Remodeling of the peripheral cardiac conduction system in response to pressure overload

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Departments of 1Regenerative Medicine and Cell Biology, 2Medicine, and 3Cardiothoracic Surgery, Medical University of South Carolina, Charleston, South Carolina; 4UCD Conway, Dublin, Ireland; 5Université de la Méditerranée, Marseille, France; and 6Ralph H. Johnson Veterans Administration Medical Center, Charleston, South Carolina

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Harris BS, Baicu CF, Haghshenas N, Kasigasan H, Scholz D, Rackley MS, Miquerol L, Gros D, Mukherjee R, O’Brien TX. Remodeling of the peripheral cardiac conduction system in response to pressure overload. Am J Physiol Heart Circ Physiol 302: H1712–H1725, 2012. First published February 3, 2012; doi:10.1152/ajpheart.00621.2011.—How chronic pressure overload affects the Purkinje fibers of the ventricular peripheral conduction system (PCS) is not known. Here, we used a connexin (Cx)40 knockout/enhanced green fluorescent protein knockin transgenic mouse model to specifically label the PCS. We hypothesized that the subendocardially located PCS would remodel after chronic pressure overload and therefore analyzed cell size, markers of hypertrophy, and PCS-specific Cx and ion channel expression patterns. Left ventricular hypertrophy with preserved systolic function was induced by 30 days of surgical transaortic constriction. After transaortic constriction, we observed that PCS cardiomyocytes hypertrophied by 23% (P < 0.05) and that microdissected PCS tissue exhibited upregulated markers of hypertrophy. PCS cardiomyocytes showed a 98% increase in the number of Cx40-positive gap junction particles, with an associated twofold increase in gene expression (P < 0.05). We also identified a 50% reduction in Cx43 gap junction particles located at the interface between PCS cardiomyocytes and the working cardiomyocyte. In addition, we measured a fourfold increase of an ion channel, hyperpolarization-activated cyclic nucleotide-gated channel (HCN)4, throughout the PCS (P < 0.05). As a direct consequence of PCS remodeling, we found that pressure-overloaded hearts exhibited marked changes in ventricular activation patterns during normal sinus rhythm. These novel findings characterize PCS cardiomyocyte remodeling after chronic pressure overload. We identified significant hypertrophic growth accompanied by modified expression of Cx40, Cx43, and HCN4 within PCS cardiomyocytes. We found that a functional outcome of these changes is a failure of the PCS to activate the ventricular myocardium normally. Our findings provide a proof of concept that pressure overload induces specific cellular changes, not just within the working myocardium but also within the specialized PCS.

The peripheral conduction system (PCS) is a network of specialized Purkinje fiber cardiomyocytes located subendocardially on the trabeculated myocardium. This cellular network precisely integrates with the working myocardium and activates coordinated ventricular contraction. Abnormalities associated with the PCS may cause ectopic sites of activation and generate arrhythmias (34). Pressure overload induces hypertrophy of working cardiomyocytes in the ventricle, a common feature of human cardiac disease, and is associated with higher morbidity and mortality (25). Thus, we hypothesized that the PCS would likely remodel when exposed to the effects of chronic hemodynamic pressure overload. To unequivocally identify PCS cells, we used the Cx40EGFP/+ transgenic mouse, where enhanced green fluorescent protein (EGFP) is knocked in under the control of the gap junction connexin (Cx)40 promoter (28). This transgenic mouse line expresses EGFP in the atria, atrioventricular node, coronary endothelium, and PCS (i.e., sites of Cx40 expression) and has been shown to have a normal anatomic phenotype with preserved electrophysiology (13, 28).

Normal intercellular propagation of electrical wavefronts occurs through gap junctions that are predominantly found at intercalated disks. Ventricular gap junctions are composed of three Cx protein subtypes: Cx43, which is the principal Cx expressed in working cardiomyocytes; Cx40, which is expressed within the PCS network (18); and Cx45, which is expressed at relatively low levels throughout the ventricle (1, 8, 9, 19, 40). It is noteworthy that coexpression of Cx40, Cx43, and, to a lesser extent, Cx45 occurs at the critical interface between the distal PCS and working ventricular cardiomyocytes. A breakdown at this key interface by, for instance, cardiac conditional knockdown of Cx43 leads to multiple ectopic activation sites (29). Indeed, since gap junctions contribute to anisotropic conduction throughout the myocardium, perturbations in their quantity or localization can significantly affect conduction. For example, remodeling of Cx43 protein has been identified during left ventricular (LV) hypertrophy (32), creating proarrhythmic substrates (37). However, the effects of pressure overload hypertrophy on changes in Cx localization within PCS cells remains unclear. Accordingly, one goal of this study was to characterize the effect of chronic pressure overload on the distribution of Cxs in the PCS.

Besides differing from working cardiomyocytes in their gap junction subtype composition, PCS cells also differ in that they possess funny current (If), also known as pacemaker current, which is required for the generation of pacemaker action potentials (38). This current is a mixed cation inward current encoded by a family of related hyperpolarization-activated cyclic nucleotide-gated channels (HCNs) (31). Cardiac HCN expression within the sinoatrial node and atrioventricular node is responsible for automaticity (2). In the mammal, HCN2 and HCN4 are found within the ventricular myocardium, and HCN4 is localized to PCS cells at low levels (14, 33). Since ventricular ectopic foci can originate within the PCS, we
hypothesized that HCN4 expression may be altered in a disease model such as pressure overload hypertrophy and that there may be changes in electrical activation patterns.

Therefore, we used the Cx40\(^{+/EGFP}\) transgenic mouse and our chronic pressure overload model to directly investigate the hypertrophic response of the PCS in terms of cell sizes, markers of hypertrophy, and changes in key Cxs (Cx40, Cx43, and Cx45) and HCN4 ion channels.

**METHODS**

The Cx40\(^{EGFP}\) transgenic mouse. The Cx40\(^{EGFP}\) transgenic mouse has one Cx40 allele knocked out with EGFP knocked in after the Cx40 promoter (28). The Cx40\(^{EGFP}\) mouse has labels on the PCS, atrioventricular node, atriun, and coronary arteries. Prior work has shown that this line has a normal anatomic phenotype with normal and preserved electrophysiology (28). The distribution of Cx40 in the mouse is identical to that in the human (20). The original Cx40\(^{-/-}\) transgenic (without EGFP) mouse has a normal phenotype, whereas homozygous Cx40 knockout mice have an embryonic lethal phenotype (4). For our experiments, the Cx40\(^{EGFP}\) transgenic mouse was maintained on a C57BL/6 mouse strain background (three generations) and genotyping was by PCR as previously described (28).

**Transaortic constriction.** Transaortic constriction (TAC) surgery was carried out on Cx40\(^{EGFP}\) mice using our established procedure (17) in 3- to 6-mo-old female Cx40\(^{EGFP}\) mice and wild-type (WT) mice to induce pressure overload left ventricular (LV) hypertrophy. A 7.0 silk suture was placed around the aorta between the carotid arteries, and a 27-gauge needle was placed inside the suture. The suture was then tightened to occlude the aorta, and the needle was removed to leave a reproducible constriction. Sham-operated (sham) mice had a suture passed under the aorta and removed. Mice were maintained for 30 days to develop sustained LV hypertrophy. Surgical survival was 78% on day 1 and 57% by day 30. We found the survival of Cx40\(^{EGFP}\) mice and WT mice to be similar. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and were in accordance with National Institutes of Health (NIH) guidelines.

**Murine echocardiography and electrocardiography.** In vivo echocardiographic measurements were obtained with a 15-MHz transducer with a Sonos 5500 system (Agilent, Santa Clara, CA) using our established protocols with heart rate maintained at 350–450 beats/min (17). End-systolic and end-diastolic LV dimensions and wall thickness were measured according to American Society of Echocardiography guidelines as applied to mice (16). LV wall thickness was measured at the interventricular septum and posterior wall. LV volumes were calculated from Simpson’s method of disks (35), and the ejection fraction was calculated as (LV end-diastolic volume − LV end-systolic volume)/LV end-diastolic volume). The pressure gradient across the aortic band was calculated as four times the peak continuous wave Doppler velocity squared. Analysis was performed with an Enconcenter workstation. To record high-resolution ECGs, anesthesia was induced in Cx40\(^{EGFP}\) mice with isoflurane and maintained with 1.5% isoflurane-5% oxygen while animals were supine on a heat mat. Three subdermal electrodes were introduced into the two forelimbs and the left hindlimb. ECGs were amplified using a Bioamp (BD Instruments) and captured, once heart rate stabilized at ~475 beats/min, at 1 and 2 kHz using Labchart software. Data analysis and interval calculations were made in Excel using eight example waveforms for each mouse.

**Fluorescent immunolabeling, confocal microscopy and Amira three-dimensional reconstructions.** Animals were anesthetized terminally, and the heart was rapidly isolated. The LV was dissected, opened along the septum, fixed in 4% paraformaldehyde, washed, cryoprotected, and embedded. Frozen sections were stained with wheat germ agglutinin (WGA) TRITC conjugate (L-5266, Sigma, St. Louis, MO) to delineate cell borders and with 4’,6-diamidino-2-phenylindole for nuclear counterstaining. For immunofluorescence, sections were incubated with anti-rabbit Cx40 (36-4900, Invitrogen, Carlsbad, CA), anti-rabbit Cx43 (C6219, Sigma), anti-rabbit Cx45 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-rabbit HCN4 antibodies (sc-28750, Santa Cruz Biotechnology). Secondary antibodies included anti-rabbit Alexa fluors 555 (Invitrogen). Immunolabeled slides were viewed with an Olympus Fluoview FV100 laser scanning confocal microscope (LSCM) using our established methods (15). Complete image sets were obtained at the same time with uniform acquisition settings to allow direct comparisons. For Amira three-dimensional reconstruction, immunostained serial sections derived from sham and TAC Cx40\(^{EGFP}\) mouse hearts were analyzed by LSCM. Sequential confocal images of PCS cardiomyocytes were acquired as ‘Z’ stacks and imported into Amira (Visage Imaging). Image stacks were volumetrically rendered in three dimensions, with fluorescent labels assigned green for EGFP and temperature glow for Cx43 immunolabeling.

**Cardiomyocyte sizes, Cx numbers, and HCN4 measurements.** To quantify changes in cardiomyocyte size, WGA-stained sections containing EGFP-positive PCS cardiomyocytes from the base, middle, and apex of the LV from four sham Cx40\(^{EGFP}\) and four TAC Cx40\(^{EGFP}\) mice were measured in a blinded fashion using LSCM. Using longitudinal sections, we determined that cell width was a reproducible and accurate measurement. A total of 362 sham fields and 392 TAC fields containing at least 1 EGFP-positive PCS cardiomyocyte were randomly selected for analysis. The average number of discretely localized and separate Cx40- and Cx43-immunolocalized particles per EGFP-positive PCS cardiomyocyte was quantitated using NIH ImageJ software (version 1.45R). In brief, confocal images were imported into Adobe Photoshop, and the EGFP signal was used to select the region of interest. The red channel (Cx fluorescence) was selected and saved as an 8-bit grayscale binary image with an identical threshold for sham and TAC cohorts. The resulting images were opened in ImageJ, the scale was set, and measurements were made using the analyze particle function. Particle size measurements were carried out using the ImageJ distribution function, with 10 bins from 0–3 μm. Data were imported into Microsoft Excel for statistical analysis and graphing. Since HCN4 channels are uniformly distributed throughout the cell membrane, we quantified thresholded immunofluorescence levels, as previously described (11) (total area of the HCN4 signal/EGFP-positive PCS cardiomyocyte) using 10 fields taken from 3 pairs of sham and TAC Cx40\(^{EGFP}\) mice.

**Colocalization analysis.** Sections of hearts from sham (n = 11) and TAC (n = 17) cohorts were double immunolabeled for Cx40 (anti-goat Cx40, sc-20466, Santa Cruz Biotechnology) and Cx43 (anti-rabbit Cx43, C6219, Sigma). Secondary antibodies included anti-rabbit Alexa fluors 555 and anti-goat Alexa fluors 633 (Invitrogen). Cx-expressing gap junction interfaces between PCS cardiomyocytes (EGFP positive) and working cardiomyocytes (EGFP negative) were identified, and high-resolution image capture of these junctional interfaces was performed by LSCM at ×63 with a ×4 digital zoom using identical settings. To enable colocalization analysis, the resulting single optical sections were separated into individual channels and modified by switching Cx40 onto the green channel (channel 2) while maintaining Cx43 on the red channel (channel 1). Images were imported into NIH ImageJ (version 1.45R), and colocalization analysis was performed (5, 15, 42). In brief, we used ImageJ plugins-colocalization analysis-colocalization highlighter to identify the actual signal overlap, presented as white pixels.

**Tissue fractionation and Western blot analysis.** Tissue was homogenized with an Ika-Werke T-25 Ultra Turrax disperser in modified RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM EGTA, 1 mM
EDTA, 2 mM sodium vanadate, 1% Nonidet P-40, and 0.1% SDS) with protease and phosphatase inhibitors for 30 s. Samples were then centrifuged at 8,000 rpm (7,649 g) in a Sorvall RC-5B Refrigerated Superspeed Centrifuge with a SS-34 rotor for 20 min at 4°C to collect the insoluble cytoskeletal and membrane fraction (pellet 1), and the remaining sample was centrifuged in a Beckman L-70 Ultracentrifuge with a 70Ti rotor for 150 min at 37,000 rpm (89,544 g) at 4°C to separate the soluble cytosolic fraction (supernatant 2) from the insoluble nuclear membrane (pellet 2). Pellets 1 and 2 were resuspended by boiling for 5 min in 2× SDS Laemmli loading buffer and briefly sonicated.

Proteins were separated by SDS-PAGE (10%) gels and transferred to Immobilon-P polyvinylidene difluoride membranes. Blots were blocked with 2% BSA at room temperature and incubated with primary antibody overnight at 4°C in 2% BSA [1:20,000 anti-rabbit Cx43 (C6219, Sigma), 1:5,000 anti-goat Cx40 (sc-20466, Santa Cruz Biotechnology), 1:5,000 anti-rabbit desmin (D8281, Sigma), and 1:20,000 anti-rabbit GAPDH (10R-G109a, Fitzgerald Industries)]. Blots were washed with Tris-buffered saline followed by an incubation with horseradish peroxidase-conjugated secondary antibodies in 2% BSA and an exposure to chemiluminescent substrate (Western Lightning ECL, Perkin-Elmer, Waltham, MA).

Quantitative real-time RT-PCR. EGFP-positive Purkinje fibers were microdissected from the subendocardium of 30-day TAC and sham Cx40EGFP/+ mice using an Olympus MVX10 Microfluorescence dissection scope. Tissue was combined from three individual hearts, collected in RNAlater solution (Ambion, Austin, TX), and homogenized. RNA was isolated with the Qiagen Fibrous Tissue RNAeasy kit. RNA was quantified using the Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA). RNAs (2 μg) were reverse transcribed into cDNA using the RT2 First-Strand Kit (SA-Biosciences, Frederick, MD). Equal volumes of cDNA were loaded into our custom-designed RT2 Profiler Arrays using the Liquid Handling epMotion System (Eppendorf, Hauppauge, NY). After PCR analysis using the Bio-Rad CFX96 Real-Time PCR system, relative gene expression was determined using the ΔΔCt method, where Ct is threshold cycle. PCR primers included the following: myosin heavy chain (MHC; NM_080728), atrial natriuretic factor (ANF; NM_008725), brain natriuretic peptide (BNP; NM_008726), Cx40 (NM_008812), and HCN4 (NM_001088192) plus loading controls.

Optical mapping of action potential propagation. To analyze the electrical activation through the myocardium at the functional level, we used optical mapping: the visual recording of action potentials in the heart using a voltage-sensitive dye. The approaches used for optical mapping in sinus rhythm were similar to those we and others have previously described for studies of the mouse heart (27), with some modifications. Mouse hearts were isolated and cannulated via the heart using a voltage-sensitive dye (di-4-ANEPPS) for contraction decoupler) in the perfusate. Hearts were stained using an increasing LV-to-body weight ratio (from 2.9 to 6.5), increased LV-to-tibial length ratio (from 3.3 to 7.2), and increased mean LV wall thickness (from 0.7 to 1.2 mm, P < 0.05), similar to WT mice. The average LV ejection fraction in both Cx40EGFP/+ and WT mice was in the normal range and was not altered after TAC. Increased heart size after TAC was evident in whole heart heart preparations (Fig. 1, A–D). Echocardiography revealed that TAC did not affect heart rate (Table 1); similarly, ECG analysis demonstrated comparable R-R intervals (Fig. 1, E and F). High-resolution ECG analysis (2 kHz) revealed that TAC caused the expected significant increase in QRS interval duration together with abnormal ST morphology (note that in mice, the S and T waves merge into a composite ST segment; Fig. 1, G and H). In addition, we identified an increase in P wave duration, likely due to left atrial enlargement.

Macroscopic heart dissection and exposure of the LV endocardial surface allowed us to unequivocally identify, by EGFP expression, the entire PCS network localized throughout the cardiac surface allowing us to unequivocally identify, by EGFP expression, the entire PCS network localized throughout the cardiac surface. Motion artifacts were prevented by the introduction of minimal levels of blebbistatin (an excitation-contraction decoupler) in the perfusate. Hearts were stained using a voltage-sensitive dye (di-4-ANEPPS) for ~10 min. The spread of depolarization across the myocardium was monitored by changes in the fluorescence signals of di-4-ANEPPS using a MiCAM2-CMOS high-speed camera (Scimedia) at 1.2 ms/frame and 833 Hz. Isochrome maps indicating the site of earliest activation were generated from these data.

Statistics. Differences in measured parameters were compared between the sham and TAC groups using a t-test and ANOVA. Excel software was used to calculate statistics and generate graphs. Data are presented as means ± SE. P values of <0.05 were considered to be statistically significant.

RESULTS

Pressure overload induces PCS hypertrophy. Pressure over-load induced by TAC for 30 days resulted in statistically identical echocardiographic, hemodynamic, and gravimetric responses in Cx40EGFP/+ and WT mice (Table 1). After 30 days of TAC, Cx40EGFP/+ mice showed increased aortic gradients from below 5 mmHg to an average of 98 mmHg. After TAC, Cx40EGFP/+ mice displayed an increased LV-to-body weight ratio (from 2.9 to 6.5), increased LV-to-tibial length ratio (from 3.3 to 7.2), and increased mean LV wall thickness (from 0.7 to 1.2 mm, P < 0.05), similar to WT mice. The average LV ejection fraction in both Cx40EGFP/+ and WT mice was in the normal range and was not altered after TAC. Increased heart size after TAC was evident in whole heart heart preparations (Fig. 1, A–D). Echocardiography revealed that TAC did not affect heart rate (Table 1); similarly, ECG analysis demonstrated comparable R-R intervals (Fig. 1, E and F). High-resolution ECG analysis (2 kHz) revealed that TAC caused the expected significant increase in QRS interval duration together with abnormal ST morphology (note that in mice, the S and T waves merge into a composite ST segment; Fig. 1, G and H). In addition, we identified an increase in P wave duration, likely due to left atrial enlargement.

Macroscopic heart dissection and exposure of the LV endocardial surface allowed us to unequivocally identify, by EGFP expression, the entire PCS network localized throughout the subendocardial surface of the heart (Fig. 2, A and B). These whole mount images, taken at the same magnification, suggested a denser or more extensive EGFP-positive Purkinje fiber network after TAC (Fig. 2, A vs. B, shown at higher magnifi-

Table 1. Gravimetric and echocardiographic measurements demonstrating LV hypertrophy after TAC

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<th></th>
<th>Wild-Type Mice</th>
<th>Cx40EGFP/+ Mice</th>
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<tr>
<td></td>
<td>Before TAC</td>
<td>After TAC</td>
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<tr>
<td>n</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Transaortic pressure gradient, mmHg</td>
<td>96 ± 3*</td>
<td>99 ± 0.4*</td>
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<tr>
<td>LV/body wt, mg/g</td>
<td>3.1 ± 0.1</td>
<td>6.6 ± 0.5*</td>
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<tr>
<td>LV/tibial length</td>
<td>8.2 ± 0.6</td>
<td>3.3 ± 0.2</td>
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<tr>
<td>LV ejection fraction, %</td>
<td>67 ± 3</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>372 ± 7</td>
<td>402 ± 22</td>
</tr>
<tr>
<td>LV mean septal and posterior wall thickness, mm</td>
<td>1.2 ± 0.1*</td>
<td>1.2 ± 0.1*</td>
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Data are shown as means ± SE; n, number of mice/group. For heart rate, n = 5 mice/group; for LV/tibial length, n = 4 mice/group. LV, left ventricular; Cx40EGFP/+ mice, connexin (Cx)40 knockout/enhanced green fluorescent protein (EGFP) knockin transgenic mice. *P < 0.05 vs. the corresponding before TAC values.
We therefore investigated PCS cell size changes from tissue sections from these same hearts, which were counterstained with WGA to demarcate cell borders. Analysis of the 362 images from sham cohorts and 392 images from TAC cohorts captured by LSCM determined that PCS cells responded to TAC with an increase in cell width by 23 ± 10% (Fig. 2E). This hypertrophic increase was statistically significant, albeit less than the response found in working cardiomyocytes (which increased by 31 ± 3%). To examine the gene expression of hypertrophic PCS cardiomyocytes, we microdissected Purkinje fiber networks from both sham and TAC Cx40EGFP/+ mice (n = 7 and n = 11, respectively) and isolated mRNA. We then used quantitative real-time RT-PCR to probe for three key markers of cardiac hypertrophy: the major structural sarcomeric protein MHC (24, 41) and two small peptide hormones, BNP (21, 30) and ANF (23, 26).

Fig. 1. Cardiac hypertrophy is associated with altered ECGs. A–D: whole mount images of hearts from a connexin (Cx)40 knockout/enhanced green fluorescent protein (EGFP) knockin transgenic mouse model (Cx40EGFP/+ mice) after sham operation (sham; A and B) or after 30 days of transaortic constriction (TAC; C and D). Note the increased heart size and left atrial enlargement. E and F: ECGs showing 3-s rhythm strips revealed normal R-R intervals but aberrant waveform morphology after TAC (E vs. F). G and H: high-resolution ECGs (2 kHz, displayed on identical axes, x = μV and y = μs) showing representative waveforms from sham (G) and TAC (H) Cx40EGFP/+ mice. Note that P wave and QRS intervals were significantly prolonged. *P < 0.05. Scale bar = 1 mm.
each case, we found a significant upregulation after TAC of 19-, 11-, and 23-fold increases, respectively (Fig. 2F).

Remodeling of PCS Cxs with chronic pressure overload. We next sought to evaluate changes in Cx expression within PCS cardiomyocytes, beginning with Cx40, since it is the key component of PCS gap junctions (12). Immunohistochemistry was performed on sections of sham and TAC cardiac ventricular tissue using either α9251-HA9251–actinin to label the adjacent working myocardium or EGFP expression to localize PCS cells. At no time did we identify upregulated/ectopic Cx40 expression in the pressure-overloaded working myocardium (Fig. 3, A and B). However, LSCM analysis revealed that Cx40 levels after TAC were increased throughout the PCS, both in areas of cell-cell contact as well as laterally along the cellular periphery (Fig. 3, C–F). To quantitate these changes, the average number of Cx40 particles was measured in EGFP-positive PCS cardiomyocytes throughout the subendocardial LV. After 30 days of TAC, Cx40 particle numbers increased significantly by 98 ± 15% compared with baseline levels (Fig. 4A). Analysis of Cx40 particle size distribution after TAC revealed significant increases in number through almost the entire size range compared with sham controls (Fig. 4B). We next asked whether the mechanism underlying this increase in Cx40 protein expression was a transcriptional process by probing Cx40 mRNA levels using quantitative RT-PCR, as described above. We identified nearly a twofold increase in Cx40 mRNA levels after TAC (Fig. 4C). To explore these findings further, we microdissected the entire EGFP-positive PCS network from sham and TAC Cx40EGFP+/mice (n = 3 for both), fractionated the tissue by centrifugation, and probed the tissue by Western blot analysis (Fig. 4D). The tissue was fractionated into samples enriched for 1) the cytoskeleton and membranes, 2) the cytosol, and 3) the nucleus and endoplasmic reticulum. Two control antibodies were used to confirm sample purity: desmin (an adhesion molecule found within intercalated disks) was found only in cytoskeletal membrane fraction lane 1 but not lanes 2 and 3, but after TAC there was less Cx40 in lane 1 but increased levels of Cx40 in both cytosol fraction lane 2 and
within the fraction containing the endoplasmic reticulum and other nuclear-associated organelles, lane 3. Thus, we have shown that PCS cardiomyocytes respond to chronic pressure overload with increased levels of Cx40 protein, which is likely caused, in part, by both upregulated transcription and translation.

We next analyzed the expression of Cx43 expression within PCS cardiomyocytes from sham and TAC hearts. By Western blot analysis, we demonstrated a significant reduction in the total amount of membrane-localized Cx43 protein after TAC (Fig. 4D). Analysis of Cx43 immunolabeling by LSCM revealed the typical abundant Cx43 expression pattern within the intercalated disk of the working myocardium (colabeled with antibodies to α-actinin) even after TAC (Fig. 5, A and B). In contrast, Cx43 immunolabeling revealed a limited number of Cx43 particles localized to PCS cells (arrowheads in Fig. 5C) (12). After TAC, Cx43 particles could still be detected but were reduced in number (arrowheads in Fig. 5D). As described above, Cx43 labels the surrounding EGFP-negative myocardium within presumptive intercalated disks (arrows in Fig. 5, C and D). To further analyze the localization of the Cx43 labeling associated with these PCS cardiomyocytes, we generated interactive three-dimensional models. These reconstructions allowed us to demonstrate that the Cx43 particles were mainly localized to the aspect of the PCS that integrates with the underlying working myocardium (Fig. 5, E and F). This is the zone or interface between PCS cardiomyocytes and the working myocardium, and we were able to detect decreased Cx43 immuno-localized particles after TAC (Fig. 5, E and F). We confirmed these findings by immunolabeling and by counting the average number of Cx43 particles identified in EGFP-positive PCS cardiomyocytes, showing that after 30 days of TAC, Cx43 particle numbers decreased by 50 ± 13% compared with baseline levels (Fig. 5G).

To further refine these novel findings, we carried out double immunostainings for both Cx40 and Cx43 and performed a high-resolution confocal analysis of the intercalated disks that form the interface between PCS cells and working cardiomyocytes. In sham tissue, we could identify the expression of Cx43 (red) and Cx40 (green) with colocalization when images were overlayed (Fig. 6, A–C). In contrast, after TAC, we identified...
Fig. 4. PCS cardiomyocytes show altered Cx40 expression patterns after TAC. A: average numbers of discretely localized and separate immunolabeled Cx40 particles were quantified from optical sections by laser scanning confocal microscopy (LSCM). After TAC, PCS cardiomyocytes show a 98 ± 15% increase in Cx40 particle numbers. *P < 0.05. B: Cx40 expression was further analyzed by particle size distribution (in 0.3-μm bins) showing increased Cx40 particle numbers throughout the size range after TAC. C: quantitative RT-PCR was performed on mRNA isolated from microdissected EGFP-positive PCS networks from control (n = 7) and TAC (n = 5) mice, revealing that PCS cardiomyocytes showed a twofold increase in Cx40 gene expression after TAC. *P < 0.05. D: Western blot analysis of fractionated microdissected PCS protein isolations revealed the redistribution of Cx40. Isolates were separated by centrifugation into the cell membrane/cytoskeletal fraction (lane 1), the cytosolic fraction (lane 2), and the nuclear fraction (also containing intracellular organelles; lane 3). Note that after TAC, Cx40 was reduced in the cell membrane (lane 1) and appeared within the cytosolic pellet (lane 2) and nuclear pellet (lane 3). Cx43 levels were significantly reduced in the cell membrane fraction (lane 1 after TAC). Desmin and GAPDH served as loading controls for cell membrane/cytoskeletal and cytosolic fractions, respectively.

a shift in the Cx expression pattern, with reduced Cx43 levels (red) and increased Cx40 (green) and an associated decrease in colocalization (Fig. 6, F–H). Data analysis with ImageJ (version 1.45R) revealed that significant colocalization was evident in sham tissue (as determined by R – 0.727 ± 0.051 and R = 0.851 ± 0.063), which was visually represented as white pixels using the colocalization highlighter and the yellow overlap within the scatterplot (Fig. 6, D and E). After TAC, we identified a statistically significant decrease in colocalization (as determined by R = 0.336 ± 0.015 and R = 0.574 ± 0.036), which resulted in fewer white pixels using the colocalization highlighter and a significant reduction of the yellow overlap within the scatterplot (Fig. 6, E and J).

Since a third Cx subtype, Cx45, is also expressed in the rodent ventricle, having been identified in both working cardiomyocytes (1, 19, 40) and PCS cells (8, 9), we explored its localization in our model system. Using immunoconfocal analysis, we identified overlapping expression of Cx45 with Cx43 in presumptive intercalated disks, albeit at lower levels, as previously reported (Fig. 7, A–D) (1, 19, 40). Similarly, we were able to visualize Cx45 protein expression within EGFP-positive PCS cells, as the published literature would suggest (Fig. 7, E–G) (8, 9). Interestingly, we identified that the limited Cx45 expression was specifically localized to those junctions present at the interface of PCS cells with working cardiomyocytes (Fig. 7, F and G). After TAC, we identified similar Cx45 staining patterns, suggesting that Cx45 is not significantly up- or down-regulated in this particular hypertrophic disease model (Fig. 7, H–J). We quantified these findings by immunolabeling and by counting the average number of Cx45 particles identified in EGFP-positive PCS cardiomyocytes, showing that after 30 days of TAC, Cx45 particle numbers did not significantly change over baseline levels (Fig. 7K). Thus, we have shown that PCS cardiomyocytes respond to chronic pressure overload with phenotypic and Cx subtype-specific changes in expression and distribution.

Upregulated HCN4 expression in the PCS after chronic pressure overload. HCN4 expression was examined by immunofluorescence and confocal microscopy in the PCS pressure overload phenotype and by quantitative RT-PCR. Within working ventricular cardiomyocytes, HCN4 protein was found to be below the limits of detection and not upregulated after pressure overload (Fig. 8, A and B). As previously described, we identified detectable levels of HCN4 in the ventricles of mice in the sham cohort, and this expression was restricted to EGFP-positive PCS cardiomyocytes cells in the ventricle (Fig. 8, C and E). (2). After 30 days of TAC, we found that HCN4 expression was uniformly increased throughout ventricular EGFP-positive PCS cardiomyocytes compared with controls (Fig. 8, C–F). Quantification of immunolocalized HCN4 protein levels revealed a significant increase in the relative signal intensity per PCS cell, from 5.3 ± 1.9 to 19.2 ± 3.8 (P < 0.05; Fig. 8G). We next analyzed HCN4 mRNA levels in control and TAC PCS tissues (microdissected), as described above. As expected, we were able to detect quantifiable levels of HCN4, but this transcript was not modified by TAC (Fig. 8H). Thus, the discrete localization of upregulated HCN4 expression within the pressure-overloaded ventricle is restricted to PCS cardiomyocytes, and this is as a consequence of a hitherto unidentified nontranscriptional event.

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Functional consequences of PCS remodeling on cardiac activation. Since the PCS facilitates the coordinated apex to base activation of the ventricular myocardium in the healthy heart, we next investigated the electrical activation of the epicardial myocardium during normal sinus rhythm in sham and TAC hearts using optical mapping (22, 27). Isochronal maps of the anterior surface of sham hearts reveal the normal apex to base activation pattern, with epicardial breakthrough occurring at the LV and right ventricular apexes (arrows in Fig. 9A) (Supplemental Material, Supplemental Data Movie Fig. 9, sham). In contrast,
isochronal maps of TAC hearts identified reproducibly abnormal activation patterns, with epicardial breakthrough occurring midway through the ventricle (arrow in Fig. 9B) (Supplemental Data Movie Fig. 9, TAC). Quantitation of this change revealed a significant shift in epicardial breakthrough patterns in TAC hearts compared with sham hearts. Analysis in the longitudinal or long-axis plane (apex, midlevel, and base) revealed that while 80% of sham hearts showed apical activation, we were unable to identify any apical activation after TAC; instead, we identified that 83% of the activations occurred at the midlevel of the heart (compared with 20% in sham hearts; Fig. 9C). In addition, we analyzed maps in the lateral or short-axis plane (left and right) and showed that in sham hearts 60% of all breakthroughs were bilateral with a 40% contribution of right-sided activation (Fig. 9D). Similar analysis of TAC hearts identified a shift to left-sided activation breakthroughs (50%) in combination with reduced bilateral (33%) and right-sided (17%) activations (Fig. 9D). These data are the first demonstration that one functional outcome of TAC-induced PCS remodeling is abnormal cardiac activation patterns during normal sinus rhythm. Analysis of myocardial conduction speed after TAC revealed significant changes and included a reduction in longitudinal velocities and an increase in transverse velocities (Fig. 9F). In turn, this lead to a significant drop in anisotropy, from 2 in sham control mice to 1.3 in TAC mice (Fig. 9G).

DISCUSSION

Pressure overload has been documented to elicit LV hypertrophy and is clinically associated with conduction abnormalities (25). We designed this study to examine how the PCS network is involved in this deleterious process by probing the pathophysiological response of these specialized cells to chronic pressure overload. To specifically visualize PCS cardiomyocytes within the ventricle, we used the established Cx40EGFP/+ transgenic mouse line (28). Prior work with this mouse line has demonstrated a normal morphological and electrophysiological phenotype, and we found a similar reproducible response to a TAC model between Cx40EGFP/+ and WT mice. Using EGFP to identify PCS cardiomyocytes, we showed that these cells responded to TAC with a hypertrophic 23% increase in cell width compared with the 31% increase seen in working cardiomyocytes. We also quantified significant upregulation of MHC, BNP, and ANF, three key markers of hypertrophy. Together, our data demonstrate that chronically pressure-overloaded PCS cardiomyocytes do undergo significant hypertrophic growth as an important part of the ventricular hypertrophic disease process.

Since LV hypertrophy is associated with alterations in Cx expression patterns, with proarhythmic potential (32, 34), we analyzed if hypertrophy affected Purkinje fiber Cxs. We demonstrated a doubling of Cx40 expression (the principle gap...
Fig. 7. Cx45 is expressed within the PCS and working myocardium and is unaltered by TAC. A–I: we performed immunoconfocal analysis for Cx45 on tissue sections from sham (A–G) and TAC (H–J) hearts. A: confocal microscopy demonstrated working LV cardiomyocytes coimmunolabel for Cx43 (red) and Cx45 (blue). Shown at higher magnification is the typical and robust cardiomyocyte expression pattern of Cx43 (B). These same cells also expressed Cx45, at lower levels (C), and with similar localization to presumptive intercalated disks (D). E: Cx45 immunolocalization demonstrated a comparable low-level Cx45 (red channel) distribution pattern within EGFP-labeled sham PCS cardiomyocytes, predominantly at the interface with the working myocardium. H: post-TAC PCS cardiomyocytes had similar numbers of Cx45 immunolabeling. Note that the EGFP-negative working myocardium expressed Cx45 (red staining). Insets show magnified regions in each image for sham (F) and TAC (I) hearts. F and G: arrowheads indicate Cx45 immunolabeling in sham hearts (F) and with the EGFP green signal removed for clarity (G). I and J: arrowheads indicate similar Cx45 staining patterns after TAC (I) and with the EGFP green signal removed (J). Nuclei were labeled blue with DAPI. Scale bar = 10 μm. K: average numbers of discretely localized and separate Cx45-immunolabeled gap junction plaques were quantified from optical sections by LCSM and showed no significant change with TAC (P > 0.05).
junction subtype in PCS cells), aberrant distribution within PCS cells, and increased localization to PCS-working cardiomyocyte cellular interfaces. Our data build on previous work that identified increased Cx40 levels (with unspecified localization) in the whole hearts of spontaneously hypertensive rats (3), and advances our knowledge by revealing the PCS to be one of the specific cellular sources for the observed changes. Interestingly, we found that Cx40 maintains its specificity within the PCS network and is not ectopically induced in ventricular working cardiomyocytes, despite pressure overload.

Fig. 8. PCS cardiomyocytes display increased hyperpolarization-activated cyclic nucleotide-gated channel (HCN4) levels after chronic pressure overload. A and B: we were unable to detect HCN4 immunolabeling within α-actinin-labeled working cardiomyocytes after sham operation (A) or TAC surgery (B). Insets show a region in each image where α-actinin green staining was removed for clarity. C and E: HCN4 immunolocalization demonstrated baseline low-level distribution within EGFP-labeled sham PCS cardiomyocytes from different areas of the LV septum. D and F: post-TAC PCS cardiomyocytes from different areas displayed increased HCN4 immunolabeling with uniform distribution. Nuclei were labeled blue with DAPI. Scale bar = 10 μm. Boxes within E and F show the HCN4 staining pattern with EGFP fluorescence removed. G: quantified thresholded immunofluorescently labeled HCN4 protein levels demonstrated a highly significant fourfold increase. *P < 0.05 vs. control. H: quantitative real-time RT-PCR was performed on mRNA isolated from microdissected EGFP-positive PCS networks from sham (n = 7) and TAC (n = 5) hearts revealed that PCS cardiomyocytes did not show a change in HCN4 gene transcription after TAC.

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Further work will be needed to reveal if the upregulation of Cx40 has any effect on the conduction velocity through the PCS network itself. However, we speculate that upregulation of Cx40 could play another, as yet, undiscovered role in this remodeling process. Recent findings in humans have shown that somatic mutations in the Cx40 gene are associated with idiopathic atrial fibrillation (11). Indeed, the gap junction subtype composition that exists within the atrial myocardium is remarkably similar to the interface of the PCS with the ventricular myocardium, i.e., Cx40:Cx43, but the effect of the identified Cx40 mutations at this key zone has not yet been explored (13, 26).

Reduced expression of Cx43 in models of cardiac hypertrophy has been described in vivo and in vitro (7, 32); therefore, we examined Cx43 expression within the PCS. We identified reduced numbers of Cx43 particles localized to the PCS cardiomyocyte-working cardiomyocyte interface, indicating that the same reduction in Cx43 seen at the intercalated disks between ventricular working cardiomyocytes also occurs in this key zone. Evidence exists that perturbations of the stoichiometry of cardiac Cxs at this critical cellular interface between the PCS and working myocardium may generate aberrant activation patterns (29). Here, we identified both upregulated Cx40 expression and reduced Cx43 levels and an associated shift in the colocalization of these two Cxs. This combination has the potential to have a profound effect on the preexisting source-sink relationship between the PCS and ventricular myocardium. Cx45 has been documented to remodel in the context of Cx43 deficiency (19) and has been observed to be upregulated in the failing heart (40). In this study, using our model of pressure overload hypertrophy, we noted a trend for the PCS to have localized Cx45 reductions, but this was not a statistically significant finding.

Abnormalities of If (or its molecular correlate, HCN4) may also have the potential to contribute to arrhythmogenesis (31). Studies of cardiomyocytes from end-stage human heart failure explants have shown elevated HCN4 expression at the mRNA and protein levels and larger amplitude If (6, 39). While these findings did not localize the increased HCN4 to any specific cell subtype (i.e., PCS vs. working cardiomyocytes), they show...
that cardiomyocytes are capable of upregulating this key channel in the context of cardiac disease. Elegant previous work in a rat hypertrophy model identified higher density and maximal conductance of \( h \), which was found to correlate with increases in HCN4 mRNA. Furthermore, this study showed these changes were more evident within cardiomyocytes isolated from the interventricular septum. Given that abundant PCS cells are organized on the interventricular septum and with our data showing elevated HCN4 in PCS cells, we speculate that PCS cells may have been the principle source of increased HCN4. In contrast to the above-mentioned studies with heart failure, our TAC model did not elicit changes in HCN4 mRNA levels, suggesting that a posttranscriptional process was responsible for the upregulated immunolocalized HCN4 protein. This observation may be attributed to differences between a pathologically dilated versus hypertrophied model. Nonetheless, changes in HCN4 expression within the PCS may lead to functional aberrations. Further investigation will be necessary to determine the mechanisms by which HCN4 protein levels increase in PCS cardiomyocytes after pressure overload and may include assessments of protein turnover rates, stability, and degradation pathways.

Analysis of cardiac function by high-resolution surface ECGs and optical mapping of epicardial ventricular activation patterns identified significantly altered electrophysiology during normal sinus rhythm. After TAC, we found significant P wave and QRS interval prolongation. We also identified altered conduction velocities through the TAC ventricular working myocardium that resulted in a decreased anisotropic ratio. Finally, we revealed a dramatic shift in cardiac activation sequence and patterns, which are indicative of a failure of the PCS network to coordinate activation of the ventricular myocardium in a normal fashion. These changes are arguably more deleterious with the potential to cause at a minimum a delay in the normal spread of activation through the myocardium and in the worst case potentially generate ventricular arrhythmias.

In conclusion, we have shown that PCS cardiomyocytes hyperpolarize, albeit to a lesser degree than working cardiomyocytes. We also demonstrate that Cx40 and HCN4, normally expressed in the PCS, are both significantly upregulated, that Cx43 significantly reduced in response to this hemodynamic stress, and that there are induced PCS-working cardiomyocyte stoichiometric changes. This was reflected electrophysiologically by the presence of aberrant activation patterns after chronic pressure overload. Given the known roles of Cx40, Cx43, and HCN4 in cardiac arrhythmogenesis, we speculate that molecular changes in their expression may contribute to the nodule for ventricular arrhythmias in LV hypertrophy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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