Supplemental Material

Methods Online

MicroRNA target prediction

The microRNA sequences were extracted from miRBase (Version 13.0), and the 3' UTR sequences of JP2 mRNAs were from the NCBI database. We used the seed region sequences (positions 2-8, from 5' to 3') of microRNAs to scan the 3' UTR sequences of JP2 mRNAs, and selected the perfect complementary regions as potential microRNA target sites. For microRNAs whose 5' 2-8 position sequences had one perfect complementary pairing site on the 3' UTR sequences of JP2 mRNAs, other potential binding sites of these microRNAs were also screened, with the requirement of at most one mismatch among the 5' 2-8 position sequences.

Rat heart failure model

All the animal experiments conformed to the Guide for Care and Use of Laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and all our investigation were approved by the Institutional Animal Care and Use Committee of Peking University.

Male Sprague-Dawley rats (body weight, 50–55 g) were used for transverse aortic constriction (TAC) surgery as described previously[1]. In short, rats were anesthetized with a ketamine-xylazine mixture (5:3, 1.32 mg/kg i.p.). Anesthesia was confirmed by complete inhibition of pedal pinch reflexes. After that, the thorax was opened and a silver clip (0.9-mm inside diameter) was placed on the ascending aorta in the TAC group, while rats in the sham group were subjected to exactly the same surgery except for implanting the clip. After surgery, we characterized heart function by echocardiography (VisualSonics Vevo770 cardiovascular ultrasound system).

Luciferase assay

For luciferase assays, the 3'UTR sequences of human and rat JP2 mRNA, as well as 3' UTRs containing mutations of putative miRNA-24 binding sites, were cloned into the open reading frame of p-MIR reporter plasmid (Ambion). For transfection of HEK293 cells, 50 ng constructed plasmid together with 10 pmol miR-24 Mimics (RiboBio Co., Ltd, China) were delivered by Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions. PhRL-TK plasmid (25 ng, Promega, Madison, WI, USA) was co-transfected with constructed plasmids as control. After 48 hours of transfection, cells were washed with PBS, and lysed in 100 μl passive lysis buffer. After adding 20 μl Luciferase Assay Reagent II, the firefly luciferase activity was recorded using a Varioskan Flash spectral scanning multimode reader (Thermo Scientific). Then, 25 μl Stop & Glo Reagent (Dual Luciferase Assay System; Promega) was added to each well to measure the *Renilla* luciferase activity as control.

Cell culture and adenovirus infection

Ventricular cardiomyocytes were isolated from adult Sprague-Dawley rats (200-250 g) as described[2,3]. Cells were plated on coverslips pre-coated with 1 μg/ml laminin (Sigma) at a density of 5×10^5/dish, and were cultured in pre-balanced M199 medium under 5% CO_2. The miR-24 precursor sequence was cloned using specific primer pairs, and inserted into a pDC315 plasmid, in which the expression of miR-24 was directed by the CMV promoter. A GFP over-expression vector was used as control. Virus was added to the culture medium at an optimized MOI for 12 hours of incubation. Cultured cardiomyocytes were used for experiments 48 hours after infection.

MicroRNA-24 and JP2 mRNA expression assay

Total RNA was extracted from cardiac tissue and cell samples using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and microRNAs were further isolated and enriched from total RNA samples using a mirVana microRNA isolation kit (Ambion). For reverse transcription of total RNA, 2 μg total RNA was added to a 50 μl reaction system containing oligo-dT(15), dNTP, RNAse OUT and Superscript III reverse transcriptase (Invitrogen), and incubated for 1 hour at 50°C. For cDNA synthesis with microRNA samples, 0.5 μg microRNAs was used as template and microRNA-specific reverse transcription primers (RiboBio Co. Ltd) were added instead of oligo-dT(15). To assess the expression of miR-24 or JP2 mRNA, 10 ng cDNA product was used for real-time PCR amplification using Brilliant II SYBR Green QPCR master mix (Stratagene), and the fluorescent signals were monitored by an Mx3000p real-time PCR system (Stratagene). The thermo-cycling program was set as: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, and finally an additional dissociation step to ensure the specificity of amplification. Each sample was measured in triplicate. The primers for microRNA sample amplification were provided by RiboBio Co. Ltd, and the primers for rat JP2 and GAPDH were: rat Jph2 (forward: 5'-AGG-CGG-GTG-CCA-AGA-AGA-AG-3'; reverse: 5'-CGA-TGT-TCA-GCA-GGA-TCA-CCA-3'); and rat GAPDH (forward: 5'-ATC-AAG-AAG-GTG-GTG-AAG-CA-3'; reverse: 5'-AAG-GTG-GAA-GAA-TGG-GAG-TTG-3'). The small nuclear RNA U6 was used as control for microRNA samples and GAPDH was used as control for JP2 mRNA quantification.

Western blot

Cardiomyocytes were homogenized in lysis buffer containing 1% sodium deoxycholate, 10 mM $Na_4P_2O_7$, 1% Triton-100, 10% glycerol, 150 mM NaCl, 5 mM EDTA·Na_2, 20 mM Tris (pH 7.4), 0.1% SDS, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor cocktail (Roche). The sample lysate was separated on 10% SDS-PAGE and then transferred to PVDF membrane. Membrane was incubated with self-made rabbit polyclonal antibody against JP2 (1 μg/ml), which specifically recognized the rat JP2 p434-p447 peptide (QEILENSESLLEPR). A horseradish peroxidase-conjugated GAPDH antibody (KangChen Bio-tech Inc., China) was used to measure the GAPDH content as loading control.

Whole-cell and loose-seal patch clamp

Myocytes were bathed in an extracellular solution containing (in mM) 137 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, 0.02 tetrodotoxin and 10 HEPES, pH 7.35 adjusted with NaOH. The $CaCl_2$ was 2 mM for mouse experiments. The pipette electrode was filled with a solution containing (in mM) 110 CsCl, 6 $MgCl_2$, 5 Na_2ATP, 15 TEACl, 10 HEPES and 0.2 Rhod-2 tripotassium (in experiments involving GFP) or fluo-4 pentapotassium (in other experiments), pH 7.2 adjusted with CsOH. I_{Ca} was activated at 10-s intervals using an EPC7 amplifier (List Medical Electronic, Germany). For loose-seal patch clamp, glass pipettes of 3-5 MΩ were filled with (in mM) 120 TEA-Cl, 20 $CaCl_2$, 10 HEPES, 0.01 tetrodotoxin and 10 μM FPL64176, pH 7.2 adjusted by TEA-OH. The membrane potential (V_m) was determined by proportionally dividing the test voltages between the pipette resistance and the seal resistance (15-20 MΩ). All experiments were performed at room temperature (25°C).

Ca^{2+} imaging

Intracellular Ca^{2+} dynamics were recorded using inverted confocal microscopes (LSM-510 or 5Live, Carl Zeiss, Germany). Line-scan images were acquired at 3.84 ms/line for whole-cell recording and 0.47 ms/line for local Ca^{2+} detection. The Ca^{2+} concentration was either reported as the fluorescence normalized to the

resting level ($R = F/F_0$), or calculated by $Ca^{2+}] = k_d \cdot R/(k_d/C_0+1-R)$, assuming a resting Ca^{2+} concentration C_0 = 100 nM and a dissociation constant k_d = 1.1 μM. The change of cell length was derived from edge-detection of the fluorescence.

TEM imaging and analysis

Isolated mouse cardiomyocytes were washed and primarily fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in PBS (pH 7.4). The cell samples were then rinsed 3 times with PBS and post-fixed with 2% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1 M sodium cacodylate buffer, as recommended by Forbes *et al*[4]. After dehydration in a graded series of alcohols, the samples were embedded in Spurr resin and sectioned with a glass knife on a Leica Ultracut R cutter.

After staining with uranyl acetate and lead citrate, the sections were observed and randomly imaged under an FEI Tecnai G^2 20 Twin system. We analyzed 2 sections per cell from at least 5 cells for each preparation, and >10 images were captured for each section. In stereological measurements[5], TEM images with a magnification factor of 14500 were covered with a 167 nm-spaced grid. The surface area of interest (S_i) in a tested volume (V_{test}) was calculated by[5]

$$\frac{S_i}{V_{test}} = \frac{C_i}{d \cdot P_{test}}$$

where C_i is the number of intersections between the grid and membrane structures of interest (open circles in Online Fig. II), d is the distance between parallel lines in the grid, and P_{test} is the total number of grid points in tested areas. The volume density was calculated as the percentage of interested volume component (V_i) in all the tested volume by the formula[5]

$$\frac{V_i}{V_{test}} = \frac{P_i}{P_{test}}$$

where P_i is the number of grid points falling within the component of interest (closed circles in Online Fig. II).

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed, where appropriate, using Student's t-test, the Mann-Whitney rank sum test, one-way ANOVA and two-way ANOVA with repeated measures. A value of P <0.05 was considered significant.

References：

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2. Zhou YY, Wang SQ, Zhu WZ, Chruscinski A, Kobilka BK, Ziman B, Wang S, Lakatta EG, Cheng H, Xiao RP. Culture and adenoviral infection of adult mouse cardiac myocytes: methods for cellular genetic physiology. *Am. J. Physiol.* 2000;279:H429-436.
3. Fu Y, Zhang GQ, Hao XM, Wu CH, Chai Z, Wang SQ. Temperature dependence and thermodynamic properties of Ca^{2+} sparks in rat cardiomyocytes. *Biophys. J.* 2005;89:2533-2541.
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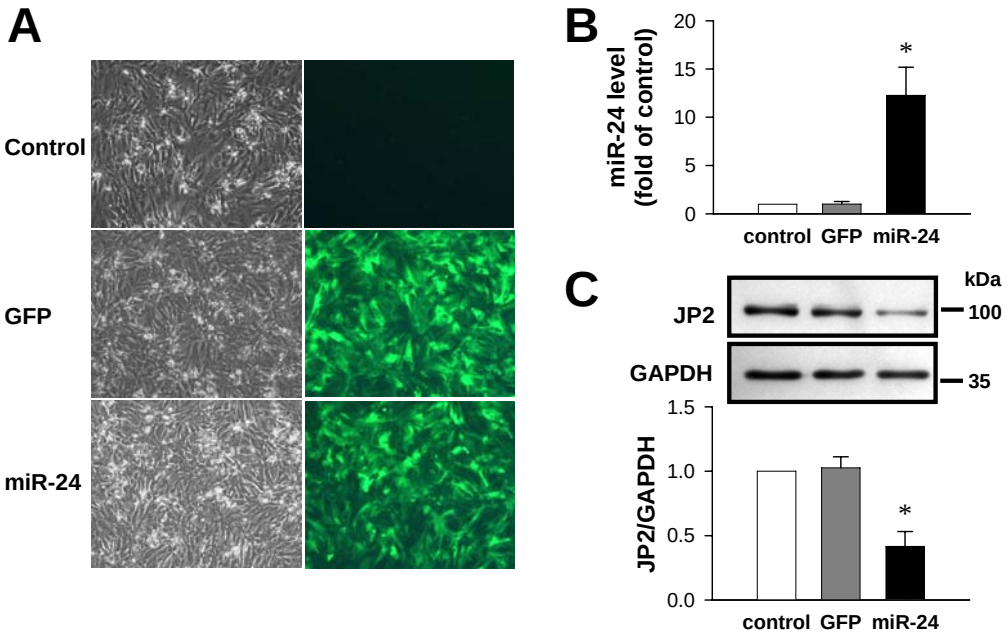
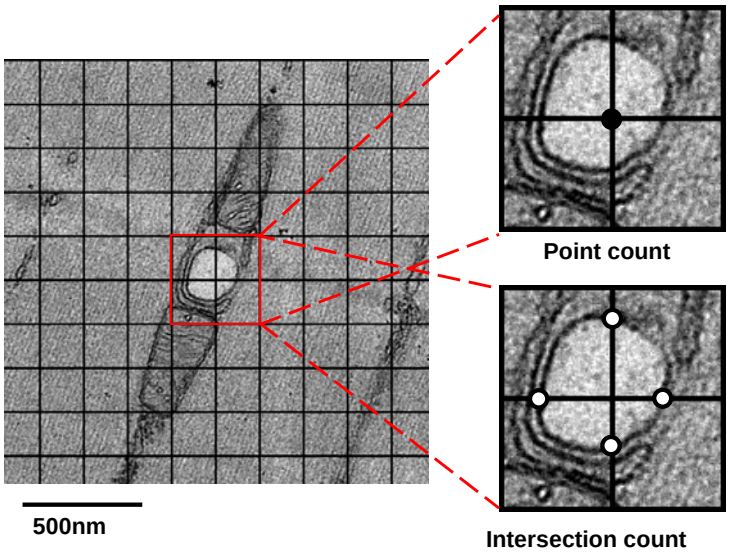
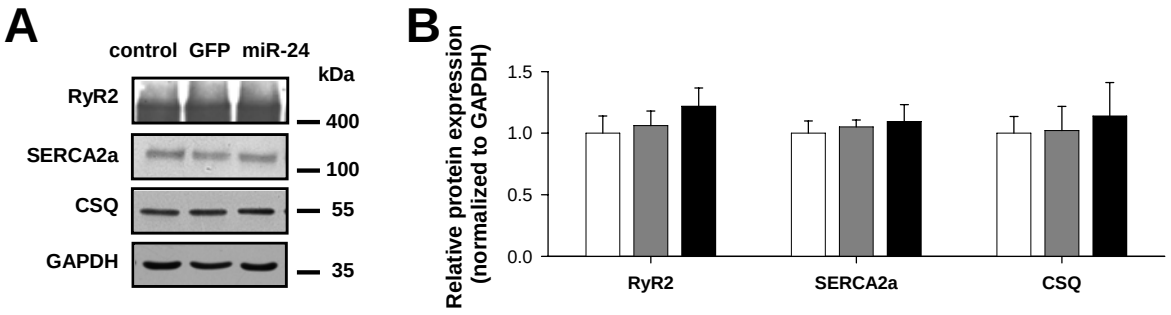
5. Mobley BA, Eisenberg BR. Sizes of components in frog skeletal muscle measured by methods of stereology. *J. Gen. Physiol.* 1975;66:31-45.



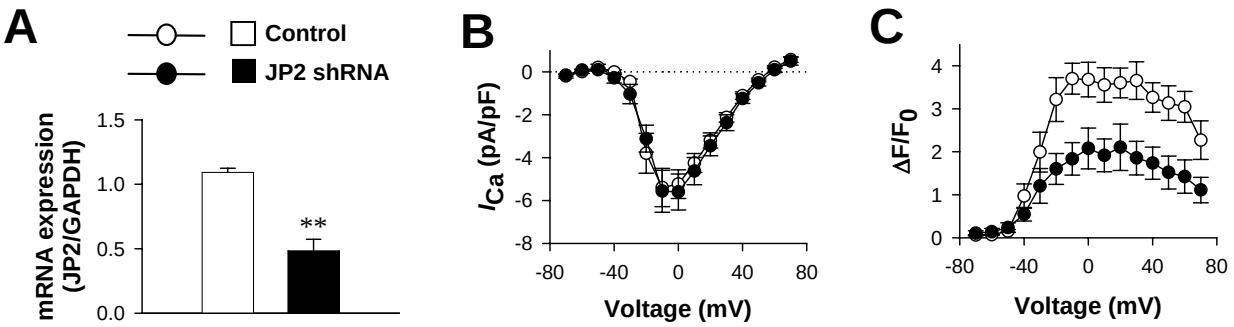
Online Figure I. MiR-24 over-expression suppressed JP2 expression in neonatal rat cardiomyocytes. A, Confocal images of cultured neonatal rat cardiomyocytes (left panels) and GFP fluorescence (right panels) without infection (Control, upper), infected by lentivirus containing GFP (middle) and lentivirus containing miR-24 and GFP (miR-24, lower). The lentivirus was produced by GeneChem Co., Ltd (Shanghai). **B,** Real-time PCR showed that miR-24 was over-expressed in the miR-24 group. n = 3/group. **C,** Western blot assay of JP2 expression. n = 3/group, **P* <0.05 *vs* control group.



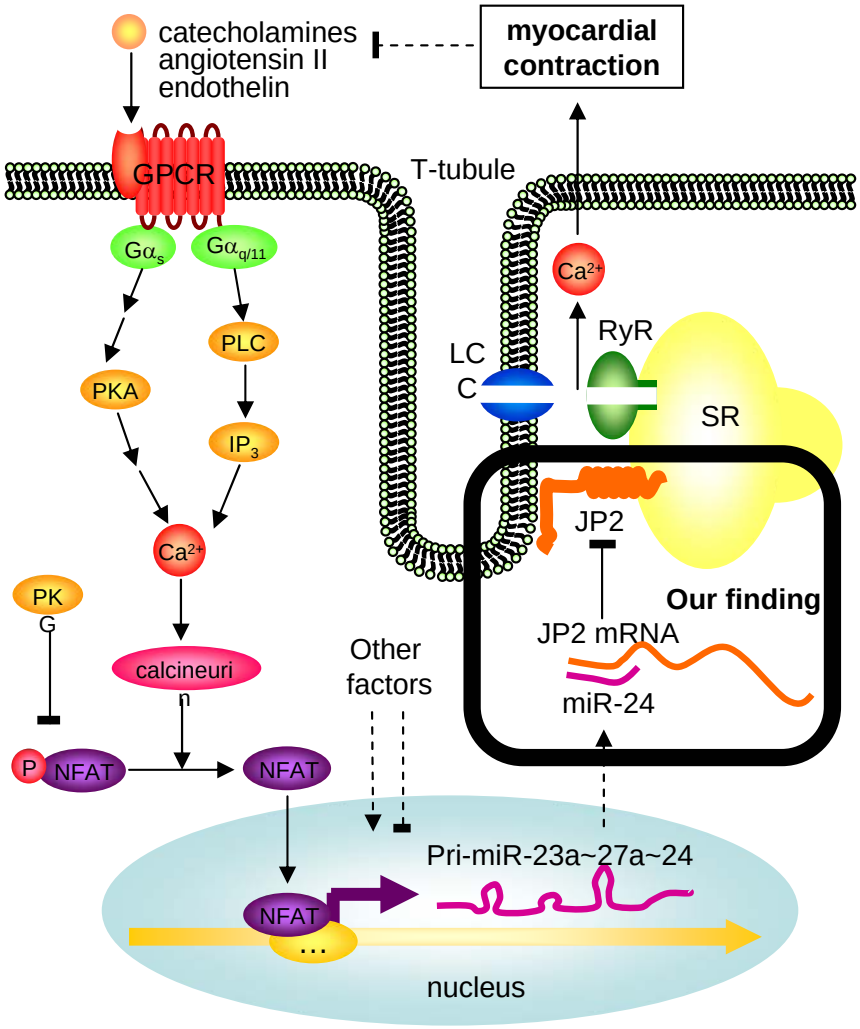
Online Figure II. A representative TEM image from a TAC rat, illustrating the stereological measurements of TT-SR junction in cardiomyocytes. The grid lines were spaced 0.167 mm apart as suggested by Mobley et al.[5] The closed circle and open circles denote examples of point counts and intersection counts, respectively.



Online Figure III. Western blot quantification of the expression of ryanodine receptor type II (RyR2), sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase type IIa (SERCA2a) and calsequestrin (CSQ) after miR-24 delivery. **A,** Typical Western blot results. **B,** Comparison of protein expression among cultured rat cardiomyocytes without infection (Control), infected by adenovirus containing GFP or miR-24. Data were normalized to GAPDH. *n*=4 in each group.



Online Figure IV. Interference of JP2 mRNA led to functional degradation of E-C coupling in rat cardiomyocytes. A, The mRNA level of JP2 was compared by real-time PCR between cells infected with adenovirus containing JP2-targeting shRNA (containing the siRNA sequence 5'-ACACCGTCCTCATCTGTAT-3') and cells infected with adenovirus containing a nonspecific control sequence (containing 5'-TTCTCCGAACGTGTCACGT-3'). The infection of cardiomyocytes by adenovirus was optimized such that JP2 expression was suppressed to a level similar to that in Fig.3B. *n*=3 in each group. **B,** The amplitude and voltage-dependence of *I*Ca density were unchanged by shRNA. **C,** The peak of Ca^{2+} transient was reduced in the JP2 shRNA group, as tested by two-way ANOVA with repeated measures. Data were from ≥ 13 cells in ≥ 4 animals in each group. **P* < 0.05 and ***P*< 0.01 vs. control group.



Online Figure V. An updated pathway linking hypertrophy/ heart failure signaling to E-C coupling. The findings of our present work are highlighted in the black box. The links from G-protein-coupled receptors (GPCR), protein kinase A (PKA) and phospholipase C (PLC) to dephosphorylation of NFAT is based on Rereference 21. The link from calcineurin-NFAT to the miR-23a~27a~24 cluster was based on Reference 23. The role of calcineurin-NFAT pathway in regulating miR-24 has not been tested directly.