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Supplemental Data

Syndecan-4 Signaling Is Required for Exercise-Induced Cardiac Hypertrophy

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MATERIALS AND METHODS

Primary Culture of Neonatal Mice Ventricular Cardiomyocytes (NMVCs)

We obtained ventricles from 13-d-old mice and isolated cardiomyocytes through digestion with trypsin-EDTA and type 2 collagenase. The tissues were cut into small pieces and digested by 0.125% trypsin at 4°C overnight. Collagenase (Sigma, USA, 1 mg/ml in DMEM) was used to further digest tissues in shaking bath at 37°C for 10 min. The cell suspension was centrifuged at 1,000 rpm for 5 min and re-suspended in 10% fetal bovine serum (FBS) (GIBCO,USA) DMEM with 1 g/L glucose (GIBCO,USA) and 10% FBS. Cells were cultured for 1 h to allow fibroblast cells to attach to the flask. NMVMs were collected from the supernatants and cultured with DMEM containing 1 g/L glucose plus 10% FBS and 1% penicillin/streptomycin (GIBCO, USA) for 24 h, The following day media was replaced with DMEM 1 %FBS, pen/strep and adenovirus were added at the indicated multiplicity of infection (MOI). Twenty-four hours later media was again replaced with DMEM 1 % FBS and penicillin/streptomycin and Inhibitors (100 nM Calphostin C, Sigma, USA, 5 uM A6730, Sigma, USA, Sigma, USA), insulin like growth farctor -1 (IGF-1, 10 nM, Sigma, USA) and Isoprenaline (ISO, 10 um, Sigma, USA) were added, incubated for additional 48 h.

Ultrasonic Cardiogram (UCG)

Cardiac function was evaluated by transthoracic echocardiography. The measurement was performed by an observer blinded to treatment. Studies were recorded with a dynamic focused 30-MHz probe echocardiography machine (VisualSonics Vevo2100, Canada). The M-mode measurements of LV dimensions were averaged from more than 3 cycles. Left ventricular end-systolic diameter (LVESD) and end-diastolic diameter (LVEDD) as well as interventricular septal thickness in diastole (IVSD) and LV posterior wall thickness in diastole (LVPWD) were measured. Percentage of left ventricular fractional shortening (%FS) was calculated as follows: %FS = (LVEDD-LVESD)/LVEDD*100. Ejection fractional (EF) was calculated as follows: $\%EF = (LVEDD^2\text{-}LVESD^2)/LVEDD^2*100$.

Histological Examination

After UCG, the mice and rats were euthanized and their hearts were obtained. The hearts were fixed with formalin, embedded in paraffin, and cut into 4 mm slices for subsequent histologic and Immunofluorescence analysis. Cross sections of the LV area between the apices and bases of the hearts were stained with hematoxylin/eosin or Masson's trichrome, and histological studies were performed, including measurements of cardiomyocytes sizes.

Sections were incubated overnight with rabbit-anti-rat synd4 antibody (1:200, Lifespan, USA); following which they were exposed to goat anti-rabbit/mouse secondary antibody conjugated with horseradish peroxidase (Dako, Northern Ireland). The level of synd4 was measured as integrated optical density (IOD). The images were analyzed by two investigators who were blinded to treatment.

Tunel Assay in Myocardium and Cardiomyocytes

Slides of myocardium were incubated with rabbit anti-mice sarcomeric actin (1:50, Abcam USA) overnight at 4°C. Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:400, Molecular Probes USA) was used for subsequent detection. Cardiomyocytes were incubated with rabbit anti-mice sarcomeric actin (1:50, Abcam USA) overnight at 4°C. Alexa Fluor 633-conjugated goat anti-rabbit secondary antibody (1:600, Molecular Probes USA) was used for subsequent detection. TUNEL assay was then performed utilizing an *in situ* cell death detection kit (Promega) following the manufacturer's instructions, and counterstaining of all nuclei was done with 4,6-diamidino-2-phenylindole (DAPI; Sigma, USA). The number of TUNEL-positive and sarcomeric actin positive cells was counted and divided by the total number of sarcomeric actin

positive cells in ten randomly selected fields of view under confocal microscopy. The experiment was performed in triplicate.

Immunofluorescence Assay in Cardiomyocytes

Cardiomyocytes were incubated with rabbit anti-mice sarcomeric actin (1:50, Abcam USA) overnight at 4°C. Alexa Fluor 633-conjugated goat anti-rabbit secondary antibody (1:600, Molecular Probes USA) was used for subsequent detection.

(^{35}S) Methionine Incorporation Assay

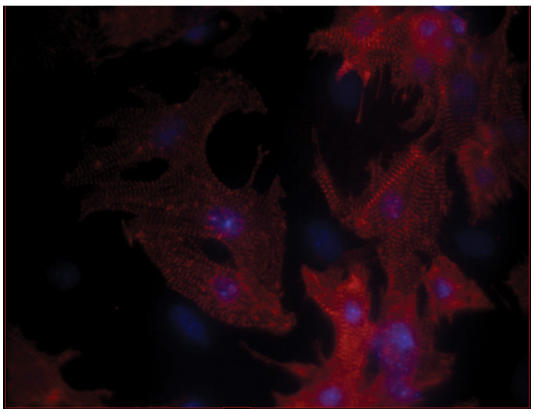
Protein synthesis in mice fetal cardiomyocytes was measured by the incorporation of [^{35}S] methionine (Amersham Pharmacia, USA). Cardiomyocytes were cultured in six-well plate, and were infected with adenovirus for 24 h. After infection, the cells were incubated with methionine-deficient DMEM medium (Sigma, USA) containing 2% FBS for 24 h, and exposed to [^{35}S] methionine at 2 uCi/mL for 24 h. The cells were washed with cold PBS, followed by cold 5% trichloroacetic acid X2, and radioactivity counted using a liquid scintillation counter.

Quantitative Real-time PCR

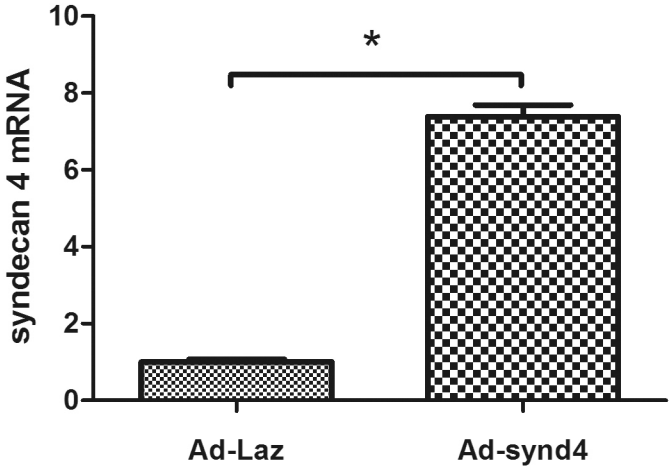
Total RNA from cardiomyocytes was prepared by Trizol (Invitrogen USA) using the protocol provided by the manufacturer, from which cDNA was generated using PrimeScript RT reagent kit (TaKaRa, Japan). mRNA expression of synd4 after MI was analyzed by quantitative real-time RT-PCR using a SYBR® Premix Ex Taq™ System (Takara, Japan) (n=3 per group). mRNA expression of genes were determined using quantitative real-time RT-PCR using a SYBR® Premix Ex Taq™ System (Takara, Japan) (n=3 per group). The primers are listed in the Supplementary Table S1. The reaction was carried out on an ABI 7500 machine. SYBR green real-time PCR assay was carried out in 20 μl PCR mixture volume consisting of 10 μl 2x Premix Ex Taq™ Mix (Takala), 0.4 μl 50x ROX™ Reference Dye, 4 μl RNA and 5.6 μl H2O. Gene amplification was carried out as follows: initial activation of Taq DNA Polymerase at 95°C for 15 min; 45 cycles in four steps: 94°C for 10 s, 55-60°C for 30 s, 72°C for 45 s and 78°C for 3 s; at the end of the amplification cycles, melting temperature analysis was carried out by a slow increase in temperature (0.1°C/s) up to 95°C. Data analysis was carried out using ABI 7500 system software. All samples were run three times and the average value of the copy number was used for further analysis. The RNA expression level was expressed as fold of the Ad-Laz group.

Supplementary Table S1. Primers for Real-Time PCR Assay.

Gene	Primer
β-MHC	5′ TGAGCGGCGCATCAAGGAGC 3′ 5′ CTTGGCACCAATGTCCCGGC 3′
α-Actin	5′ CCCAGGGCCAGAGTCAGAGCAGCAG 3′ 5′ GCTCTGGGCCTCATCACCCACG 3′
MCAD	5′ GTGGCCTTGGCCTGGGAACG 3′ 5′ ATGGCCGCCACATCAGAGCC 3′
α-MHC	5′ GCTGCAGAAGCTGGAGGCCC 3′ 5′ CTCGTGCTGCACCTTGCGGA 3′
LDH	5′ TCAGGCGGCTACACGTACACGGAGA 3′ 5′ TGCTGGAGATCCATCATCTCGCCC 3′
HIF-1α	5′ AGACAACGCGGGCACCGATTC 3′ 5′ GTGGGGAAGTGGCAACTGATGAGC 3′
GLUT1	5′ ATGGCAGGCTGTGCTGTGCTC 3′ 5′ GCCGCACAGTTGCTCCACAT 3′
Hexokinase2	5′ CAGCTCTGTGGCGCAGGCAT 3′ 5′ TATCTCTGCCCGGCCTCCCG 3′
PDK1	5′ TCTATGCGCGCTTCTCGCCG 3′ 5′ ACCAGCTGTACGGATGGGGTCC 3′
PPARα	5′ CGGAGTGCAGCCTCAGCCAAGTTGA 3′ 5′ ATGTTGGATGGATGTGGCCAGGCA 3′
mCPT	5′ TCCGAAGCAGGAGCCCCCTCA 3′ 5′ ATACCCAGTGCCATGACCGGCT 3′
synd4	5′ CAGGGCAGCAACATCTTTGA 3′ 5′ CACGATCAGAGCTGCCAAGA 3′
ANF	5′ ACGCAGCTTGGTCACATTGC 3′ 5′ CCACTAGACCACTCATCTAC 3′



Supplementary Figure S1. Representative micrographs showed candiomyocytes were identified by immunofluorescent staining with an anti-sarcomeric actin antibody.



Supplementary Figure S2. qPCR analyses showed increased synd4 mRNA expression in Ad-synd4-infected NMCMs. The data are derived from 5 independent experiments performed in triplicate and are normalized to the GAPDH content (n-5).