A critical role of cardiac fibroblast-derived exosomes in activating renin angiotensin system in cardiomyocytes

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ONLINE-ONLY SUPPLEMENTS

I. Methods

Reagents

Telmisartan (Cat#:.S1738), an angiotensin II receptor type 1 (AT_1R) blocker, and Akt inhibitor MK-2206 2HCL (Cat#: S1078) were purchased from Selleck Chemicals, USA. PD 123319 ditrifluoroacetate (Cat#: sc-204835), an angiotensin II receptor type 2 (AT_2R) antagonist, was purchased from Santa Cruz Biotechnology, USA. ERK inhibitor U0126 (Cat#: HY-12031), JNK inhibitor SP600125 (Cat#: HY-12041), and p38 inhibitor SB023580 (Cat#: HY-10256) were purchased from MedChem Express (MCE), USA.

Animals and Treatments

Male C57BL/6N mice were purchased from Charles River Laboratories and kept on a 12-h light/dark cycle in a temperature-controlled room with free access to food and water. Fifty two mice at age of 8 weeks were randomly divided into six groups which were treated with vehicle, angiotension II (Ang II) (Cat#: A9525, Sigma-Aldrich, USA), GW4869 (Cat#: sc-218578, Santa Cruz, USA), and dimethyl amiloride (DMA) (Cat#: A4562, Sigma-Aldrich, USA) as follows: (***a***) Control group (n=10) was treated with vehicle saline alone. (***b***) Ang II group (n=10) was treated with subcutaneous delivery of Ang II dissolved in saline (0.9% NaCl) via Alzet osmotic minipumps (Model 2002 ,DURECT, Cupertino, California, USA) at a dose of 2.4 mg/kg body weight/day with an infusion rate of 0.5 µl/h as described elsewhere [1, 2]. (***c***) Ang II plus GW4869 group (n=10) was treated with the subcutaneous delivery of Ang II and intraperitoneal (i.p.) injection of GW4869 (2.5 mg/kg body weight) daily in 0.9% saline as previously reported [3]. (***d***) Ang II plus DMA group (n=10) was treated with the subcutaneous delivery of Ang II and i.p. injection of DMA (1 µmol/kg body weight) daily in 0.9% saline [4]. (***e***) GW4869 (n=6) and (***f***) DMA (n=6) groups were treated with the same amount of GW4869 and DMA alone as described above. Systolic blood pressure was measured noninvasively by the tail-cuff instrument (IITC Inc., Woodland Hills, California, USA) as previously described [2, 5]. The mice were euthanized 2 weeks after the treatment. After clearing blood with saline, the hearts were harvested, dried on gauze, weighed, dissected, and fixed in 10% formalin or frozen in liquid nitrogen. Another set of experiments (n=10, per group) aforementioned was executed with an endpoint at 5 days after the treatment for isolation and culture of adult mouse cardiac fibroblasts. All animal experiments were performed according to National Institute of Heath Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Usage Committees (IACUC) at Shandong University and the University of South Carolina.

Histology and Immunochemistry

Paraffin sections (5 μm) of the hearts were prepared using a rotary microtome (Leica RM2030, Germany) and stored at room temperature until staining. Histology and immunohistochemistry was performed as previously described [1]. Briefly, cardiomyocyte cross-sectional area was measured via wheat germ agglutinin (WGA) (Invitrogen Corp., Carlsbad, CA, USA) staining. For myocardial fibrosis, tissue sections were stained for collagen with a Masson's Trichrome Kit (Poly Scientific, Bay Shore, NY, USA).

Cell Culture and Treatments

Neonatal rat cardiac myocytes and fibroblasts were isolated and cultured as previously described [5]. Briefly, the hearts from 1- to 3-day old Sprague Dawley (SD) rats were finely minced and digested with type II collagenase (200 units/ml; Worthington Biochemical Corp., Lakewood, NJ, USA). Dispersed cells were placed in culture flask for 80 min at 37°C in a CO_2 incubator. During this time, only the fibroblasts became attached to the culture flask, the unadherent cells were mainly cardiomyocytes. The isolated cardiomyocytes were seeded onto gelatin-coated plastic culture dishes at a density of 5×10^4 cells/cm^2 in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% horse serum, 5% new-born calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). The fibroblasts attached to the culture flasks during the primary culture were maintained in high glucose (4.5 g/L) DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/m l). Cardiac fibroblasts at passage (P)2 or 3 were used for the experiments.

Adult mouse cardiac fibroblasts were also isolated form male C57BL/6N mice at ages of 8-9 weeks as previously reported [6] with a minor modification. Briefly, the heart was removed, rinsed and minced in cold PBS. The minced heart tissues were incubated with 15 ml of collagenase IV (4 mg/ml, Cat#: C0130, Sigma-Aldrich, USA) and deoxyribonuclease I (10 U/ml, Cat#: D5025, Sigma-Aldrich, USA) in Krebs-Henseleit bicarbonate (KHB) buffer (pH 7.2) (Sigma-Aldrich) with agitation at 37°C for 40 min. The remaining tissues were digested further in 15 ml of trypsin (0.125%, Cat#: 15050, Thermo Fisher, USA) in Krebs-Henseleit bicarbonate (KHB) buffer (pH 7.2) (Sigma-Aldrich) with agitation at 37°C for 20 min. The remaining tissues were subjected to the digestion and sampling steps again until all the tissue was dissolved. All supernatant was added to 30 ml of full growth medium (4.5 g/L glucose DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml)) and the resulting solution was subjected to centrifugation at 1000 rpm (201 ×g) at room temperature for 5 min. The cell pellet was resuspended, filtrated through a 40-μm filter, and plated onto gelatin coated culture dishes at a density of 2×10^4 cells/cm^2 in full growth medium in a CO_2 incubator at 37°C

(passage 0; P0). When near confluent, the cells were passaged at 1:3 to gelatin coated culture dishes (passage 1). The subsequent subculture was performed at a sub-confluent state (60~80%). The characterization of cardiac fibroblasts was performed at passage 2. Of note, to determine the effect of GW4869 and DMA on Ang II-induced exosome release of cardiac fibroblasts in the heart, mouse adult cardiac fibroblasts (P0) from the mice after the treatment as indicated were used for isolation of the released exosomes. Cells at P2 were used for the other experiments.

Almost 100% of the cultured neonatal rat and mouse adult cardiac fibroblasts express the fibroblast markers Vimentin and S100A4/fibroblast-stimulating factor/fibroblast-specific protein-1 (FSP-1) [7] without detectable expression levels of cardiomyocyte specific Troponin-I and alpha myosin heavy chain (αMHC) [5], smooth muscle alpha actin (SMA) [8], and endothelial cell marker CD31 (Supplementary Fig. 1), indicating that our primary culture of cardiac fibroblasts is established.

For some experiments, the co-culture of neonatal rat cardiac fibroblasts and myocytes was performed using transwell 24-well plates (Cat#: 3413, Corning, Costar, USA) by cardiac fibroblasts (CF) in the top wells and cardiomyocytes in the bottom wells, and a 0.4-μm porous membrane between the wells. Briefly, the lower wells were pre-coated with 0.1% gelatin, and then neonatal rat cardiomyocytes (1×10^5 per well) were cultured in the lower wells. Cardiac fibroblasts (5×10^4 per well) were plated in the upper wells. CF and cardiomyocytes were starved for 24 h in serum-free high glucose DMEM prior to treatment, and then co-cultured with cardiomyocytes in serum free DMEM containing Ang II (1 μM) and GW4869 (10 or 40 μM) for 48 h. Since a ten-time higher dose of AT_1R blockers or AT_2R antagonists is usually used to block Ang II-mediated activation of these receptors on the cell in vitro including primary cultured cardiac fibroblasts and cardiomyocytes [9-11], we chose 10 μM of Temisartan or PD123319 in our experiments. The protein synthesis in cardiomyocytes was measured by [^{3}H]-Leucine uptake assay.

For isolation and quantification of released exosomes, confluent monolayers of neonatal rat cardiac fibroblasts (P2, 4×10^6 cells in a T-75 flask for each group, n=3) were washed with PBS 3 times, and then were cultured with high glucose (4.5 g/L) DMEM supplemented with 5% exosome-depleted FBS with or without Ang II (1 μM), ET-1 (0.1 μM), TGFβ1 (10 ng/ml), insulin (0.1 μM), LPS (0.1 μg/ml), or H_2O_2 (500 μM) for 48 h. Confluent monolayers of mouse adult cardiac fibroblasts (P2, 3.5×10^6 cells in a T-75 flask for each group, n=3) were washed with PBS 3 times, and then were cultured with high glucose (4.5 g/L) DMEM supplemented with 5% exosome-depleted FBS with or without Ang II (1 μM) for 48 h. In addition, cardiac fibroblasts (P0,

2×10^6 cells in a T-75 flask for each group, n=3) of the mice after treatment were cultured in high glucose (4.5 g/L) DMEM supplemented with 10% exosome-depleted FBS for 96 h. The cell number reached up to 3×10^6 per flask 96 h after the culture without significant difference between the experimental groups. All culture medium was subjected to isolation and purification of exosomes.

All cell culture experiments were repeated at least three times with a sample size of 3~6 for each group.

Preparations of Conditioned Medium

Confluent HEK293 cells or neonatal rat cardiac fibroblasts (P2) grew with full growth medium were washed with phosphate buffer (PBS) 3 times and then were cultured with serum-free DMEM for 24 h. The conditioned medium was prepared as previously described [12] with a minimal modification. The supernatants were collected and pre-cleared by successive centrifugations at 300 × g (10 min), 2,000 × g (10 min), and 18,000 × g (30 min) at 4°C to eliminate cells and debris, and then filtered with a 0.22 μm filter unit (Cat#: SLGP033RB ,Millipore, USA). The filtered solutions referred as to conditioned medium (CM) and were transferred to a sterile bottle and stored in -80°C. For concentrated CM of cardiac fibroblasts (CF), the filtered culture medium was further concentrated at 1,000 × g for 30 min at 4°C using a 100 kDa centrifugal filter unit (Cat#: Amicon Ultra-15, Millipore, USA) which cut off molecules with molecular weight > 100 kDa. The concentrated solutions were designed as CF CM (>100 kDa). To deplete exosomes in CF CM (>100 kDa), the concentrated solutions were further centrifuged at 110,000 × g for 12 h in a ultracentrifuge (Beckman Coulter, Optima L-100XP, USA) at 4°C. The pellets were discarded, and the supernatants were exosome depleted CF CM (>100 kDa).

Immunofluorescence Staining and Measurement of Cardiomyocytes Surface Area

Neonatal rat cardiomyocytes were seeded onto gelatin-coated glass slides at a density of 2×10^4 cells/cm^2. After treatments as indicated, the cells were subjected to immunofluorescence staining as previously described [5]. Morphology of the cardiomyocytes was visualized by the immunofluorescence staining for sarcomeric actin with a mouse α-actinin antibody (Cat#: A7811, 1:500, Sigma-Aldrich, USA). For each slide, 6 fields were randomly chosen to photograph and cell sizes were measured by Image Proplus 6.0 software (Media Cybernetics Inc., USA). Cardiac fibroblasts were stained for Vimentin, cardiac Troponin-I, smooth muscle alpha actin (SMA), and CD31 using anti-Vimentin (Cat#:V2258,1:40 ,Sigma-Aldrich, USA), anti-Troponin-I (Cat#:

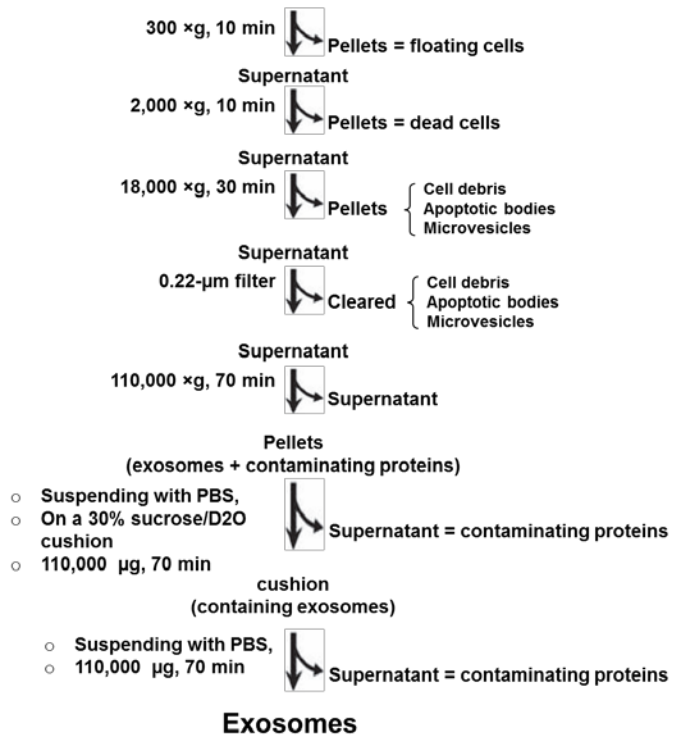
ab47003, 1:400, Abcam, USA), anti-SMA (ab5694, 1:100,abcam, USA), and anti-CD31 (Cat#:ab28364,1:50,abcam, USA).

[^{3}H]-leucine Incorporation

Neonatal rat cardiomyocytes seeded in 24-well plates were treated with different stimuli as indicated for 48 h. [^{3}H]-leucine (Cat#: NET135H, PerkinElmer, USA) was added to the media (final concentration 1 μCi/ml) during the last 6 h of the treatment. After being washed with ice-cold PBS two times, the cells were precipitated with ice-cold 5% trichloroacetic acid (TCA) for 30 min and then washed with ice-cold 5% TCA two times followed by two additional washes with ice-cold PBS. Finally, the cells were lysed with 0.2 ml per well of 0.5 M NaOH for 30 min at 37°C. The radioactivities of [^{3}H]-leucine in cell lysates were measured by a scintillation counter (SN-6930, Rihuan photoelectric device Co.Ltd, Shanghai Institute of Atomic Nucleus)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Quantitative Real Time (Q-PCR)

Total RNAs of the cardiomyocytes were extracted using Trizol reagent (Cat#: 15596, Invitrogen, USA), and reverse transcription reactions were performed with 0.5 μg RNA using a Revert Aid TM First Strand cDNA Synthesis Kit (Cat#: K1622, Fermentas, Lithuania). Expression levels of target genes were normalized by concurrent measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Primers that were used for quantitative (Q-PCR) are summarized in Supplementary Table 1.



Inserted Fig. I: A flow chart for isolation and purification of exosomes from culture medium.

Isolation and Purification of Exosomes

Exosomes were isolated and purified using a multi-step centrifugation protocol as described previously

[13-15] with slight modifications. All centrifugation steps were carried out at 4ºC and depicted in inserted Fig. I. Briefly, the culture media were collected and pre-cleared by successive centrifugations at 300 × g (10 min), 2,000 × g (10 min), and 18,000 × g (30 min) to eliminate dead cells, debris and microvesicles respectively. After being filtered with a 0.22 μm filter unit (Cat#: SLGP033RB Millipore, Ireland), the supernatant was centrifuged at 110,000 × g for 70 min with an ultracentrifuge (Beckman Coulter, Optima L-100XP, USA). The exosome pellet was diluted with PBS and centrifuged on a 30% sucrose/D_2O density cushion at 110,000 × g for 70 min. The exosomes-containing fraction was collected, diluted with PBS and ultracentrifuged again at 110,000 × g for 70 min. The final pellet was resuspended in 200 μl PBS. The exosomal protein concentration was measured using an enhanced BCA assay kit (Cat#: P0010S, Beyotime, China).

Transmission Electron Microscopy (TEM) Analysis

Exosome sample for TEM were prepared as described previously [3,4] with slight modifications. Briefly, immediately after the last ultracentrifuge step, the exosome pellets were suspended with 200 mM phosphate buffer (pH 7.4) containing 2% paraformaldehyde (PFA, w/v). The fixed exosomes were dropped onto a formvar-carbon–coated grid and left to dry at room temperature for 20 min. After washing with PBS (pH 7.4) three times, the exosomes were re-fixed in 1% glutaraldehyde for 5 min. After washing eight times with distilled water, samples are contrasted first in 0.4% (w/v) uranyl acetate for 5min, and then contrasted and embedded in a mixture of 4% uranyl acetate and 2% methyl cellulose in a ratio of 1:9 for 10 min on ice. The excess liquid was blotted by filter paper so that a thin film is left behind over the exosome side of the grid. The grid was dried at room temperature for 10 min and viewed at 80 kV using a transmission electron microscope (JEOL, JEOL1200, Japan).

Quantitation of Exosome Release

The amount of exosomes was quantified by measuring protein concentrations, protein levels of exosome markers, TSG101 and CD63 [16, 17], and activities of acetylcholinesterase (AChE), an exosome specific enzyme [18], of exosomes isolated from equal numbers of cultured cardiac fibroblasts. The protein concentration was determined by bicinchoninic acid (BCA) assay, the protein expression of TSG101 and CD63 was measured by Western blot, and the acetylcholinesterase activity was assessed as previously reported [19]. Briefly, exosomes were resuspended in 40 μl of PBS and incubated with 1.25 mM acetylthiocholine and 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) in a final volume of 300 μl. After 30 min of incubation at 37°C, absorbance at 412 nm was measured to quantify the enzymatic activity.

Proteomics Analysis of Exosomes and Bioinformatic Analysis

The protein expression profile in exosomes derived from neonatal cardiac fibroblasts was analyzed by Shanghai Applied Protein Technology (APT), China, via liquid chromatograph (LC)-tandem mass spectrometry (MS/MS) proteomics as previously described protocol with modifications [20-22]. Briefly, exosomes were lysated with SDT buffer (4% SDS, 150 mM TrisHCl, pH8.0). Total exosome proteins (20 µg) of each group were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After being stained with colloidal Coomassie, the gel band pieces were excised, reduced in 500 µM of DTT. Trypsin digestion was done at 37°C for 18 h with trypsin at a final ratio of 1:50 (trypsin: protein). Experiments were performed on a Q Exactive mass spectrometer that was coupled to an Easy nLC MS system (Proxeon Biosystems, now Thermo Fisher Scientific). Two µg of resulting tryptic peptide mixtures were loaded onto the C18-reversed phase column (Thermo Scientific Easy Column, 10-cm long, 75-µm inner diameter, 3-µm resin) in Buffer A (0.1% formic acid) and separated with a linear gradient of Buffer B (80% acetonitrile and 0.1% formic acid) at a flow rate of 400 nL/min over 140 min. MS data was acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for higher energy collisional dissociation (HCD) fragmentation. Survey scans were acquired at a resolution of 70000 at m/z 200 and resolution for HCD spectra was set to 17500 at m/z 200. The instrument was run with peptide recognition mode enabled.

The sequence data of exosomal proteins were retrieved in batches from the UniProtKB database (Release 2014_11) in FASTA format. The retrieved sequences were locally searched against the SwissProt database (rat) using the NCBI BLAST+ client software (ncbi-blast-2.2.28+-win32.exe) to find homologue sequences so that the identified sequences can be functionally annotated. The top 10 blast hits with an E-value less than 1e-3 for each query sequence were retrieved and loaded into Blast2GO (Version 2.8.0) for gene ontology (GO) mapping and annotation. Following annotation and annotation augmentation steps, the studied proteins were blasted against Kyoto Encyclopedia of Genes and Genomes (KEGG) genes (rat) to retrieve their KEGG orthology identifications and were subsequently mapped to pathways in KEGG. For GO and KEGG analysis, we applied a similar protocol to the one used previously with modification [23-25].

Exosome Uptake Assay in live cells

The exosomes derived from neonatal rat cardiac fibroblasts (CFs) were labelled with a PKH67 Green Fluorescent Cell Linker Kit (Cat#: MINI67-1KT, Sigma-Aldrich, USA) according to the

manufacturer's protocol. Firstly, 20 μg purified CF exosomes were suspended with 250 μl Diluent C, and 1 μl PKH67 dye was diluted in another 250 μl Diluent C. The two solutions were mixed gently for 4 min and then 3.5 ml of 1% BSA was added to bind the excess PKH67 dye. After ultracentrifugation at 110,000 × g for 70 min, the generated pellets were PKH67-labelled CF exosomes and suspended in 100 μl PBS. As the control, 4 ml of 0.5% BSA solution was subjected to PKH67 labeling and then ultra-centrifuged at 200, 000 × g for 2 h. The generated BSA pellet was suspended in 100 μl PBS.

Neonatal rat cardiomyocytes were seeded onto 3.5-cm diameter cell culture dishes with glass bottoms (Cat#: 801001, NEST, China) at the density of 2×10^4 cells/cm^2 in high glucose DMEM supplemented with 8% horse serum, 5% new-born calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). PKH67-labelled CF exosomes (100 μl per dish) or BSA ((100 μl per dish) were then added to the culture medium and incubated at 37ºC for 5-120 min as indicated. The free exosomes were removed by PBS washes for 3 times the uptaken exosomes were visualized by a live cell confocal microscopy (UltraVIEW, PerkinElmer, USA)

Angiotensin II Measurement

The concentrations of angiotensin II (Ang II) in exosomes or culture medium of rat neonatal cardiomyocytes were measured by an Ang II enzyme immunoassay (EIA) Kit with a minimum detectable concentration of Ang II at 21.19 pg/ml (Cat#: RAB0010, Sigma-Aldrich,USA) according to the manufacturer's protocol. Exosomes isolated and purified from rat neonatal cardiac fibroblasts were suspended in PBS and then broken by the treatment of freeze-thaw cycle for three times (-80°C for 10 min and 37°C for 10 min per cycle). Briefly, 100 μg of CF exosomes (0.5 μg/μl) or 100 μl of the supernatants from cultured rat neonatal cardiomyocytes were added to a well of a 96-well microplate. A new standard curve using Ang II provided by the Kit was prepared for each experiment. Absorbance of samples was measured by a microplate reader at a wavelength of 450 nm. AngII concentrations were calculated using the standard curve. Each experiment (n=3-4) was repeated at least three times.

Western Blot

Equal amounts of purified exosomes or cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk solution and then incubated with the first and second antibodies. The first antibodies used included anti-CD9 (Cat#: ab927260, 1:1000, Abcam, USA), anti-CD63 (Cat#: sc-25183, 1:200, Santa Cruz Biotechnology, USA), anti-HSP70 (Cat#: sc32239, 1:200,

Santa Cruz Biotechnology, USA), anti-Calnexin (Cat#: sc11397, 1:1000, Santa Cruz Biotechnology, USA), anti-TSG101 (Cat#: sc136111, 1:150, Santa Cruz, USA), anti-phospho Erk1/2(Cat#: 9101, 1:1000, Cell Signaling, USA), anti-Erk1/2 (Cat#: 4695,1:1000, Cell Signaling, USA), anti-phospho-SPAK/JNK (Cat#: 9255, 1:1000, Cell Signaling, USA), anti-SPAK/JNK (Cat#: 9258, 1:1000, Cell Signaling, USA), anti-phospho-p38 MAPK (Cat#: 4631, 1:1000, Cell Signaling, USA), anti- p38 MAPK (Cat#: 9212, 1:1000, Cell Signaling, USA), anti-phospho-Akt (Cat#: 4058,1:2000, Cell Signaling, USA), anti- Akt (Cat#: 9272, 1:2000, Cell signaling, USA), anti-GAPDH (Cat#: G9545,1:2000, Sigma-Aldrich, USA).

Statistical Analysis

Data are presented as means ± SD. Statistical analyses were performed by one-way ANOVA by use of SPSS (SPSS Inc, Chicago, IL, USA). Probability (P) values of < 0.05 were considered statistically significant.

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II. Supplementary Tables, Figures and Legends

Supplementary Tables

Supplementary Table 1. The protein expression profile in exosomes released from neonatal rat cardiac fibroblasts. The exosomes (20 μg) released from neonatal rat cardiac fibroblasts (CFs) treated with vehicle control (CTL) in 4.5 g/L DMEM supplemented with 5% exosome-depleted FBS for 48 h were subjected to proteomics analysis (n=3). These exosomes (EXO) are designated as CTL CF EXO. Protein mass spectrometry identifies 792 proteins in CTL CF EXO.

Supplementary Table 2. The signaling pathways which are potentially regulated by exosomes released from neonatal rat cardiac fibroblasts. KEGG analysis of all proteins in exosomes released from vehicle control-treated neonatal rat cardiac fibroblasts identified by protein mass spectrometry (n=3). CTL CF EXO; exosomes released from vehicle control-treated neonatal rat cardiac fibroblasts.

Supplementary Table 3. The proteins in exosomes released from neonatal rat cardiac fibroblasts which potentially regulate PI3K/Akt pathway. KEGG analysis of all proteins in exosomes released from vehicle control-treated neonatal rat cardiac fibroblasts identified by protein mass spectrometry (n=3) which regulate PI3K/Akt pathway. CTL CF EXO; exosomes released from vehicle control-treated neonatal rat cardiac fibroblasts.

Supplementary Table 4. The proteins in exosomes released from neonatal rat cardiac fibroblasts which potentially regulate MAPK pathway. KEGG analysis of all proteins in exosomes released from vehicle control-treated neonatal rat cardiac fibroblasts identified by protein mass spectrometry (n=3) which regulate MAPK pathway. CTL CF EXO; exosomes released from vehicle control-treated neonatal rat cardiac fibroblasts.

Supplementary Table 5. The effect of Ang II stimulation on protein expression profile in exosomes released from neonatal rat cardiac fibroblasts. The exosomes (20 μg) released from neonatal rat cardiac fibroblasts (CFs) treated with Ang II (1 μM) in 4.5 g/L DMEM supplemented with 5% exosome-depleted FBS for 48 h were subjected to proteomics analysis (n=3). These

exosomes (EXO) are designated as Ang II CF EXO. Protein mass spectrometry identifies 798 proteins in Ang II CF EXO.

Supplementary Table 6. The effect of Ang II stimulation on the protein expression in exosomes released from neonatal rat cardiac fibroblasts which potentially regulate MAPK pathway. KEGG analysis of all proteins in exosomes released from Ang II (1 μM)-treated neonatal rat cardiac fibroblasts identified by protein mass spectrometry (n=3) which regulate MAPK pathway. Ang II CF EXO; exosomes released from Ang II-treated neonatal rat cardiac fibroblasts.

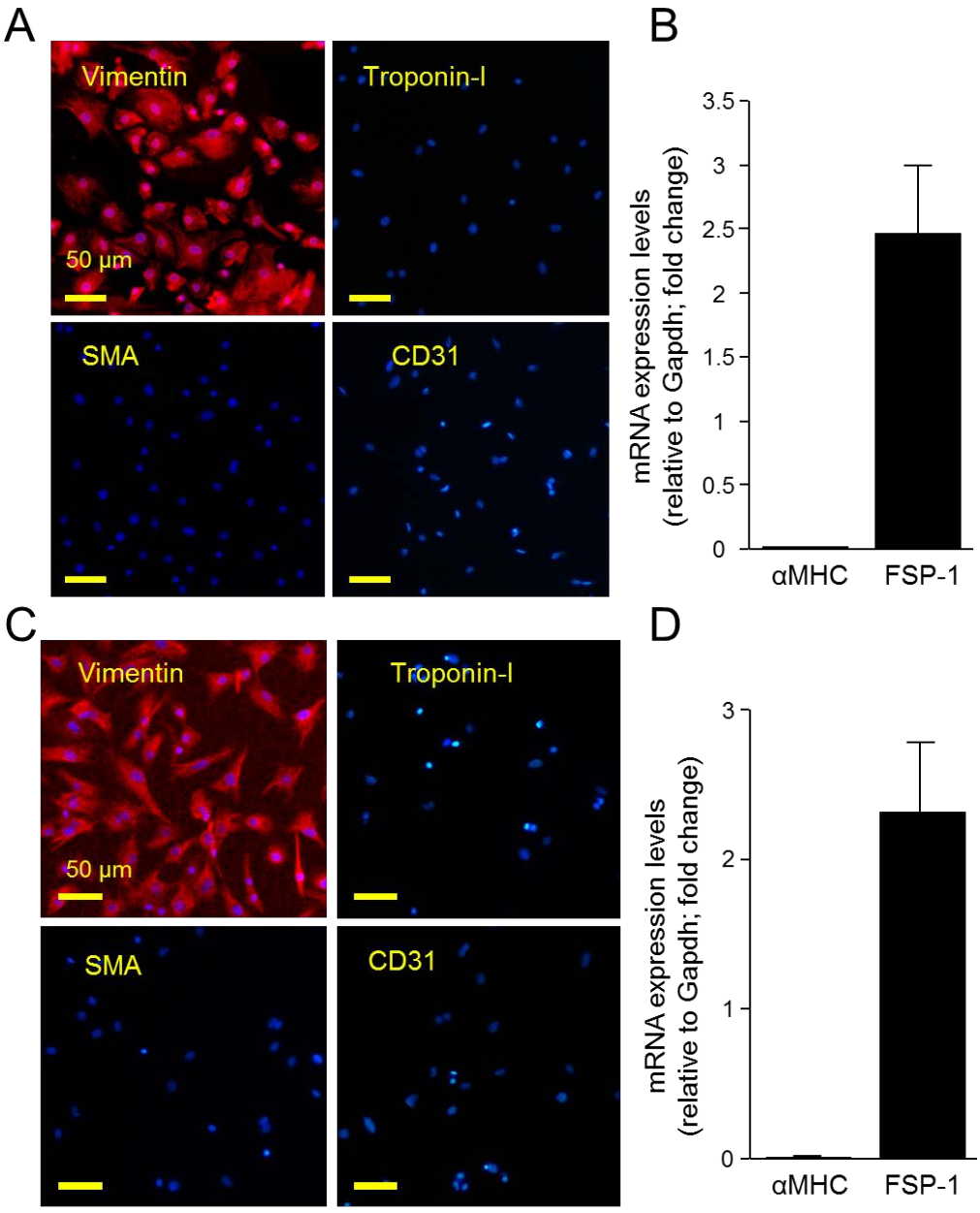
Supplementary Table 7. The effect of Ang II stimulation on the protein expression in exosomes released from neonatal rat cardiac fibroblasts which potentially regulate PI3K/Akt pathway. KEGG analysis of all proteins in exosomes released from Ang II (1 μM)-treated neonatal rat cardiac fibroblasts identified by protein mass spectrometry (n=3) which regulate PI3K/Akt pathway. Ang II CF EXO; exosomes released from Ang II-treated neonatal rat cardiac fibroblasts.

Supplementary Table 8. Identified proteins in exosomes released from neonatal rat cardiac fibroblasts which potentially upregulate renin-angiotensin system (RAS) leading to cardiomyocyte hypertrophy via activation of PI3K/Akt and MAPK pathways.

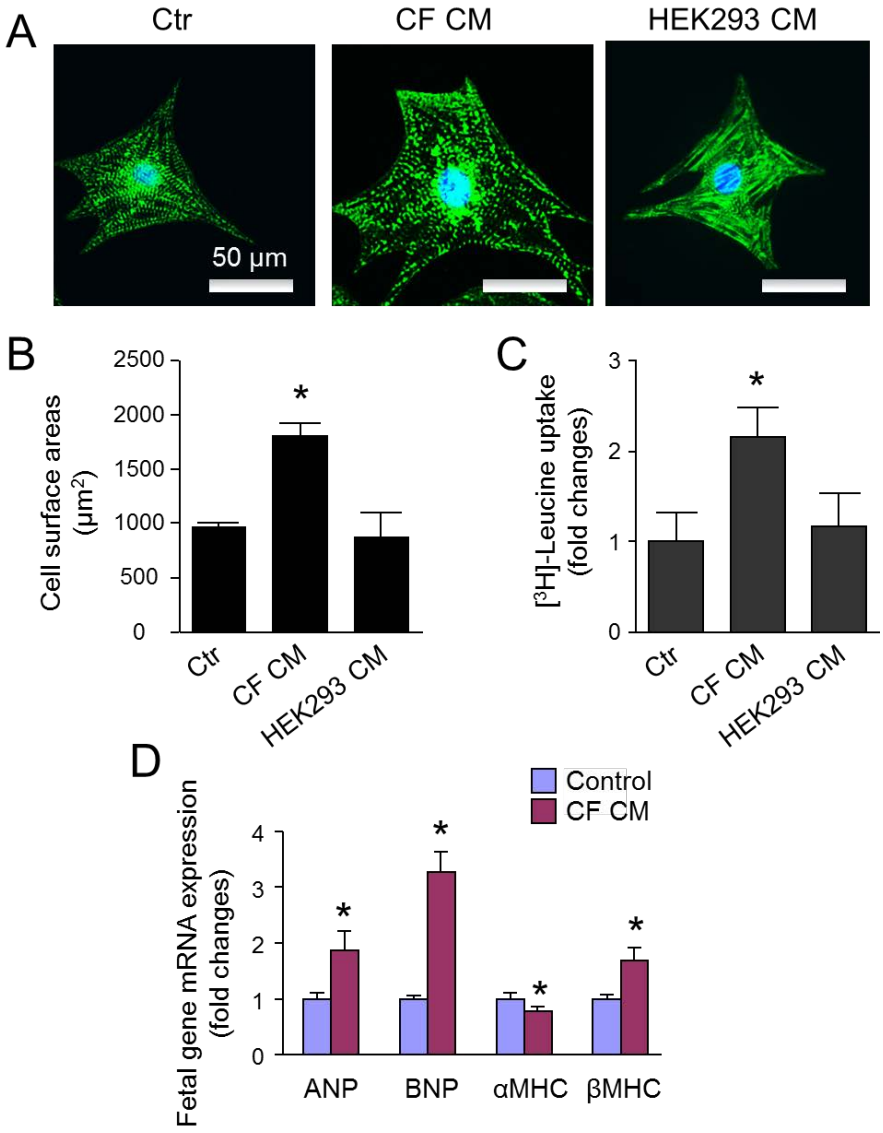
Supplementary Table 9. Primers for qPCR

Gene	Gene accession number	Forward	Reverse	Product length
ANF (rat)	NM_012612.2	ATACAGTGCGGTGTCCAACA	CGAGAGCACCTCCATCTCTC	209
BNP (rat)	NM_031545.1	GGAAATGGCTCAGAGACAGC	CGATCCGGTCTATCTTCTGC	164
αMHC (rat)	NM_017239.2	ACAGAGTGCTTCGTGCCTGAT	CGAATTTCGGAGGGTTCTGC	151
βMHC (rat)	NM_017240.2	TTGGCACGGACTGCGTCATC	GAGCCTCCAGAGTTTGCTG	60
SERCA2a (rat)	NM_001110139.2	CAAGTTTGTGGCCCGAAACT	AAGGA GAACACCCTTACATTTCTG	82
Gapdh (rat)	NM_017008.4	ACCACAGTCCATGCCATCAC	CAATGT TCCACCACCCTGTTGCTGTA	84
AT_1aR (rat)	NM_030985.4	TCTCTCAGCTCTGCCACATTC	TTGGGGCAGTCATCTTGGATT	219
AT_2R (rat)	NM_012494.3	TGCTCTGACCTGGATGGGTA	AGCTGTTGGTGAATCCCAGG	98
Renin (rat)	NM_012642.4	CTGTGCATACTGGCTCTCCA	GAACCCGATGCGATTGTTAT	126
Agt (rat)	NM_134432.2	CATCTTCCCTCGCTCTCTG	GCCTCTCATCTTCCCTTGG	175
ACE (rat)	NM_012544.1	ATGCCTCTGCGTGGGACTTC	TACTGCACGTGGCCCATCTC	112
ACE2 (rat)	NM_001012006.1	AATCGTAGGCTCTGGGCTT	TTCGATCAACTGGTTTCGG	198
FSP-1 (rat)	NM_012618.2	GGA AGGACAGACGAAGCTGCATT	TTGT CTCACAGCCAACATGGAAGA	218
FSP1 (mouse)	NM_011311.2	TTCCAGAAGGTGATGAG	TCATGGCAATGCAGGACAG	91
αMHC (mouse)	NM_010856.3	CCAATGAGTACCGCGTGAA	GAAGA ACAGTCATGCCGGGATGAT	254
nSMase1 (rat)	NM_031360.2	AGCACTAGCGGCCGATCT	TCATGGTTGGAGTGCTTCGG	187
nSMase2 (rat)	NM_053605.1	AGGACTGGCTGGCTGATTTC	CCGTTTATGTCCAGCAGGGT	229
nSMase3 (rat)	NM_001167806.2	GAATGGCAGCTCCCGTTTCT	TGTTGTAGGTGAGGGAACGC	122
Gapdh (mouse)	NM_001289726	ATGTTCCAGTATGACTCCAC TCACG	GAAGACACCAGTAGACTCC ACGACA	171

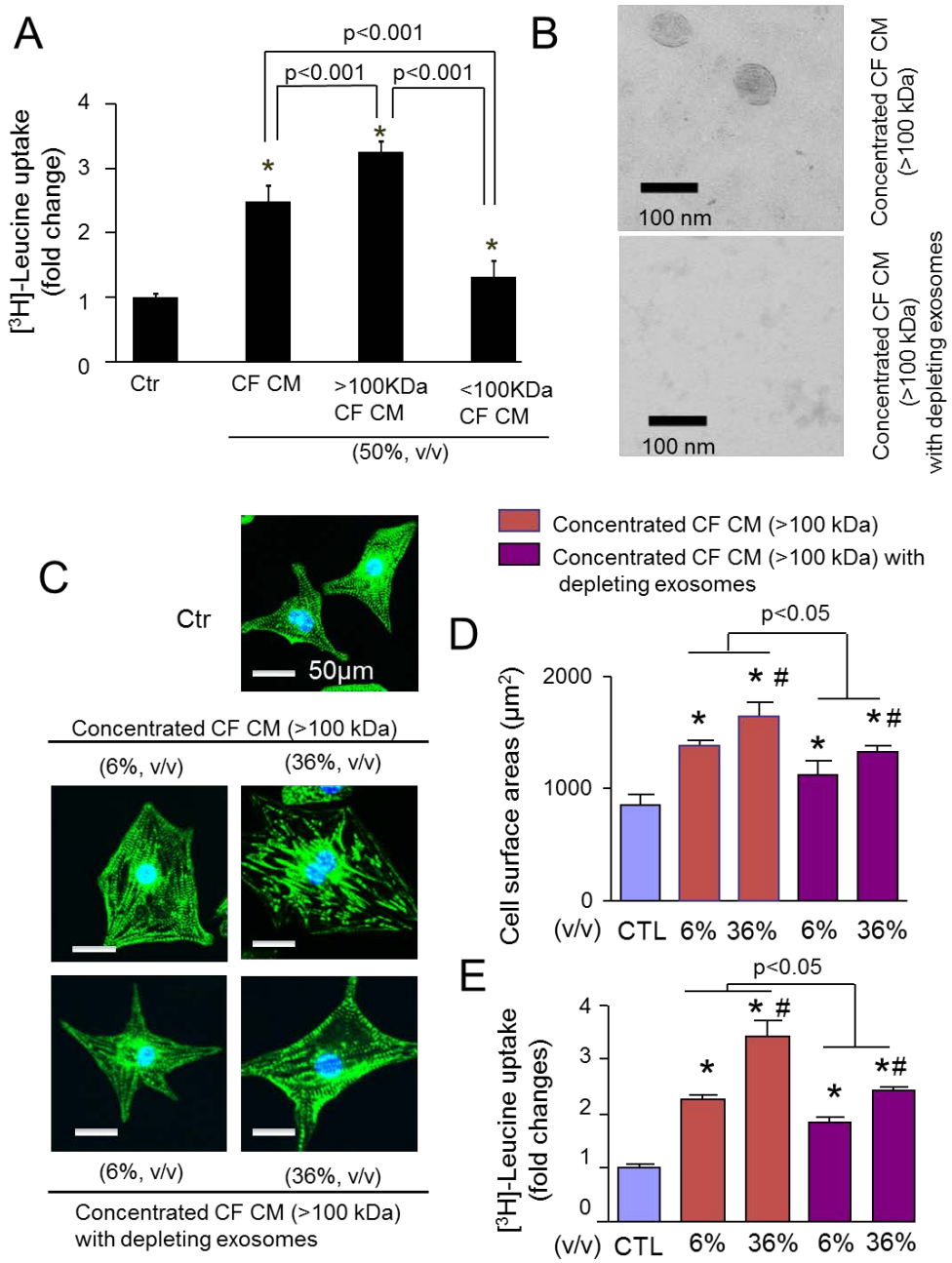
Supplementary Figures and Legends



Supplementary Fig. 1. Purity of rat neonatal cardiac and mouse adult fibroblast cultures. Immunofluorescence staining of primary (*A*) rat neonatal and (*C*) mouse adult cardiac fibroblasts. Cardiac fibroblasts were fixed with 4% paraformaldehyde in PBS and stained for (*A, C*) Vimentin (fibroblast marker), Troponin-I (cardiomyocyte marker), smooth muscle alpha actin (SMA, smooth muscle marker), and endothelial cell marker (CD31), and nucleus (DAPI). (n=4 experiments). Scale bar is 50 µm. *B, D,* mRNA expression of αMHC and FSP-1 in (*B*) rat neonatal and (*D*) mouse adult cardiac fibroblasts analyzed by qPCR. n=4.

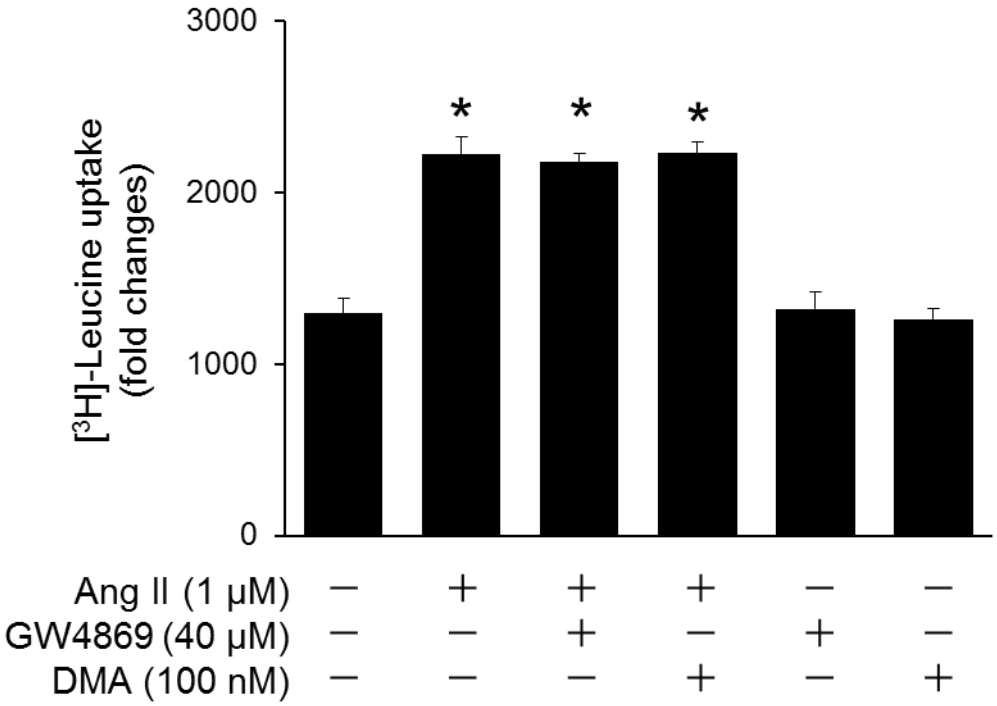


Supplementary Fig. 2. Cardiac fibroblast-conditioned medium induces pathological cardiomyocyte hypertrophy**.** Neonatal rat ventricular cardiomyocytes were incubated with serum free DMEM (100%, volume/volume; v/v, indicated as control), neonatal cardiac fibroblast (CF)-conditioned medium (CM) (50%, v/v) or HEK293 CM (50%, v/v) for 48 h, and then subjected to (*A* and *B*) cell surface area measurement (n=4), (*C*) [^{3}H]-leucine uptake assay (n=4), (*D*) qPCR analysis of fetal gene expression (n=4). Green indicates α-actinin and scale bar = 50 µm in panel A. *p<0.05 vs. Control (Ctr).

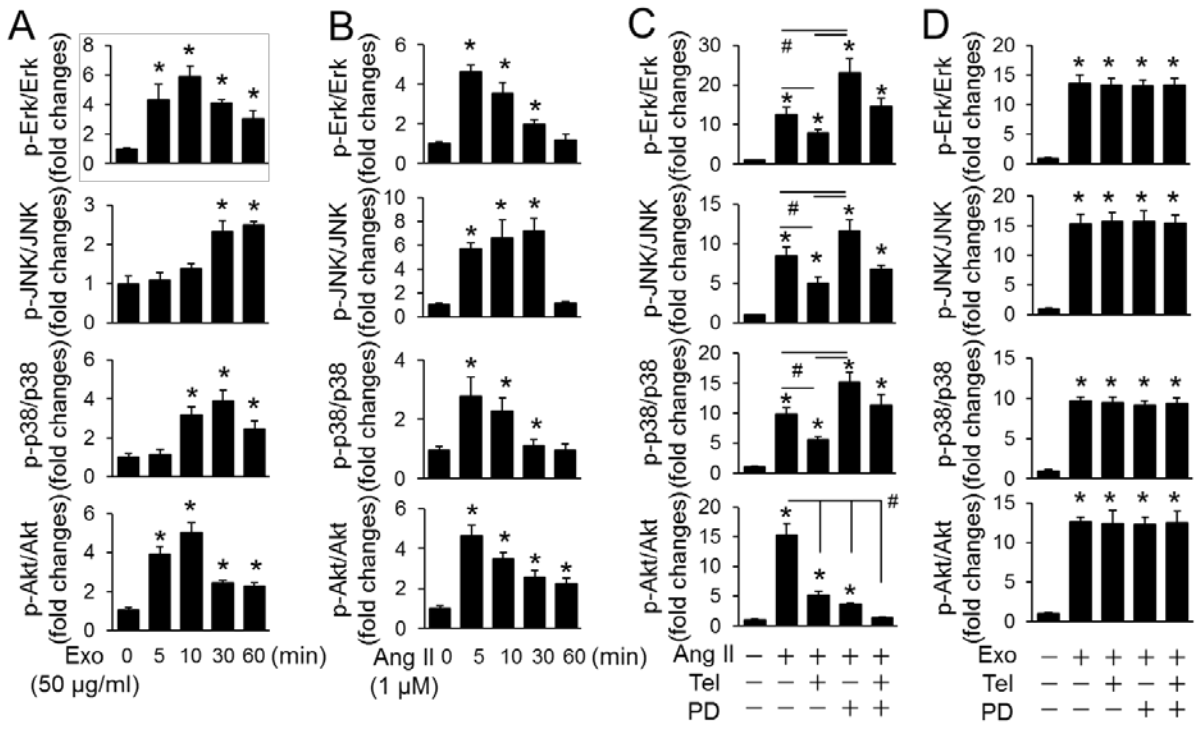


Supplementary Fig. 3. Cardiac fibroblast-derived exosomes are the major effectors of cardiac fibroblast-conditioned medium in inducing cardiomyocyte hypertrophy. Cardiac fibroblast-conditioned medium was further fractioned into <100 kDa (<100 kDa CF CM) and >100 kDa (>100 kDa CF CM) fractions using a 100 kDa molecular weight cut off filter membrane. The >100 kDa CF CM was further ultracentrifuged overnight to deplete exosomes. Neonatal rat cardiomyocytes were incubated with serum free DMEM (100%, volume/volume; v/v, indicated as control) or different CF CM fractions (50%, v/v) as indicated for 48 h. (*A*) The effects of CF CM,

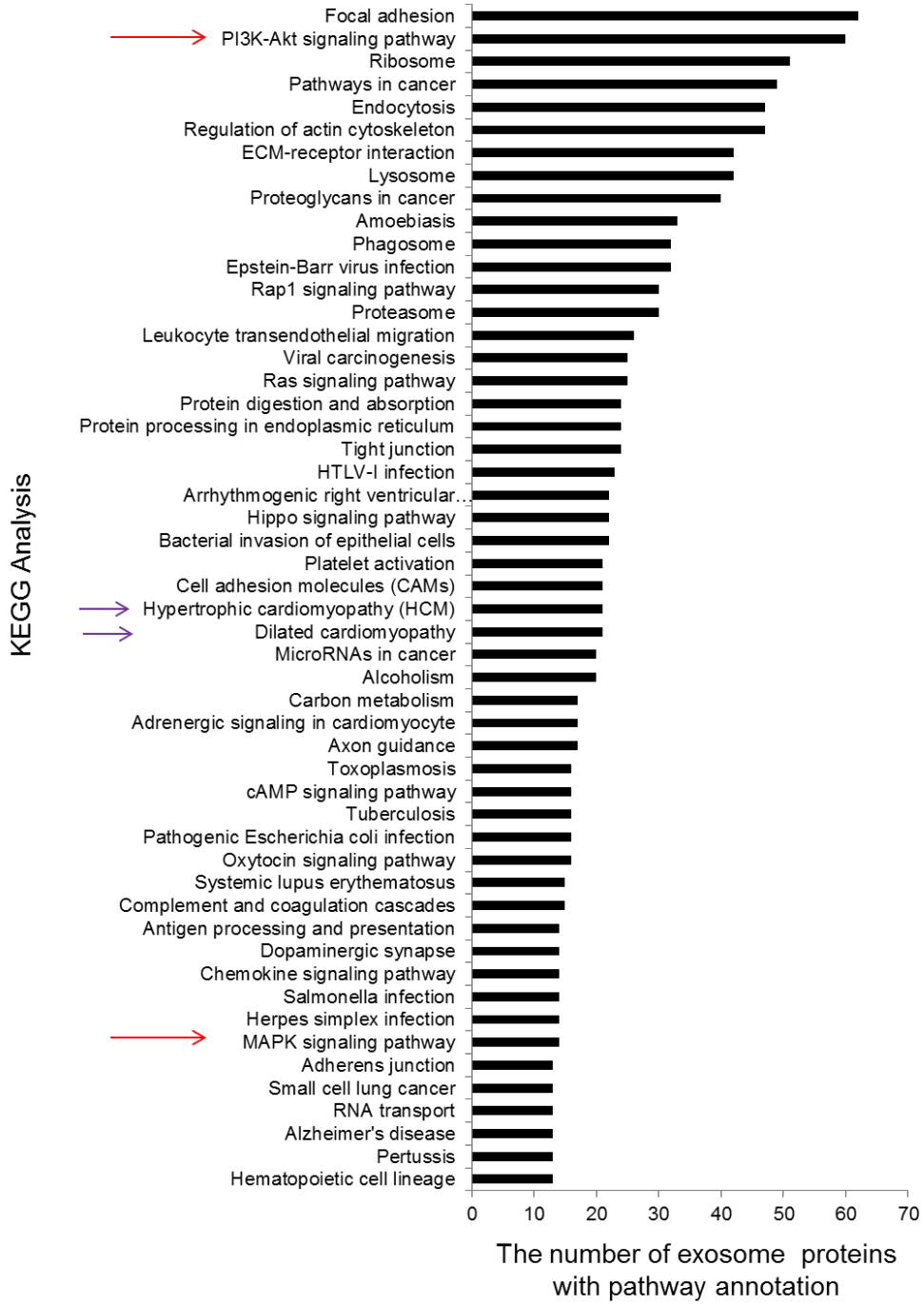
>100 kDa and <100 kDa CF CM fractions on [^{3}H]-Leucine incorporation in cardiomyocytes (n=4). *p<0.05 vs. Control (Ctr). (*B*) Transmission electron microscopy (TEM) analysis of >100 kDa CF CM with or without depleting exosomes. Scale bar = 100 nm. (*C*) Representative immunofluorescence staining of α-actinin (Scale bar = 50μm). (*D*, *E*) The effects of >100 kDa CF CM and exosome-depleted >100 kDa CF CM on cardiomyocyte hypertrophy, which were assessed by measuring cell surface areas and [^{3}H]-Leucine incorporation, respectively (n=4). *p<0.05 vs. Control (Ctr), #p<0.05 vs. 6% CF CM (>100 kDa).



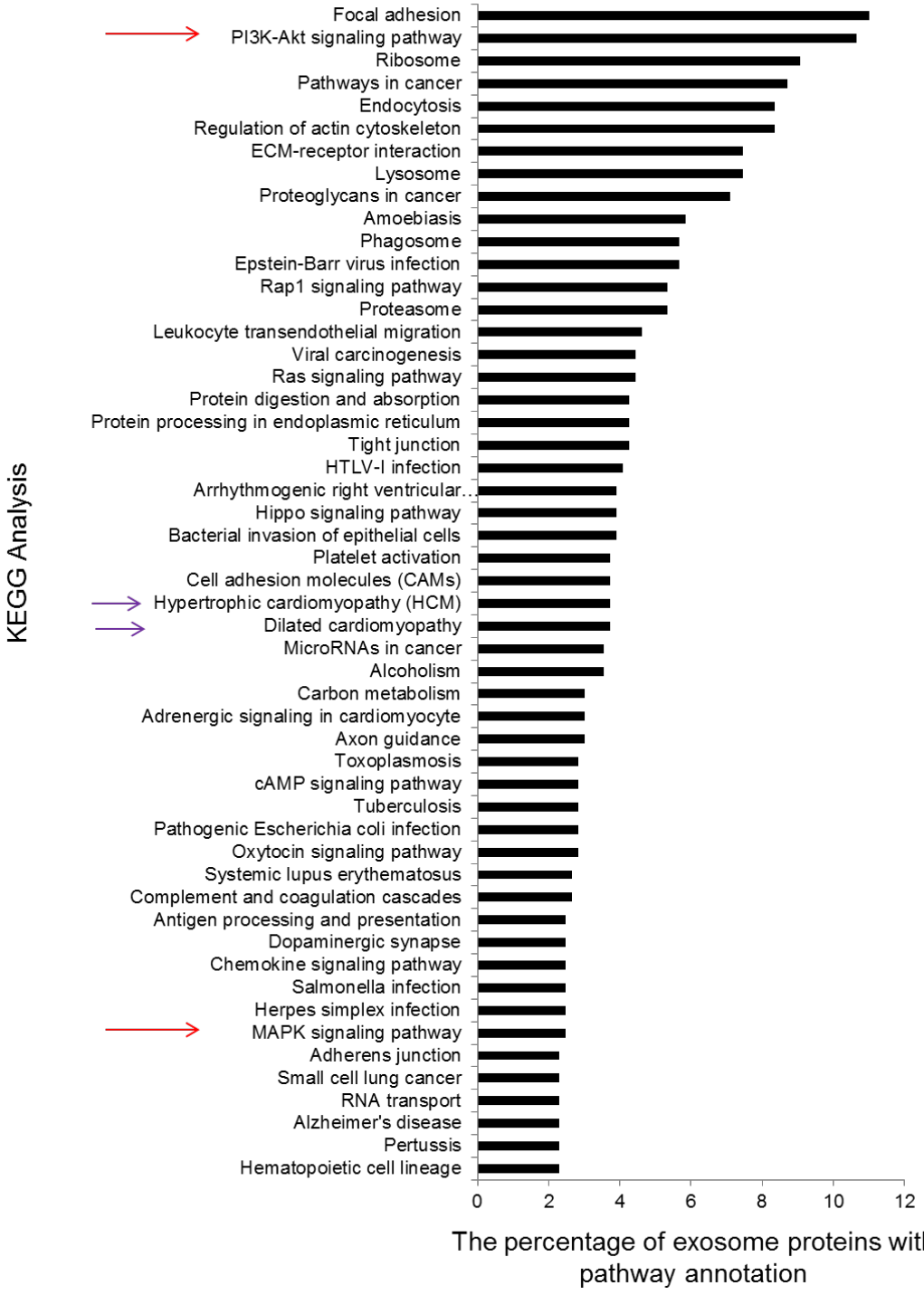
Supplementary Fig. 4. The effects of exosome inhibitors on Ang II-induced cardiomyoycte hypertrophy. Neonatal rat cardiomyocytes were treated with or without Ang II (1 μM), GW4869 (40 μM) and DMA (100 nM) as indicated for 48 h. n=4, *p<0.05 vs. vehicle-treated control (-).



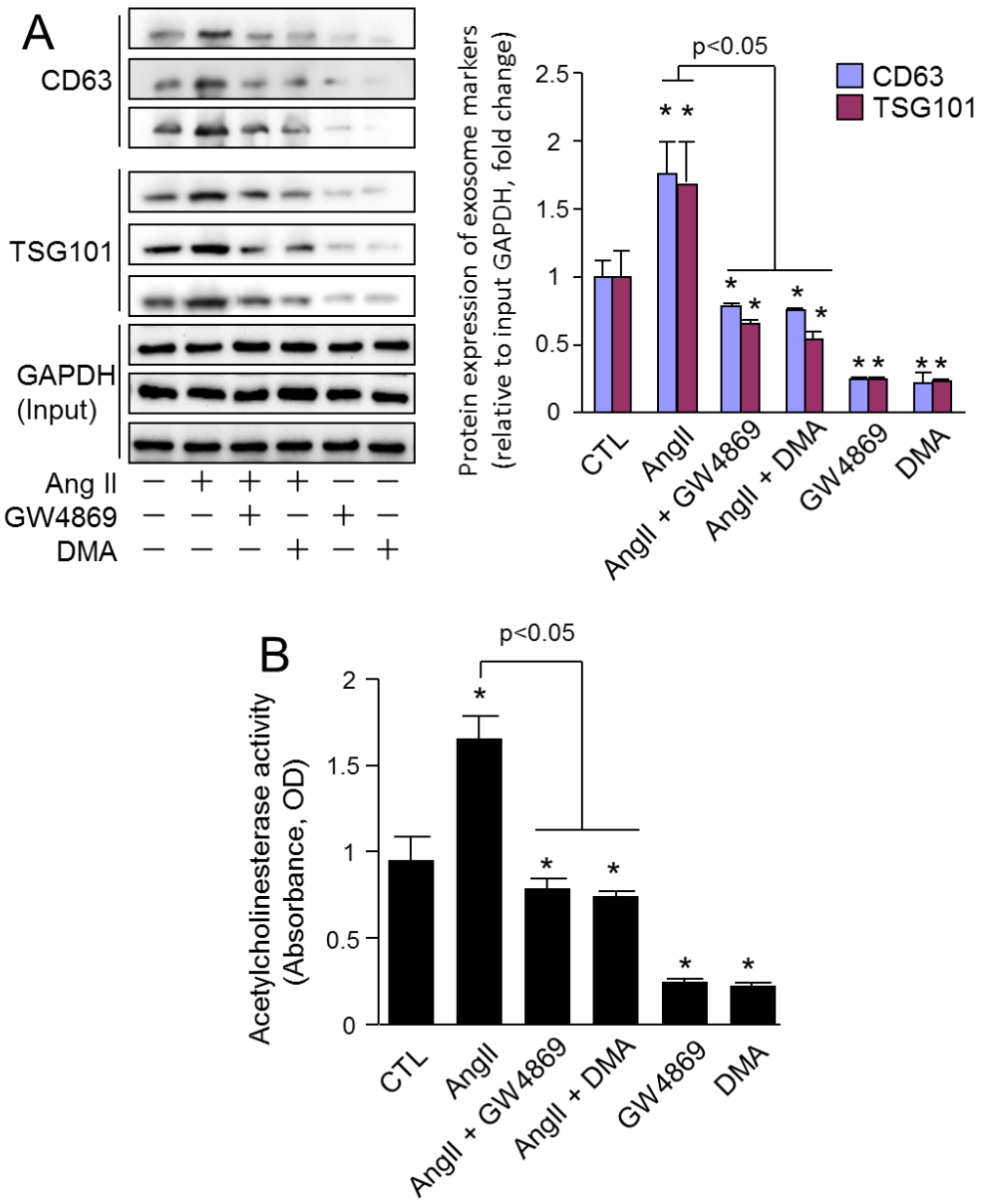
Supplementary Fig. 5. The effects of cardiac fibroblast-derived exosomes on pro-hypertrophic signaling in cardiomyocytes. Semi-quantification of Western blot analysis. (*A*) Exosome (Exo)-induced activation of mitogen-activated protein kinases (MAPKs) and Akt. Neonatal rat cardiomyocytes were treated with (5, 10, 30 and 60 min) and without (0 min) Exo (50 μg/ml) derived from neonatal rat cardiac fibroblasts as indicated. The results are representatives of 4 separated experiments. *p<0.05 vs. non-treated cells (0). (*B*) Ang II-induced activation of MAPKs and Akt. Neonatal rat cardiomyocytes were treated with (5, 10, 30 and 60 min) or without (0 min) Ang II (1 μM) as indicated. The results are representatives of 4 separated experiments. *p<0.05 vs. vehicle-treated control cells (0). (*C*) The effects of Telmisartan (Tel) and PD123319 (PD) on Exo-induced activation of MAPKs and Akt. Neonatal rat cardiomyocytes were treated with Exo, Tel (10 μM), and PD (10 μM) as indicated for 20 min. The immunoblots are representatives of 4 separated experiments. *p<0.05 vs. vehicle-treated control cells (0). #p<0.05 vs. indicated groups. (*D*) The effects of Tel and PD on Ang II-induced activation of MAPKs and Akt. Neonatal rat cardiomyocytes were treated with Ang II, Tel (10 μM), and PD (10 μM) as indicated for 5. The immunoblots are representatives of 4 separate experiments. *p<0.05 vs. vehicle-treated control cells (0).



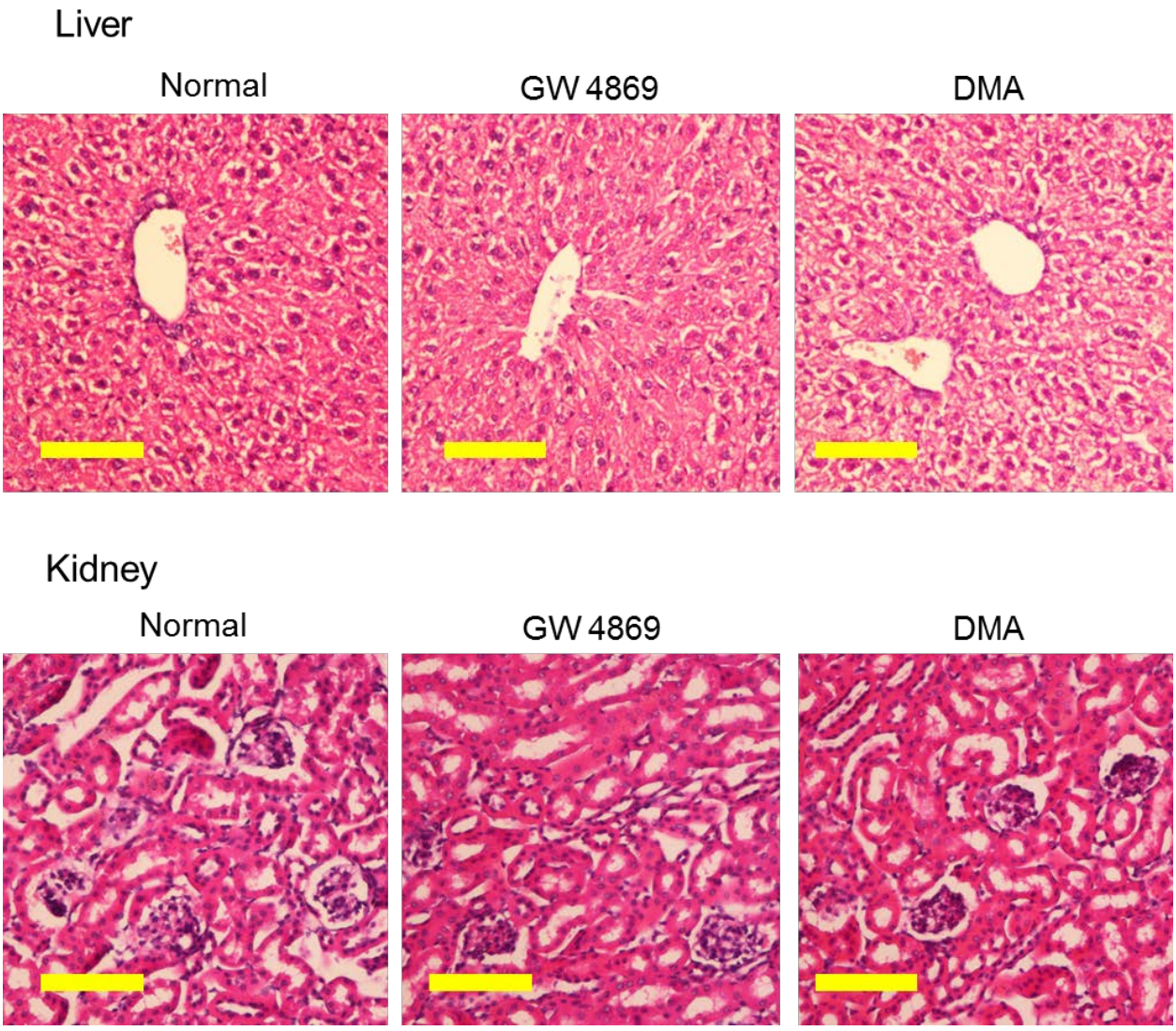
Supplementary Fig. 6. KEGG analysis of exosome proteins. Exosomes released from cultured neonatal rat cardiac fibroblasts (CFs) without any treatment were subjected to proteomics analysis and followed KEGG analysis. n=3. The number of CF exosome proteins linked to different signaling pathways is presented.



Supplementary Fig. 7. KEGG analysis of exosome proteins. Exosomes released from cultured neonatal rat cardiac fibroblasts (CFs) without any treatment were subjected to proteomics analysis and followed KEGG analysis. n=3. The percentage of CF exosome proteins linked to different signaling pathways is presented.



Supplementary Fig. 8. The effect of GW4869 and DMA on Ang II-induced exosome release from cardiac fibroblasts in the heart. Adult male C57BL/6N mice (n=10) at 8 weeks of age were treated with Ang II, GW4869, and DMA for 5 days, and then cardiac fibroblasts were isolated for measuring exosome release as described in "Methods". *A*, Western blot analysis of CD63 and TSG101 in total released exosomes from 3 × 10^6 CFs of each group (n=3). *p<0.05 vs. vehicle-treated control (CTL). Input: 10 µl of whole CF lysates of each group. *B*, Acetylcholinesterase activity of total released exosomes from 3 × 10^6 CFs of each group (n=3). *p<0.05 vs. vehicle-treated control (CTL).



Supplementary Fig. 9. The impact of GW4869 and DMA on liver and kidney morphology. H&E staining of the liver and kidney tissue sections after 14 day systemic treatment of Ang II, GW4869, and DMA. The results are representatives of 3 separated experiments. Scale bars are 100 µm.