Online Supplemental Information

T-tubule remodeling during transition from hypertrophy to heart failure

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Online Extended Methods

Animal Model

Animal experiments were performed according to the protocol approved by the University of Iowa Institutional Animal Care and Use Committee. Male SD rats (~60 gram) were subjected to pressure overload by TAB surgery as described [1]. Briefly, three and half week old male SD rat pups (~60 gram) were anesthetized with ketamine/xylazine (40 / 5 mg/kg respectively) by intraperitoneal injection. The rats were then intubated with a 16 gauge tube, and ventilated with a small rodent ventilator (Harvard Apparatus, USA). A thoracotomy was created between the second and third intercostal space, and the aortic arch visualized. A titanium clip was placed on the aortic arch between the brachiocephalic and left common carotid arteries, causing a pressure gradient of ~80 mmHg (81±18 mmHg, N=12). The chest wall was then closed, and the pneumothorax was evacuated. In sham operated animals, the aortic arch was visualized but not banded. The rats were then allowed to recover and then returned to their cages.

Echocardiography of cardiac function

Transthoracic echocardiograms were performed in the University of Iowa Cardiology Animal Phenotyping Core Laboratory, using a Sonos 5500 Imager (Phillips Medical Systems, Andover, MA) [2]. Ketamine HCl (25 mg/kg i.p.) was used to induce a

semiconscious state. 2D images were acquired in LV short- and long-axis planes with an 8-MHz sector-array probe, yielding 100 frames per second. LV mass, volumes, and ejection fractions were calculated with the area-length method [2]. Regions demonstrating akinesis or dyskinesis were visually identified, planimetered, and expressed as percentages of total LV end-diastolic silhouette.

In situ confocal imaging of epicardial myocyte t-tubule structure on intact heart

Intact rat hearts were Langendorff-perfused at room temperature with 0 Ca^{2+} Tyrode's solution (NaCl 137, KCl 5.4, HEPES 10, Glucose 10, $MgCl_2$ 1, NaH_2PO_4 0.33, pH adjusted to 7.4 with NaOH, oxygenated with 95% O_2 and 5% CO_2 during experiments), containing 2.5 μM FM 4-64, a lipophilic fluorescence indicator of membrane structure (Invitrogen Inc., USA) for 20 min. FM 4-64 gave the same in situ t-tubule staining on intact heart as Di-8-ANEPPS (Data not shown). The hearts were placed in the perfusion chamber attached on the stage of a confocal microscope (Figure 2A), and perfused with indicator free / Ca^{2+} free solution (with oxygenation). The membrane structure of epicardial myocytes was analyzed in situ with confocal microscope (LSM510, Carl Zeiss MicroImaging Inc., Germany). The microscope was equipped with 63x (NA=1.4) oil immersion lens. The optical pinhole was set to 1 airy disc (<1 μm axial resolution) during confocal imaging. Each t-tubule image frame contains 202x202 μm^2 area of myocytes (Figure 2A). Ten to fifteen images from different locations of each ventricular free walls (not including apex and interventricular area) were acquired, and power values from each ventricle were averaged to represent the global t-tubule structure of each ventricle.

Image Processing

T-tubule images were analyzed offline with custom routines composed with IDL image analysis program (ITT VIS Inc., Colorado) [3]. Background noise in confocal images was filtered with a threshold value retrieved from image intensity histogram. (Figure 2A, t-tubule image with background noise filtered) [3-4]. FFT routine (coded in IDL) was utilized to perform a Fast Fourier Transformation (FFT) and convert two dimensional images from the spatial domain into the frequency domain (Figure 2C upper panel). This allowed us to determine which pixels contain the most important information, whether repeating patterns occur and how strong (power) the repeating patterns are. The power spectrum obtained from two dimensional FFT of confocal t-tubule images characterized the magnitude of the regular organization of t-tubules, and was used to evaluate t-tubule

remodeling. The central bright signals (Figure 2C upper panel), corresponding to 0 μm^{-1} spatial frequency in lower panel represent low frequency noise. The first peak of spatial frequency components (pointed by blue dashed arrow) centered at ~0.5 μm^{-1} corresponded to the ~2 μm spacing interval of t-tubules, representing the regularity of myocyte t-tubule system. The second and third peaks are the harmonics of the first peak (Figure 2C). The peak power (only the first peak in this study) was measured as the absolute value using Gaussian fitting with pClamp 10 data analysis software (Clampfit, Molecular Devices, USA) (as shown in Figure 2C lower panel). Changes in power (we used the magnitude at the frequency of first peak in this study) were analyzed at different stages of disease.

Western blotting assay of juntophilin-2 (JP-2)

The rat left ventricles were harvested, quickly rinsed in 0 Ca^{2+} Tyrode's solution, immediately frozen in liquid nitrogen and stored at -80 ^{o}C. Frozen tissues were homogenized in lysis buffer (50 mM Tris, PH 7.5, 150 mM NaCl, 10mM NaF, 1mM Na3VO4, 5 mM EGTA, 5 mM EDTA, 0.5% Triton X-100, 0.5% Na Deoxycholate, 0.1% SDS), containing protease inhibitors (Sigma P8340). Tissue lysates were then centrifuged at 12,000 X g for 3 min to remove insoluble debris. Protein concentrations were determined using the Pierce BCA assay (Pierce, Thermo Scientific). Samples (10 μg) were separated by SDS-PAGE (10% acrylamide) and transferred to PVDF membranes. Primary antibodies that recognizes JP-2 (Santa Cruz, sc-51313) and GAPDH (Cell Signaling, #2118) were used. HRP linked anti-Goat IgG (1:5000) and anti-rabbit IgG (1:10000) were used to visualize bound primary antibodies with the SuperSignal chemiluminescence substrate (Pierce, Thermo Scientific), and imaged with Fuji life science imaging system (LAS-3000, Fujifilm, Japan). The protein bands were quantified using Image J software (version 1.43d).

JP-2 shRNA knockdown and t-tubule assay in cultured mouse ventricular myocytes

The shRNA lentiviral particles carrying JP-2 gene silencing sequence were purchased from Santa Cruz Biotechnology Inc. JP-2 shRNA (m) lentiviral particles contain the following 3 constructs
CCAGTGGGAATACCTTTGATTCAAGAGATCAAAGGTATTCCCACTGGTTTTT;
CTCAGAATCACGCACATCATTCAAGAGATGATGTGCGTGATTCTGAGTTTTT;

CTGACTTGACCCTCATCTATTCAAGAGATAGATGAGGGTCAAGTCAGTTTTT. Cultured adult mouse ventricular myocytes, as previously described [5], were transfected with JP-2 shRNA or scrambled control lentiviral particles at a concentration of 1.0×10^5 infectious units of virus (IFU) per ml. 64 hours after viral infection, proteins were extracted and quantified using western blotting assay. Myocyte t-tubule images were acquired with confocal microscope with the same configurations for different group studies.

Statistics

Data were expressed as mean ± SE. Student's t tests were applied when appropriate. $p<0.05$ was considered statistically significant. The Chi-Square test and regression / correlation analysis were performed with Winks 4.62 statistics software (TexasSoft, Cedar Hill, Texas).

Online Table I. Echocardiographic results of anesthetized rats

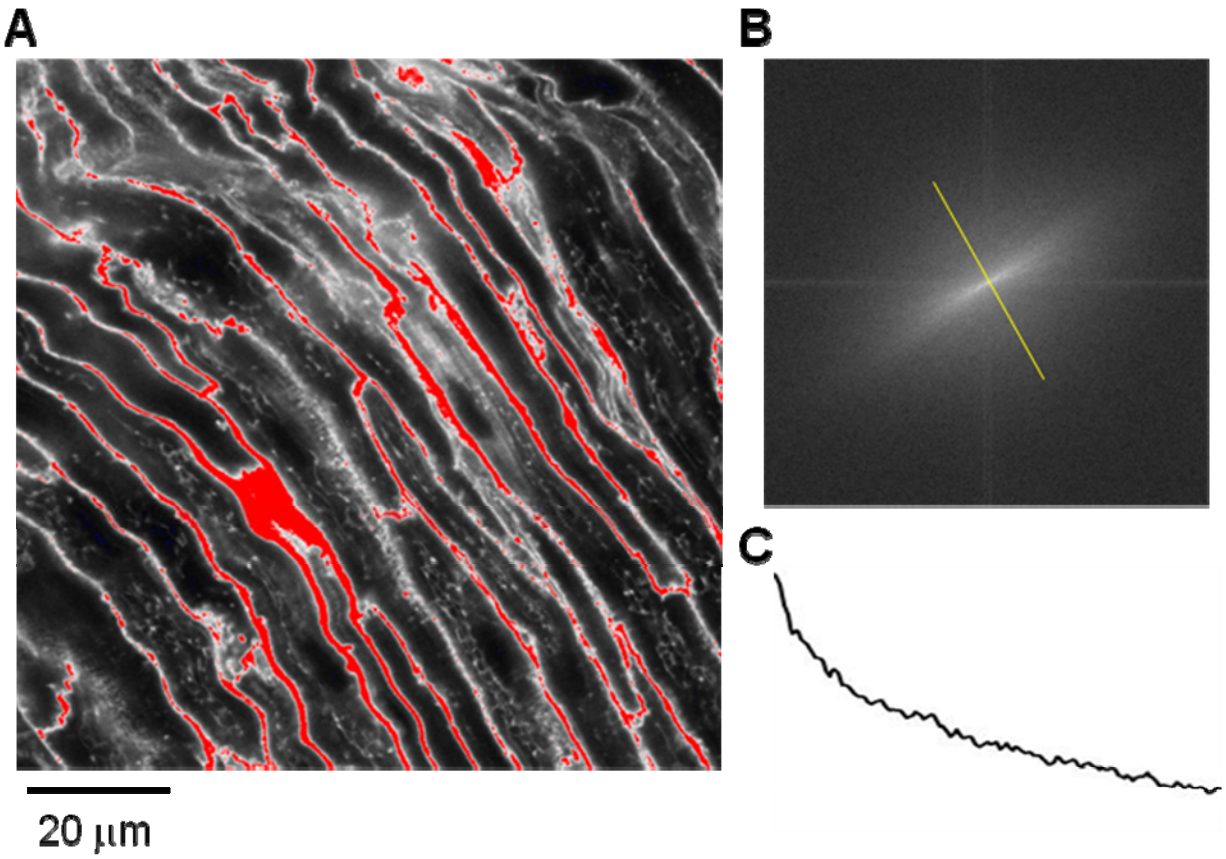
	Sham	Hypertrophy	HF
N	7	12	14
Age (weeks after TAB)	10.0 ± 0.8	9.2 ± 0.48	9.9 ± 1.0
BW (g)	373 ± 26	369 ± 18	346 ± 19
HW (g)	1.49 ± 0.06	2.41 ± 0.11 **	3.25 ± 0.21 **, II
HW/BW (mg/g)	4.01 ± 0.15	5.84 ± 0.18 **	9.00 ± 0.40 **, II
LVMass (mg)	684 ± 28	981 ± 65 **	1090 ± 58 **
LVMass/BW (mg/g)	1.86 ± 0.10	2.81 ± 0.12 **	3.13 ± 0.20 **
EDV (μl)	334 ± 24	374 ± 17	651 ± 59 **, II
ESV (μl)	62 ± 9	72 ± 9	341 ± 41 **, II
Vol/Mass (μl/mg)	0.49 ± 0.02	0.38 ± 0.02 *	0.62 ± 0.05 *, II
EF (%)	81.8 ± 1.2	79.3 ± 2.2	52.8 ± 2.3 **, II

Notes:

N, heart number;
Age (weeks), weeks after banding surgery;
HR (bpm), heart rate (beats per minutes);
BW (g), body weight; HW (mg), heart weight (wet) ;
HW/BW (mg/g), heart weight to body weight ratio;
LVMass (mg), left ventricular mass;
LVMass / BW (mg/g): left ventricular mass to body weight ratio;
EDV (μl): end diastolic volume;
ESV (μl): end systolic volume;
Vol/Mass: end diastolic volume to LV mass ratio
EF (%): ejection fraction.

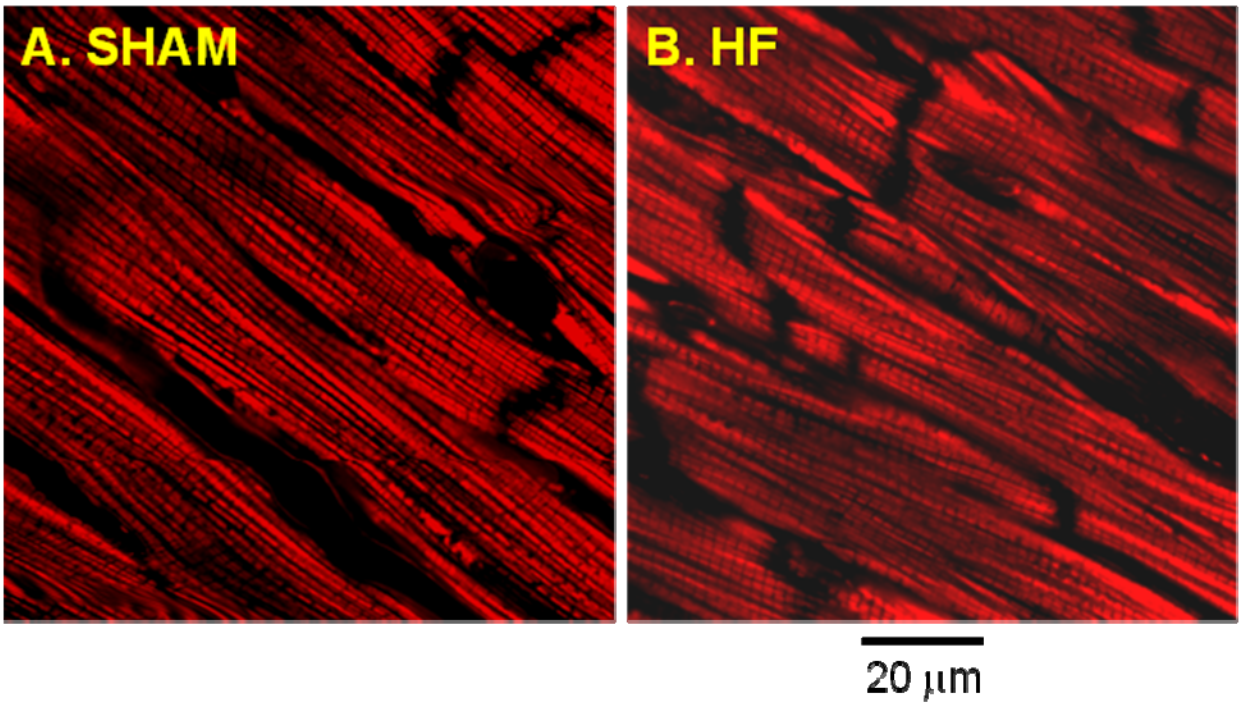
*, $p<0.05$ vs sham; **, $p<0.01$ vs sham; I, $p<0.05$ vs hypertrophy;
II, $p<0.01$ vs hypertrophy.

Online Figure I.



Online Figure I. In situ t-tubule imaging in atrial cells. A. A typical confocal image from right atrial epicardium displaying irregular, sparse t-tubule network in atrial myocytes. **B&C**, Power spectrum analysis shows no dominant peak, consistent with the poor regularity of t-tubule system in these atrial myocytes.

Online Figure II.



Online Figure II. In situ mitochondria imaging in left ventricular myocytes of intact heart. Mitochondria membrane potential probe, TMRE (2.5 μM) were loaded through retrograde Langendorff perfusion system. After 20 minutes of loading, the hearts were then placed into a perfusion chamber attached to confocal microscope equipped with a 63x optical lens (NA=1.3). The excitation wavelength is 561 nm, and emission wavelength >575 nm. **A.** A typical confocal image of mitochondria arrays in normal left ventricular myocytes from a sham operated heart. **B.** In situ mitochondria imaging from a failing heart (pericardial effusion, heart weight = 2.92 g, heart weight / body weight ratio = 9.7 mg/g, Lung weight = 2.45 g, lung weight / body weight ratio = 8.1 mg/g, EF=53%). No significant difference was found in the organization and membrane potential of mitochondria between sham and failing hearts.

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