre-cleared rat or mouse heart lysate in 1 ml of lysis buffer for 90 minutes, rocking at 4 °C. A separate sample was incubated with lysis buffer only as a control. Unbound proteins were removed by washing 2 times with lysis buffer. The final pellet was resuspended in Laemmli sample buffer and probed by western blotting as described above.

**Immunofluorescence**

Either EPDCs or NRVMs were plated to ~ 70% confluency on coverslips in 12-well dishes and treated as described above. After 72 hours, coverslips were washed with PBS and fixed with 4% PFA (room temp) for 5 minutes. After 3 subsequent washes in PBS, coverslips were exposed for 2 hours to a blocking buffer consisting of 4% BSA and 1% Triton X-100 in PBS, followed by an overnight incubation with primary antibody at 4°C in a humid box. Coverslips were subsequently rinsed 3 times with PBS, and incubated with secondary antibody for 45 minutes at room temperature in a dark, humid box. Following 3 PBS washes, coverslips were mounted using Permafluor mounting media (THERMO electron corporation), and kept at 4°C for 24 hours. Preparations were examined at 63X and 100X oil immersion magnifications. PKP2a&b mouse monoclonal primary antibody (Biodesign) was diluted 1:50 in blocking buffer. Cx43 polyclonal rabbit primary antibody (Chemicon) was diluted 1:400 in blocking buffer. Pan-Cadherin polyclonal rabbit primary antibody (Sigma) was diluted 1:500 in blocking buffer. E-cadherin monoclonal mouse primary antibody (BD Transduction Laboratories) was diluted 1:500 in blocking buffer. Desmoplakin polyclonal rabbit primary antibody (Serotec) was diluted 1:500 in blocking buffer. Desmin polyclonal rabbit primary antibody (Scy Tec) was diluted 1:100 in blocking buffer. Secondary antibodies used were Alexa fluor 594 donkey anti-mouse, and Alexa fluor 488 donkey anti-rabbit (Molecular Probes), diluted 1:800 in blocking buffer. Hoescht dye for nuclear staining was diluted 1:1000 in blocking buffer. Images were taken and processed using an Axioplan 2 bright field microscope (Zeiss) equipped with 63X and 100X Plan Apo lenses, and Axioplan 2E imaging with structural illumination (apotome).
Dye transfer in NRVM pairs
Dye transfer through gap junctions was assessed in PKP2 silenced and unsilenced NRVM cell pairs. A glass microelectrode was sealed on one cell of a pair, and patch break allowed access to the intracellular space (whole-cell configuration). The patch pipette was filled with a Ca\(^{2+}\) and Mg\(^{2+}\)-free 1X Phosphate buffered saline (Cellgro) in which the fluorescent dye Lucifer yellow (LY; 1 mM) was added. The dye was allowed to passively diffuse into the impaled cell, and junctional transfer to neighboring cells was monitored by fluorescence microscopy. During the experiment, cells were maintained in Tyrode’s solution ((mmol/L) NaCl 140, KCl 5.4, CaCl\(_2\) 1.8, NaH2PO4 0.33, HEPES 5.0; pH 7.4 (NaOH)). LY flux was imaged at 30-60 second intervals using a 12-bit 1.3 million pixels CCD digital camera (QImaging Retiga 1300). A calibration curve was constructed by measuring the fluorescence intensity of electrode tips containing different concentrations of LY at a fixed exposure time.\(^{6}\) Background intensity was subtracted from the total fluorescence intensity measured. This resulted in a linear concentration-intensity curve, indicating that the concentration of LY was directly related to the intensity of fluorescence.

Dye Transfer in monolayers of EPDCs
To demonstrate loss of function of Cx43 in non-contractile cells, dye transfer experiments were performed in clusters of confluent cells with epithelial morphology, obtained during the preplating step in the process of dissociation of neonatal hearts. These clusters were identified within culture dishes that were either left untreated, treated with stealth RNAi to silence PKP2, or treated with the scrambled construct. Methods for dye transfer and image acquisition were similar to what has been described for NRVMs (see above). However, in this case, dye diffusion was measured within the entire cluster and the extent and rate of diffusion was quantified as per the number of “cell layers” crossed by the dye, that is, the minimum number of cells that would be interposed between the site of injection and the particular cell being analyzed. Cells were considered “dye positive” when the fluorescence intensity recorded was at least three times larger than the background fluorescence.
RESULTS

Effect of PKP2 silencing on desmoplakin and on desmin.

Loss of PKP2 expression has been associated with significant cellular redistribution of desmoplakin.\(^7\) To further validate our experimental model, we assessed whether viral transfer of shRNA-PKP2 in NRVMs correlated with the loss of detectable PKP2 and with remodeling of DP. Online figure 1 (right columns) shows the immunolocalization of PKP2 (red) and DP (green) in NRVMs either in control (center column) or after treatment with 100 MOI of shRNA-PKP2 (far right column). Cells were incubated for five days after shRNA treatment (or untreated) prior to fixation. Images of untreated NRVMs show the characteristic co-localization of junctional proteins at the sites of cell apposition. Cells treated with shRNA showed loss of PKP2 expression. More importantly, we observed significant redistribution of desmoplakin, as predicted from results obtained in PKP2-deficient mice.\(^7\) In separate experiments (Online figure 1, left columns), we confirmed that PKP2 silencing also disrupted desmin distribution, as it would be expected from the loss of PKP2 and redistribution of DP.\(^7\) Indeed, in control conditions, desmin presented a well-organized staining, more intense at the site of cell apposition. Yet, this pattern was significantly modified in cells treated with shRNA-PKP2.

Immunostaining for E-cadherin in cells retained during the preplating step.

Cells obtained in the preplating step presented with various morphologies. We found that both Cx43 and PKP2 staining was most intense in cells with an epithelial-like appearance.\(^2,8-9\) Those cells tended to organize in clusters, and their intercellular connections were readily permeable to Lucifer yellow (see Figure 5 of manuscript). To further characterize these cells, we tested for the presence of E-cadherin. Results are presented in online figure 2. In the selected frame, two morphologies are apparent. Cells marked with red arrows were negative for E-cadherin, whereas cells marked with black arrows were positive for this protein. Interestingly, Cx43 staining was prevalent in the E-cadherin positive cells, and absent from the other cells at this same exposure. The cellular morphology, the intense staining for PKP2 and Cx43 and the fact that these cells were E-cadherin-positive strongly suggests that these clusters corresponded to epicardium-derived cells.\(^2,8-9\)
RNAi knockdown of PKP2 in EPDCs obtained from preplating

EPDCs were either left untreated, or treated with both lipofectamine and the PKP2 silencing construct, or lipofectamine and a “scrambled oligonucleotide”, as described in the methods section. Online figure 3 shows our results. Cells either untreated (UNT, 2 left lanes), or treated with the scrambled oligonucleotide (SCR, center lanes) revealed a strong signal at ~ 98kD, representing PKP2. However, cells treated with the RNAi construct revealed a diminished 98kD signal, suggesting that PKP2 had been knocked down. These results are consistent with our Western blot experiments using EPDCs obtained from explant preparations, as well as those using NRVMs.

Cadherin distribution after PKP2 silencing

To determine whether desmosomal disruption also altered the distribution of cadherin, we performed co-localizations using an antibody recognizing PKP2 and a pan-cadherin antibody. Results are shown in Online figure 4. In all cases, the first column depicts the phase image, the second depicts PKP2 signal; the third, cadherin signal, and the fourth, merged images. Panels A-H depict images obtained from rat neonatal myocytes. Two conditions were tested: untreated, and treated with the silencing construct. Panels I-T were obtained from EPDCs and in this case we tested cells that were either untreated, treated with the “scrambled” construct, or treated with the siRNA. There is a significant difference between the distribution of cadherin and that of either desmoplakin (Figure 1 of the supplement) or Cx43 in cells where PKP2 expression has been inhibited. Indeed, though the cells seem to retract from one another after PKP2 silencing (though notice the filipodia-like structures in EPDCs), cadherin could still be detected at or near the plasma membrane. This is in contrast with the loss of membrane localization observed with the desmoplakin or the Cx43 antibodies, where the protein was detected mostly in the intracellular space. These results suggest that the remodeling of Cx43 observed after PKP2 silencing was not a generalized response common to all proteins involved in either mechanical or electrical coupling.

Co-immunoprecipitation of PKP2 and Cx43 from heart lysates.

Data presented in the manuscript (Figure 7) show that a fragment of the PKP2 protein can recover Cx43 from a heart lysate, and vice versa. Here, we show initial experiments
demonstrating that cardiac PKP2 can also co-immunoprecipitate with Cx43. Results are shown in Online figure 5 of this supplement. Protein A Sepharose beads were coated with antibodies to the C-terminal domain of Cx43 and incubated with precleared rat heart lysates; the recovered precipitate was probed with an antibody to the C-terminal domain of PKP2 (left two lanes). Negative controls were carried out with heart lysates exposed to non-coated beads (right two lanes). Additionally, 1:30 dilutions of the precipitated supernatants were subjected to the same SDS-PAGE (not shown). An immuno-reactive signal, of the predicted mobility for PKP2, was detected in the Cx43 precipitates (left two lanes) and the supernatants, but not in the negative controls (right two lanes). The data indicate that PKP2 can co-exist with Cx43 in the same macromolecular complex.

Together with the data in Figure 7, the results support the notion of a multimodal interaction between Cx43 and PKP2, perhaps through more than one common partner, and involving more than one structural domain. However, future experiments will be necessary to assess the structural constraints and the possible intermediaries that modulate the PKP2-Cx43 interaction.

Previous studies have shown changes in Cx45 content in ventricular myocytes obtained from failing hearts.\textsuperscript{10} We have performed Western blots to determine whether Cx40, or Cx45 could be detected in our cell preparations, and whether changes in the content of these proteins were observed following PKP2 silencing. Experiments were conducted in neonatal rat ventricular myocytes that were either untreated, treated with a virus containing shRNA-PKP2 or treated with a virus containing the control shRNA (cont-shRNA). Detection was maximized by use of femto-chemiluminescence (Pierce Femto kit). Panel A shows a Western blot for detection of Cx40. The first lane contained a sample obtained from N2a cells transfected with Cx40 cDNA, as a positive control. No signal was obtained from the samples collected from NRVMs, regardless of whether or not they were subjected to viral treatment. The latter was consistent with previous studies indicating the absence of Cx40 protein, and of the corresponding functional channels, in the vast majority of neonatal ventricular myocytes (Beauchamp et al, 2004). Panel B shows the results of a Western blot for Cx45. A weak signal for a Cx45-immunoreactive
protein was detected in NRVMs, likely reflecting the low concentration of Cx45 in our preparations. Quantitative analysis (calibrated against an actin control) indicated that the band obtained from the sample treated with shRNA-PKP2 was 1.6X more dense than the one obtained from cells treated with the control construct. Though this could reflect an increase in Cx45 following PKP2 silencing, it should be emphasized that these bands are of a rather low intensity and as such, quantification is limited by the poor signal-to-noise ratio. Overall, our results concur with those of others suggesting that Cx45 represents only a fraction of the total amount of connexin present in these cells. Our results further suggest that changes in PKP2 expression may lead to an increase in the abundance of Cx45, similar to that observed in failing human hearts (Yamada et al, 2003). Cx45 may provide an additional pathway for cell-cell communication following remodeling of Cx43 after PKP2 silencing.
REFERENCES


**ONLINE FIGURE LEGENDS**

Online Figure 1. Immunolocalization of desmin (DSM; panels on the left) or desmoplakin (DP; panels on the right) in neonatal rat ventricular myocytes that were either untreated (UNT) or treated with shRNA for PKP2 (shRNA). Plakophilin-2 (PKP2) was labeled in red, and DSM and DP were labeled green. Bottom panels correspond to merged images. Top panels are the corresponding DIC images.

Online Figure 2. Immunolocalization of E-Cadherin (E-CAD) and Connexin43 (Cx43) in cells retained in the pre-plating phase of dissociation of neonatal rat hearts. Notice that cells of epithelial morphology (black arrows) were positive for both E-CAD and Cx43, whereas cells marked with red arrows were E-CAD negative and, under the same exposure, were also negative for Cx43.

Online Figure 3. Western blot for plakophilin-2 (PKP2) and actin (as loading control) obtained from epicardium-derived cells that were either untreated (UNT), transfected with a silencing construct (RNAi), or transfected with a construct containing the same bases as the RNAi but in a modified order (SCR). This construct was predicted to have no ability to silence PKP2 or other known proteins.

Online Figure 4. Immunolocalization of plakophilin-2 (PKP2; second column) and cadherin (CAD; third column) in either neonatal rat ventricular myocytes (NRVM; top two rows) or epicardium-derived cells (EPDCs; bottom three rows) that were either kept in control conditions (panels A-D in NRVMs; I-L in EPDCs), treated with a construct designed to silence PKP2 (panels E-H for NRVMs; Q-T for EPDCs) or with a “scrambled” construct (panels M-P). Column on the left displays DIC images; column on the right corresponds to merged images from the co-localization experiments.

Online Figure 5. Co-immunoprecipitation of PKP2 with Cx43 from rat heart lysates. The two lanes on the left show results obtained from two different rat hearts when Cx43 was immunoprecipitated. The next two lanes are negative controls, including Sepharose-A beads but omitting anti-Cx43 antibodies, showing that there was no unspecific precipitation of PKP2 protein. Western blot results for PKP2 are shown on top. Western blot results for Cx43 are shown in the bottom.

Online Figure 6. Western blots for Cx40 (Panel A) and Cx45 (Panel B). Panel A shows (from left to right lanes) N2a cells transfected with Cx40 DNA, untreated NRVMs (UNT), and NRVMs treated with PKP2 shRNA (shRNA). Though a signal corresponding to Cx40 was detected in the N2a sample, no signal was detected in either untreated NRVMs, or NRVMs treated with PKP2-shRNA. Panel B depicts Cx45 signal in untreated NRVMs (UNT, left lane) and NRVMs treated with PKP2-shRNA (shRNA; right lane). We noted a slight increase in the density of the Cx45 signal following PKP2-shRNA However, these signals were close to the level of detection and as such, are more amenable to errors introduced by the poor signal-to-noise ratio.
ONLINE FIGURE 3

![Western Blot Image]

- **PKP2**
  - UNT: Strong signal
  - SCR: Moderate signal
  - RNAI: Weak signal

- **Actin**
  - UNT: Strong signal
ONLINE FIGURE 5

anti Cx43 Ab

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- PKP2
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