Influence of plakophilin-2 haploinsufficiency on expression of plakophilin-2 and connexin43 in mice

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Introduction

ARVC is a genetically heterogeneous and progressive disease, and a leading cause of sudden cardiac death in the young. This disease is characterized by progressive degeneration of the right ventricular myocardium [1]. The hypothesis that ARVC is a disease of the desmosome is strengthened more and more, as mutations in the genes encoding the desmosomal proteins plakogobin (PKG), plakophilin-2 (PKP2), desmoplakin (DSP), desmoglein-2 (DSG2) and desmocollin-2 (DSC2) are associated strongly with ARVC patients. Here, the focus lays on PKP2. Mutations in this gene are the most common mutation found in ARVC, contributing to 70% of all familial ARVC cases [2]. However, mutations in PKP2 have an incomplete penetrance, as these mutations can be seen in people that are not diagnosed with ARVC. PKP2 is an important protein in the desmosome, providing lateral association between the desmosomal cadherins DSG2 and DSC2 [3]. The protein also has a role in the modulation of gap junctions (GJs). *In vitro*, loss of PKP2 caused connexin43 (Cx43) remodelling [4].

To date, only one article has been published that concerns PKP2 transgenic mice [3]. PKP2 null mice were shown to die at day E10.75, which was explained by the fact that loss of PKP2 caused defective cell-cell adhesion at the intercalated disc. This caused blood leakage into the pericardial cavity. PKP2 was thus shown to be a key organiser of cardiac architecture during embryogenesis.

This study can be named a continuation of the Grossman study. In mouse hearts, differences in expression of PKP2 and the GJ protein connexin43 (Cx43) were assessed between wildtype (wt) and PKP2 heterozygous mice.

Methods

Transgenic mice

Several C57/bl6 mice were sacrificed, both female and male, PKP2 heterozygous and wt. Before sacrifice, ECGs were recorded. The lungs, kidneys and liver were cut out, weighed and separately frozen in liquid nitrogen. A tip of the tail was also preserved. The tibia was cut out and measured in length. The hearts were perfused in a Langendorff setup and the conduction velocity and arrhythmia inducibility were measured on left and right ventricle (LV and RV), as well as left and right atrium (LA and RA) using a grid. The results of this technique will however not be analyzed in this report.

After that, some of the hearts were frozen as a whole, while eight of them were separated in LV, RV, LA, RA and septum. For this experiment, only the separated hearts were used. The mice whose hearts were separated and analyzed are numbered as follows (f = female, m = male, wt = wildtype, PKP2/+ = PKP2 heterozygous):

- 506567, f, wt
- 506568, f, PKP2/+
- 506570, f, wt
- 506573, f, PKP2/+
- 506575, m, wt
- 506576, m, PKP2/+
- 506577, m, wt
- 506578, m, PKP2/+

In this report, the numbers will be abbreviated to its last two figures. From each heart, both right (RV) and left ventricle (LV) were analyzed, which resulted in 16 samples.

Protein isolation

Total protein was isolated from RV and LV, which made 16 samples in total. First, lysis buffer was made. This buffer contained the following compounds:

- RIPA consists of:
    - 0.6 g Tris/HCl pH 7.4
    - 2.2 g NaCl
    - 0.44 g Na_2HPO_4
    - 2.5 mL 1% Triton X-100
    - 2.5 mL Na-deoxycholaat (deoxycholic acid)
    - 0.25 g SDS
    - 0.99 g EDTA
    - 0.53 g NaF
    - Adjusted with H_2O to 250 mL
- 40 µL Aprotinine
- 40 µL PMSF.

After that, the heart ventricles were ground using mortar and pestle, which were cooled in liquid nitrogen. The powder that resulted was put in an Eppendorf tube. Lysis buffer was then added to the samples. As the right ventricles were smaller than the left ventricles, 100 µL of buffer was added to right ventricle samples and 200 µL to left ventricle samples. After 30 minutes of incubation, the samples were centrifuged at 4 °C and 40,000 rpm for 8 minutes. The supernatant was pipetted from the pellet and put in new Eppendorf tubes. This was put into the -20 °C freezer, awaiting BCA analysis.

BCA analysis

The BCA analysis was executed to assess the total amount of protein in each sample. First, a master mix of bicinchoninic acid and 4% copper(II)sulphate was made, in a 98:2 ratio. Then 3 μL of cell lysate from the 16 samples was pipetted into Eppendorf tubes, and 0.997 mL of the master mix was added to make 1 mL.

Also, a calibration line with 0, 5, 10, 20 and 40 μg BSA (1 mg/mL) was made, and this was filled up with master mix up to 1 mL. All the tubes were incubated for 30 minutes at 30°C, and then the absorbance was measured at 562 nm.

SDS-PAGE

The samples were prepared for SDS-PAGE. For this purpose, every cell lysate had to be diluted to make the same protein concentration in every sample, 200 μg in 200 μL. This is the amount needed for 10 samples. These 200 μL consisted of cell lysate, 40 μL of sample buffer and H_2O (milliQ). 5x sample buffer contains the following components:

- 2.5 mL 25% β-mercaptoethanol
- 1 mL 2.5M Tris pH 6.8
- 5.0 mL 50% glycerol
- 1.0 g 10% SDS
- A dash of bromine phenol blue
- Adjusted to 10 mL with H2O.

A pipetting scheme was made, using the results of the BCA analysis, to assure that the protein concentration is the same in each sample (not shown). Because the amount of 67 RV sample was too small to make enough for 10 samples, 100 μL instead of 200 μL was made.

SDS-PAGE was executed using Biorad's mini protean 3 system. For this experiment, two gels were made, for one gel can contain 10 samples and 16 samples had to be examined. A 10% running gel was made as follows:

- 3.8 mL H_2O
- 3.4 mL acrylamide:bis (30%)
- 2.6 mL Tris pH 8.8
- 0.10 mL 10% APS (added penultimately)
- 0.10 mL 10% SDS
- 0.010 mL Temed (added lastly).

This was put in the Biorad systems. On top of the running gel, water saturated with isobutanol was added to prevent dehydration and make sure the border of the gel forms a straight line. When the running gel was polymerized, the isobutanol layer was removed and a 4% stacking gel was added. This contained the following ingredients for two gels:

- 3.05 mL H_2O
- 0.625 mL acrylamide:bis (30%)
- 1.25 mL Tris pH 6.8
- 0.05 mL 10% APS (added penultimately)

- 0.05 mL 10% SDS
- 0.005 mL Temed (added lastly).

This was allowed to dry for 15 minutes with the comb in the gel. Meanwhile, 1 L of 1x electrode buffer was put in the Biorad system. This was made by diluting the 10x electrode buffer that consisted of:

- 30 g Tris
- 144 g glycine
- 10 g SDS
- Adjusted to 1 L with H_2O.

The comb was then taken out of the gel carefully. The samples were cooked at 96 °C for 3 minutes, and centrifuged shortly to assure that the sample was completely at the bottom of the tube. Now the samples were ready for loading, which was done according to the following scheme:

Table 1 Gel loading scheme. Of all samples20 µL was pipetted.

Lane	Gel 1 *Contents*	Gel 2 *Contents*
1	Marker 5 µL	Marker 5 µL
2	67 LV (wt)	75 LV (wt)
3	67 RV (wt)	75 RV (wt)
4	70 LV (wt)	77 LV (wt)
5	70 RV (wt)	77 RV (wt)
6	68 LV (+/-)	76 LV (+/-)
7	68 RV (+/-)	76 RV (+/-)
8	73 LV (+/-)	78 LV (+/-)
9	73 RV (+/-)	78 RV (+/-)
10	Marker 3 µL	(empty)

This was put on 75 V for 2.5 hours, and on 150 V for the last fifteen minutes.

Western immunoblotting

For every blot 4 Whatman papers and 1 nitrocellulose membrane were prepared, each measuring 8.5 by 6.5 cm. These were all submerged 5 minutes in blot buffer before use. The blot buffer contained the following ingredients:

- 5.81 g Tris
- 2.93 g glycine
- 0.275 g SDS
- 200 mL methanol
- Adjusted with H_2O to 1 L.

In the Hoefer TE 77 semi-dry transfer the blotting was executed. A pile was made consisting of two Whatman papers, followed by the membrane and the gel, and finished by again two Whatman papers. The system was put on 43 mA per gel and 8 V for 70 minutes, with a heavy bottle on top. When the blotting was finished, the membranes were taken out and washed with ddH_2O.

Ponceau staining

The membrane was coloured for 10 minutes with Ponceau Red staining. It appeared that the blotting was successful, the loading was however not equal. The membrane was scanned so the differences in loading can be analyzed, and after that washed with TBST. TBST contained these ingredients:

- 12.12 g 20 mM Tris, pH 8.0
- 43.83 g 150 mM NaCl
- 0.05% Tween-20 (v/v)
- Adjusted to 5L with H_2O.

Antibody incubation

The blot was blocked with 5% Protifar (baby milk) in TBST for one hour on a rotating table. After that, it was washed once with TBST, and the first antibody was added. The Cx43 blots were incubated with 4 µL anti-Cx43 antibody in 1 mL TBST (1:250), and the PKP2 blots were incubated with 2 µL anti-PKP2 antibody in 1 mL TBST (1:500). Incubation took place at 4 °C on a rotating table for two days.

After that, the blots were washed three times with TBST. The second antibody, goat anti-mouse, was incubated for 2 hours at 4 °C on a rolling table (1:7000). Then the blots were washed again three times with TBST.

ECL and computer analysis

The blots were put on a plastic sheet, and the two fluids of the ECL (enhanced chemiluminiscence) were mixed in a 1:1 ratio. 1 mL of the mixture was put on each membrane. The membranes were covered with another sheet, put in a light-impermeable box, and transported to the darkroom. In this box a film was put on the blots for 2 minutes, 1 minute, 30 or 15 seconds, and developed in the development machine. Then, for each sample the best film was chosen to analyze. These were the films on which the bands were neither overexposed nor too faint. From these bands the intensity was calculated on a pc using the programme ImageJ.

Results

The results of BCA analysis are shown in Table 2 Calibration line values and absorbance values and protein concentration values for all cell lysate samplesTable 2.

Table 2 Calibration line values and absorbance values and protein concentration values for all cell lysate samples

Sample	Absorbance (AU)	Mean value	Protein concentration (μg/μL)
Calibration 5	0.138	0.125	5
	0.111		
Calibration 10	0.212	0.222	10
	0.231		
Calibration 20	0.45	0.453	20
	0.456		
Calibration 40	0.858	0.866	40
	0.874		
Calibration 80	1.389	1.443	80
	1.496		
67 RV	0.25	0.2725	4.152191
	0.295		
68 RV	0.2	0.2135	3.253185
	0.227		
70 RV	0.358	0.3415	5.203572
	0.325		
73 RV	0.437	0.4075	6.20924
	0.378		
75 RV	0.475	0.4355	6.635887
	0.396		
76 RV	0.43	0.4445	6.773024
	0.459		
77 RV	0.403	0.431	6.567319
	0.459		
78 RV	0.654	0.587	8.944353
	0.52		
67 LV	0.647	0.6975	10.62809
	0.748		

68 LV	0.421	0.4295	6.544463
	0.438		
70 LV	0.54	0.658	10.02621
	0.776		
73 LV	0.409	0.412	6.277808
	0.415		
75 LV	1.01	1.259	19.18388
	1.508		
76 LV	0.953	0.948	14.44505
	0.943		
77 LV	0.835	0.85	12.95179
	0.865		
78 LV	0.779	0.8605	13.11178
	0.942		

The values ‘calibration 5’ to ‘calibration 80’ were used to construct a calibration line, as shown in figure 1.

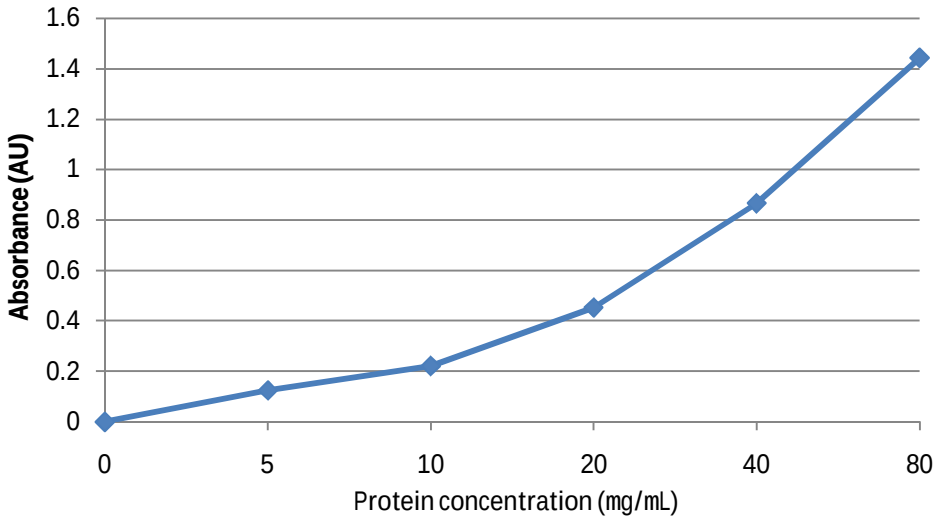

Figure 1 Calibration line

After that, the slope of every segment of the graph was calculated, and these values were mediated, which gave an avarage slope of 0.021876. Using this value, the protein concentration in each sample was calculated by the following formula: (absorbance/slope)/3. The results are shown in table 1. The division by 3 comes from the fact that 3 μL of cell lysate was added.

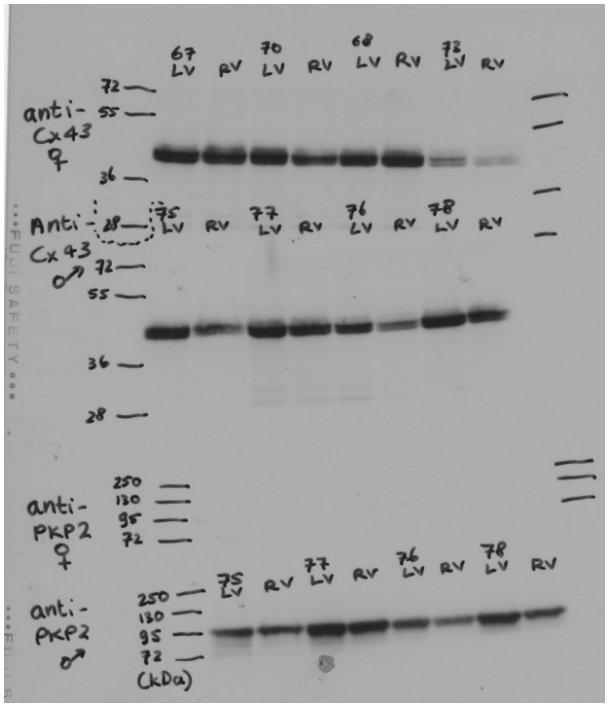

Figure 2 Westernblot, 30 s. Of this blot, the anti-Cx43 signal from male and female was analysed, as well as the anti-PKP2 signal from male mice.

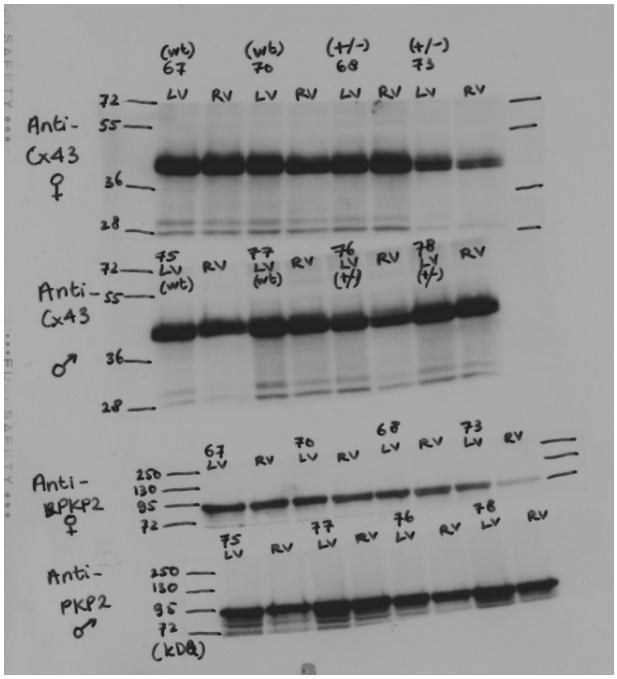

Figure 3 Westernblot, 2 minutes. Of this blot, only the anti-PKP2 singal from female mice was analysed.

Next, the western blots as shown in Figure 2 and Figure 3 were analyzed on ImageJ. The intensities of every band were divided by the intensity of a part of the corresponding lane from the Ponceau staining. These ratios were

averaged in such a way that eight values were obtained: Cx43 in female and male mice, wildtype and PKP2 haploinsufficient; and PKP2 in female and male mice, wildtype and PKP2 haploinsufficient. A graph was made based on these results, as seen in Figure 4. This shows a clear decrease in Cx43 and PKP2 between wildtype and PKP+/- mice. As expected, the PKP2 levels decreased by approximately a half. A difference between male and female mice in the expression of Cx43 or PKP2 was however not found.

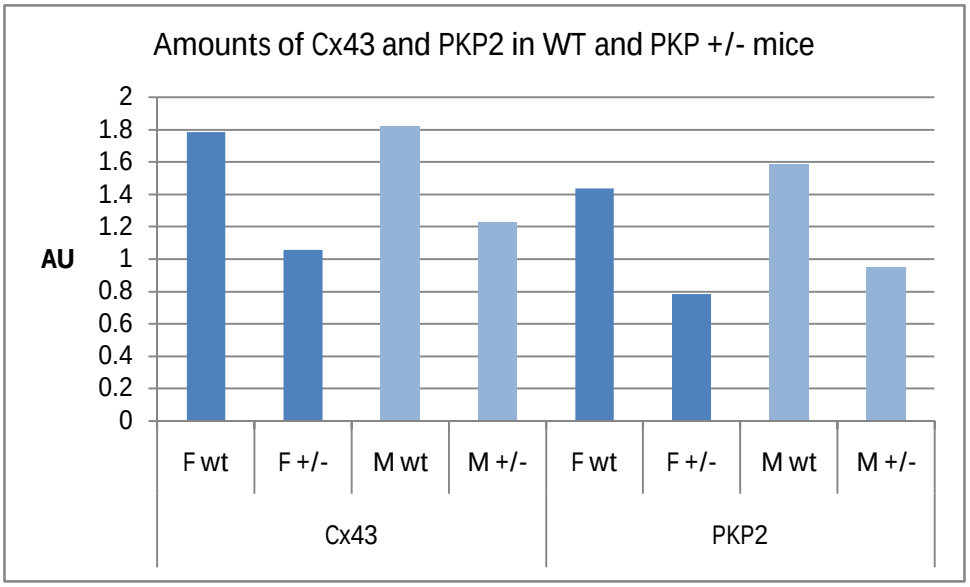

Figure 4 Amounts of Cx43 and PKP2 in WT and PKP +/- mice. F, female; M, male; wt, wildtype; +/-, PKP2 haploinsufficient

Discussion

In this report, the influence of PKP2 haploinsufficiency on the PKP2 and Cx43 expression was examined on a protein level. The haploinsufficiency caused a reduction of PKP2 levels of approximately 50%, as expected. PKP2 is a desmosomal protein, and it has been previously reported that loss of this protein causes GJ remodelling. Cx43 is an important protein in the GJ, as six Cx43 molecules form one circular canal in the plasma membrane called a connexon. Two connexons of adjacent cells can together form a GJ. Saffitz *et al.* showed already a correlation between the amount of Cx43 and GJs [5]. This was confirmed by Oxford *et al.* who also showed that loss of PKP2 caused a reduction in Lucifer yellow-permeable GJs. Also, Cx43 and PKP2 were shown to coexist in a macromolecular complex [4]. In the experiments from this report, Cx43 was indeed decreased. This implicates a loss of electrical coupling. Whether this is actually the case can be determined by the pacing experiments conducted on the mouse hearts. These experiments exceed however the scope of this report.

In a previous publication, differences in PKP2 and Cx43 in the membrane fraction between male and female mice was reported [6]. Overall expression did not differ significantly between the two groups. In these experiments, the total protein amount was analysed in these experiments, and there were no significant differences in Cx43 and PKP2 expression between male and female mice. It would be interesting to investigate further whether there are differences in membrane fraction of Cx43 and PKP2 for these mice.

Literature

1. Rampazzo, A., et al., *Mutation in human desmoplakin domain binding to plakoglobin causes a dominant form of arrhythmogenic right ventricular cardiomyopathy.* Am J Hum Genet, 2002. **71**(5): p. 1200-6.
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