Online Data Supplement

Expended Materials and Methods

Generating and purifying recombinant adenoviruses:

A Cx43siRNA 1.0-CMV shuttle vector with a hairpin structure including a 21 nucleotide pre-designed Cx43siRNA sequence (purchased from Ambion) was constructed according to the manufacture's instruction. The correct inserted sequence was verified at the University of Illinois at Chicago (UIC) Research Resources Center (RRC). A positive control (GAPDH shuttle vector) and a negative control shuttle vector (Neg, containing a "scrambled" siRNA sequence that lacks sequence homology to Cx43 and GAPDH genes) were attached with a pSilencer adeno 1.0-CMV kit purchased from Ambion the RNA Company. The Adenoviral (Ad) backbone (that included a LacZ sequence for monitoring transfection efficiency, Ambion) and Cx43siRNA, GAPDHsiRNA, and NegsiRNA vectors were liberalized by restriction enzyme digestion, and transfected into HEK-293 cells (ATCC) to produce recombinant adenovirus particles. The viral particles were harvested with a simple freeze-thaw procedure, and then a medium scale viral amplification was performed by infecting HEK-293 cells. After testing the gene silencing effects of the recombinant adenoviruses, adenoviruses were further expanded and purified by ultracentrifugation through a standard cesium chloride gradient approach.

Arrhythmogenic rabbit nonischemic HF model

New Zealand White rabbits (~3.5 kg) underwent induction of HF by aortic insufficiency followed by thoracic aortic constriction. Progression of HF was assessed

by LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD) and LV fractional shortening (FS) [FS (%) = (LVEDD - LVESD) / LVEDD] using 2-D echocardiography (under sedation with ketamine 0.35mg/kg), as previously described.[1] Animals were studied 12 ± 3 months later when severe heart failure developed (LVESD > 1.2 cm with LV FS ~ 25-30%). HF and age-matched control rabbits (n=17, 15) were euthanized by sodium pentobarbital (80-100 mg / kg i.v.) prior to cardiac excision. The LV free wall from 9 HF rabbits was either flash frozen in liquid nitrogen for immunoblotting or fixed with 4% paraformaldehyde (3 control and 3 HF rabbit hearts), followed by embedding in paraffin for immunostaining studies. 8 HF rabbit hearts were used for LV myocytes isolation.

Rabbit LV myocytes isolation

LV myocytes from HF and age-matched control rabbits were isolated as previously described with modification.[2] In brief, control and HF rabbit hearts were rapidly excised, and the left main coronary artery was canulated by a cut-off tip of a 4-French coronary catheter that was connected to a Langendorff perfusion apparatus (37°C). The heart was perfused with Tyrode's solution including collagenase (0.45-0.75 mg / ml) until it became flaccid (20-30 minutes). LV free wall tissue was minced in Tyrode's solution with 0.09% bovine serum albumin immediately after the digestion. Cells were centrifuged at 50 x g for 5 min to get rid of remaining collagenase solution and non-myocytic cells. After washing with a low (50μM) Ca Tyrode's solution, [Ca] was gradually raised to 1.2mM. Cells were then cultured (5% CO_2, 37°C) in a supplemented M199 culture medium (mmol / L, 25 $NaHCO_3$, 5 creatine, 5 taurine, 2 L-carnitine, 0.1

ascorbic acid, 5 bromo 2-deoxyuridine, and 0.2%BSA) with 2% penicillin-streptomycin (Invitrogen).

Adenoviral infection in isolated LV myocytes from control and HF rabbits

Freshly isolated myocytes from control and HF rabbits were first seeded on laminin-coated 4-well culture dishes with supplemented M199 medium. Cultured cells were infected with AdCx43siRNA (or with AdGAPDHsiRNA (positive control) or AdNegsiRNA (negative control)) or AdCx43WT (with AdLacZ as negative control) for 1hr, and then continually cultured in fresh culture medium (5% CO2, 37°C) without adenovirus for 24 or 48 hrs. In an attempt to maintain stable Cx43 protein content and phosphorylation status, cultured myocytes were continually paced at 0.5Hz (25V, 5msec interval) using a cell culture pacing device including a stimulator and a culture dish electrode system (IonOptix Corporation).

Adenoviral infection efficacy was determined by assessing β-galactosidase (encoded in adenoviral vector as a reporter gene) expression using X-gal staining. In brief, adenoviral infected myocytes were fixed for 30 min at 4ºC with 4% paraformaldehyde in 1x PBS containing (mM) 2 MgCl2, 5 EGTA (pH 8.0), and 0.02% NP-40. Cells were then washed and stained for 16 h at 37ºC for β-galactosidase in staining buffer containing (mM) 5 K3Fe(CN)6, 5 K4Fe(CN)6, 2 MgCl2, 0.01% SDS, 0.02% NP-40, and 1 mg/ml X-Gal.

Immunohistochemical and immunocytochemical staining

Immunostaining was carried out with polyclonal Cx43-T, monoclonal Cx43-NP antibodies (as above), and N-cadherin (Zymed) antibodies. Suspended isolated rabbit myocytes were attached to laminin-coated glass slides for 30 minutes, followed by fixing with 4% paraformaldehyde in PBS for 20 minutes. Rabbit LV tissue specimens were fixed with 10% formalin solution for 24 hrs, and then embedded in paraffin. Then, LV tissue sections (8 μm thickness) were de-waxed with xylene and hydrated through graded ethanol solutions.

Immunohistochemistry was performed using fluorescence labeling technique. In brief, cells were blocked in 10% goat serum and 0.2% bovine serum albumin with 0.2% Triton X100. Tissue sections (2 sections from each of 3 control and 3 HF rabbit hearts) were placed in 3% hydrogen peroxide and then blocked in 10% normal goat or donkey serum. The slides with fixed cells or tissue sections were then incubated with primary Cx43-T, Cx43NP, or N-cadherin antibodies and fluorescent conjugated secondary antibodies (Molecular Probe) as described previously.[2] Images were collected from a laser scanning confocal microscope (Carl Zeiss).

References:

1. Pogwizd SM. Nonreentrant mechanisms underlying spontaneous ventricular arrhythmias in a model of nonischemic heart failure in rabbits. *Circulation.* 1995;92(4):1034-1048.

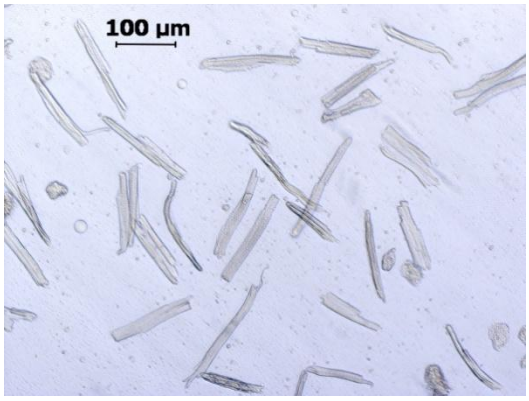
2. Ai X, Pogwizd SM. Connexin 43 downregulation and dephosphorylation in nonischemic heart failure is associated with enhanced colocalized protein phosphatase type 2A. *Circ Res.* 2005;96(1):54-63.

Figure 1

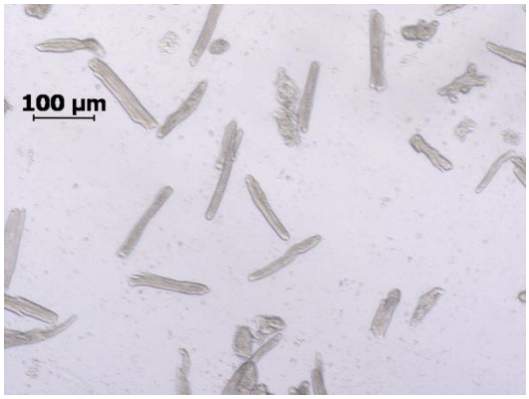
Microscopy images (10x, scale bar=100μm) of control adult rabbit LV myocytes that were freshly-isolated (**A**), cultured 48hrs with pacing (**B**) and cultured 48hrs without pacing (**C**).

Fig.1

A. Freshly isolated myocytes



B. Cultured myocytes with pacing (48hrs)



C. Cultured myocytes without pacing (48hrs)