Supporting Information

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SI Materials and Methods

Materials, Animals, and Animal Models. Antibodies for Western blot experiments were purchased as the following: P-JNK1/2 (Cell Signaling; 4668S); P-MEK1/2 (Cell Signaling; 9154); T-MEK1/2 (Cell Signaling; 9122); P-ERK1/2 (Bioworld; BS5016); T-ERK1/2 (Bioworld; BS3627); T-JNK1/2/3 (Bioworld; BS3630); P-P38 (Bioworld; BS4766);T-P38 (Bioworld; BS3566); P-AKT (Bioworld; BS4009); T-AKT (Bioworld; BS2987); GAPDH (Cell Signaling; 2118); RGS 2 (Santa Cruz Biotechnology; SC-7678); RGS 4 (Santa Cruz Biotechnology; SC-6204); mouse RGS 5 (Santa Cruz Biotechnology; SC-28491); human RGS 5 (Invitrogen; tmp_1249); P-Smad 2 (Cell Signaling; 3108L); T-Smad 2 (Cell Signaling; 3103); T-Smad 3 (Cell Signaling; 9513); T-Smad 4 (Cell Signaling, 9515); and Lamin B1 (Santa Cruz Biotechnology; SC-6217). The [^{3}H]-leucine and [^{3}H]-proline were purchased from Amersham. The BCA protein assay kit was purchased from Pierce. TGF-β1 was purchased from R&D Systems. FCS was obtained from HyClone. Cell culture reagents and all other reagents were obtained from Sigma. CTGF-luc report constructs were described previously (1).

All protocols were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. Human Rgs5 cDNA construct containing full-length human Rgs5 cDNA was cloned downstream of the cardiac MHC promoter. Transgenic mice were produced by microinjection of the α-MHC-Rgs5 construct into fertilized mouse embryos (C57 background). Four independent transgenic lines were established and studied. Transgenic mice were identified by PCR analysis of tail genomic DNA. Primers are as follows: 5′-ATCTCCCCCATAAGAGTTTGAGTC-3′ and 5′-CACAAAGCGAGGCAGAGAATCC-3′. Expected band size of PCR will be 500 bp. Functional data and gene expression levels were analyzed in pairs of α-MHC-Rgs5 (TG) and litter-mate nontransgenic (i.e., WT) male mice ranging in age from 7 to 8 weeks. Male Rgs5-KO mice (RGS5$^{-/-}$, C57 background) and their WT littermates (provided by MRC) aged 8 to 10 wk were used in the studies. Genotyping was performed by PCR. AB was performed as described previously (2). Doppler analysis was performed to ensure that adequate constriction of the aorta had been induced. U0126 (1 mg/kg every 3 d) suspension was freshly prepared and administered at a constant volume of 1 mL/100 g body weight by i.p. injection every 3 d. The dose of U0126 was determined from preliminary experiments (0.1–1 mg/kg/d) that demonstrated a greater than 90% inhibition of ERK1/2 activation in the heart at this dose. The control group for these experiments was given the same volume of PBS solution. The internal diameter and wall thickness of the LV were assessed by echocardiography at the indicated time after surgery or infusion. Hearts and lungs of the killed mice were dissected and weighed to compare HW/BW (mg/g) and LW/BW (mg/g) ratios in different groups.

Blood Pressure and Echocardiography. A microtip catheter transducer (SPR-839; Millar Instruments) was inserted into the right carotid artery and advanced into the left ventricle. After stabilization for 15 min, the pressure signals and heart rate were recorded continuously with an Aria pressure–volume conductance system coupled with a Powerlab/4SP A/D converter, stored, and displayed on a personal computer as described previously (1). Echocardiography was performed by Sonos 5500 ultrasound (Philips) with a 15-MHz linear array ultrasound transducer. The LV was assessed in both parasternal long-axis and short-axis views at a frame rate of 120 Hz. End-systole or end-diastole was defined as the phase in which the smallest or largest area of LV, respectively, was obtained. LVEDD and LVESD were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the midpapillary muscle level.

Quantitative Real-Time RT-PCR and Western Blotting. Real-time PCR was used to detect the mRNA expression levels of hypertrophic and fibrotic markers. Total RNA was extracted from frozen, pulverized mouse cardiac tissue using TRIZol (Invitrogen) and synthesized cDNA using oligo(dT) primers with the Advantage RT-for-PCR kit (BD Biosciences). We quantified PCR amplifications using SYBR Green PCR Master Mix (Applied Biosystems) and normalized results against GAPDH gene expression. Cardiac tissue and cultured cardiac myocytes or fibroblasts were lysed in RIPA lysis buffer. Nuclear protein extracts were isolated as described previously (2). Fifty micrograms of cell lysate was used for SDS/PAGE, and proteins were then transferred to an Immobilon-P membrane (Millipore). Specific protein expression levels were normalized to the GAPDH protein for total cell lysate and cytosolic proteins or the Lamin-B1 protein for nuclear proteins on the same nitrocellulose membrane. Quantification of Western blots was performed by Odyssey infrared imaging system (Li-Cor Biosciences). The secondary antibodies IRdye 800 antirabbit and IRdye 700 antimouse (Rockland) were used at 1:2,500 and 1:5,000, respectively, in Odyssey blocking for 1 h. The blots were scanned with the infrared Li-Cor scanner, allowing for simultaneous detection of two targets (antiphospho and anti–total protein) in the same experiment.

Histological Analysis. Hearts were excised, washed with saline solution, and placed in 10% formalin. Hearts were cut transversely close to the apex to visualize the left and right ventricles. Several sections of heart (4–5 μm thick) were prepared and stained with H&E for histopathology or PSR for collagen deposition and then visualized by light microscopy. For myocyte cross-sectional area, sections were stained for membranes with FITC-conjugated WGA (Invitrogen) and for nuclei with DAPI. A single myocyte was measured with an image quantitative digital analysis system (NIH Image, version 1.6). The outline of 150 myocytes was traced in each group.

Recombinant Adenoviral Vectors and Cultured Neonatal Rat Cardiac Myocytes and Fibroblasts. We used replication-defective adenoviral vectors encoding for the entire coding region of Rgs5 gene (Open Biosystems) under the control of the cytomegalovirus promoter, and as a control, a similar adenoviral vector encoding for the GFP gene (AdEasy XL adenoviral Vector system; Strategene). We ordered three rat shRGS5 constructs from SuperArray (cat. no. KR42418G) and then generated three Ad-shRGS5 adenoviruses and selected one that led to a significant decrease in RGS5 levels for further experiments. Ad-shRNA was used as control. We infected cardiomyocytes with Ad-Rgs5 and Ad-GFP as well as Ad-shRGS5 and Ad-shRNA at a multiplicity of infection of 100, resulting in 95% to 100% of cells expressing the transgenes without toxicity. Primary cultures of cardiac myocytes were prepared as described previously (3). Cells from the hearts of 1- to 2-d-old Sprague-Dawley rats (Charles River Laboratories) were seeded at a density of 1×10^6/well onto six-well culture plates coated with fibronectin (Becton Dickinson) in plating medium consisting of F10 medium supplemented with 10% FCS and penicillin/streptomycin. After 48 h, the culture medium was replaced with F10 medium containing 0.1% FCS and BrdU (0.1 mM). Viability was determined by cell number,

frequency of contractions, cellular morphology, and trypan blue exclusion. Cultures of neonatal rat ventricular nonmyocytes, which have been shown to be predominantly fibroblasts, were prepared. All experiments were performed on cells from the first or second passages which were placed in DMEM containing 0.1% FCS for 24 h before the experiment. The purity of these cultures was greater than 95% cardiac fibroblasts as determined by positive staining for vimentin and negative staining for smooth muscle actin and von Willebrand factor. For the cell infection, 1×10^6/well cardiac myocytes or cardiac fibroblasts were cultured in six-well plates and exposed to 2×10^8 pfu each of virus in 1 mL of serum-free medium for 24 h. The cells were then washed and incubated in serum-containing media for 24 h.

Protein and Collagen Synthesis Assays, and Surface Area. Protein and collagen synthesis were assessed by [^{3}H]-leucine and [^{3}H]-proline incorporation as described previously (1). Briefly, cardiac myocytes were infected with different adenoviruses for 24 h and subsequently stimulated with Ang II (1 μM) and coincubated with [^{3}H]-leucine (1 μCi/mL) for the indicated time. At the end of the experiment, the cells were washed with Hanks solution, scraped off the well, and then treated with 10% trichloroacetic acid at 4 °C for 60 min. The precipitates were then dissolved in NaOH (1 N) and subsequently counted with a scintillation counter. For collagen synthesis, cardiac fibroblasts were infected with different adenoviruses and then made quiescent by being cultured in 0.1% FCS DMEM for 24 h, and subsequently incubated with TGF-β1 and 5 μCi/mL [^{3}H]-proline for the indicated time. Cells were washed with PBS solution twice, treated with ice-cold 5% trichloroacetic acid for 1 h, and washed with distilled water twice. Cells were then lysed with 1 N NaOH solution and counted in a liquid scintillation counter. The count representing the amount of newly synthesized collagen was normalized to the cell number. For the surface areas, the cells were fixed with 3.7% formaldehyde in PBS solution, permeabilized in 0.1% Triton X-100 in PBS solution, and stained with α-actinin (Sigma) at a dilution of 1:100 by standard immunocytochemical techniques.

Reporter Assays. Cardiac myocytes or cardiac fibroblasts were seeded in triplicate in six-well plates. Cells were transfected with 0.5 μg of luciferase reporter constructs and internal control plasmid DNA using 10 μL of lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. After 6 h of exposure to the DNA–lipofectamine complex, cells were cultured in medium containing 10% serum for 24 h and then incubated with serum-free medium for 12 h. Cells were infected with different adenoviral for 24 h and then treated with Ang II for cardiac myocytes or TGF-β1 for fibroblasts. Cells were harvested using passive lysis buffer (Promega) according to the manufacturer's protocol. The luciferase activity was normalized by control plasmid. All experiments were done in triplicate and repeated at least three times.

1. Cai J, et al. (2009) Targeted expression of receptor-associated late transducer inhibits maladaptive hypertrophy via blocking epidermal growth factor receptor signaling. *Hypertension* 53:539–548.
2. Tang Q, et al. (2009) Lysosomal cysteine peptidase cathepsin L protects against cardiac hypertrophy through blocking AKT/GSK3beta signaling. *J Mol Med* 87:249–260.
3. Bian ZY, et al. (2010) LIM and cysteine-rich domains 1 regulates cardiac hypertrophy by targeting calcineurin/nuclear factor of activated T cells signaling. *Hypertension* 55: 257–263.

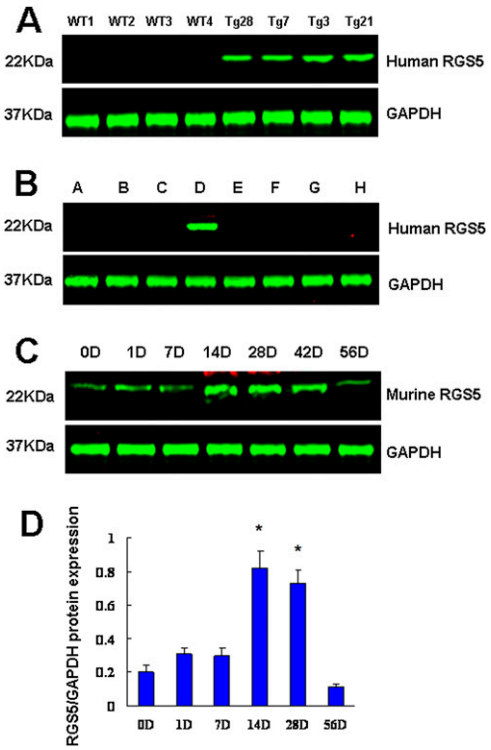

Fig. S1. Characterization of human Rgs5 transgenic mice. (*A*) Representative Western blots of human Rgs5 protein in the heart tissue from four lines of TG and WT mice. (*B*) Representative Western blot of human Rgs5 protein from different tissue of TG mice as indicated (A, lung; B, muscle; C, brain; D, heart; E, kidney; F, spleen; G, liver; H, testis). (*C* and *D*) Representative Western blots and quantitative results of endogenous Rgs5 protein levels in the heart from WT and TG mice after AB at time points indicated ($n = 7$). *$P < 0.01$ for 0 d group.

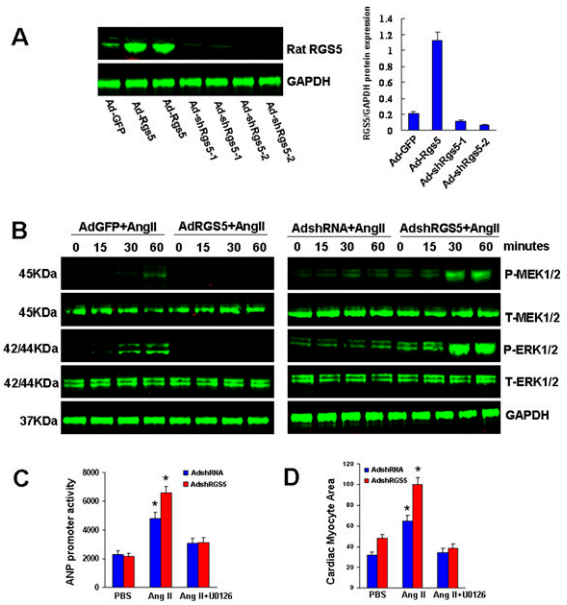

Fig. S2. Effect of Rgs5 on MEK-ERK1/2 signaling pathway. (*A*) The protein expression level of Rgs5 after infection with Ad-Rgs5 or Ad-shRgs5. *Left*: Representative blots. *Right*: Quantitative results. (*B*) Representative blots of MEK1/2 and ERK1/2 activation after treated with Ang II for indicated time in different adenovirus infected primary cardiac myocytes. The results were reproducible in three separate experiments. (*C* and *D*) The effect of U0126 on the promoter activity of ANF and enlargement of myocytes area induced by Ang II after infection with adenovirus. Cardiac myocytes were pretreated with U0126 1 h and treated with Ang II for 48 h after infection with Ad-shRNA or Ad-shRgs5 for 24 h. The results were reproducible in three separate experiments as mean ± SEM. **P* < 0.01 for PBS solution-treated groups.

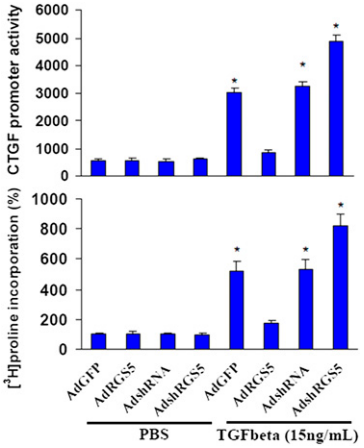

Fig. S3. The effects of Rgs5 on collagen synthesis Rgs5 was shown to inhibit TGF-β1–induced [^{3}H]-proline incorporation and the promoter activity of CTGF. Assays for [^{3}H]-proline incorporation and luciferase were performed as described in *Materials and Methods*. Values are mean ± SE. Results were reproducible in three separate experiments.

Table S1. Anatomic and cardiac function data show effects of RGS5 on cardiac hypertrophy induced by AB at 4 wk in transgenic mice and WT litter-mates

Parameter	WT sham	TG sham	WT AB	TG AB
No.	$n = 8$	$n = 8$	$n = 8$	$n = 8$
BW , g	27.3 ± 0.4	28.2 ± 0.4	27.7 ± 0.5	28.3 ± 0.3
HW/BW , mg/g	4.23 ± 0.13	4.17 ± 0.09	6.60 ± 0.17*	4.51 ± 0.12†
LW/BW , mg/g	4.03 ± 0.10	4.07 ± 0.10	6.08 ± 0.25*	4.63 ± 0.09*†
CSA , μm^2	182 ± 1.7	197 ± 2.1	391 ± 3.1*	258 ± 3.8†
SBP , mmHg	109.5 ± 1.9	110.2 ± 1.3	142.2 ± 2.0*	146.7 ± 1.8*
HR , beats/min	459 ± 5.3	462 ± 6.4	473 ± 4.3	457 ± 6.4
LVEDD, mm	3.57 ± 0.01	3.57 ± 0.01	4.31 ± 0.05*	3.78 ± 0.03†
LVESD, mm	2.24 ± 0.06	2.09 ± 0.01	3.22 ± 0.01*	2.52 ± 0.01†
PWT, mm	1.21 ± 0.01	1.23 ± 0.02	2.21 ± 0.04*	1.36 ± 0.01†
IVSD , mm	0.66 ± 0.01	0.65 ± 0.01	1.23 ± 0.03*	0.73 ± 0.01†
FS , %	47.0 ± 1.8	42.0 ± 0.3	25.0 ± 0.9*	33.0 ± 0.7*†
LVEDP , mm Hg	9.4 ± 0.1	9.8 ± 0.1	19.3 ± 0.5*	13.4 ± 0.6*†
LV dP/dT_{max} , mmHg/s	8,585.1 ± 148.3	9,221.9 ± 267.4	6,272.4 ± 148.2*	7,922.8 ± 165.7†
LV dP/dT_{min} , mm Hg/s	−7,839.1 ± 110.5	−7,834.3 ± 170.9	−4,603.9 ± 134.8*	−6,949.1 ± 127.1†

HR, heart rate; CSA, cardiomyocyte cross-sectional area; PWT, posterior wall thickness; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; IVSD, left ventricular septum, diastolic; FS, fractional shortening; SBP, systolic blood pressure. All values are mean ± SEM.

*$P < 0.01$ for WT sham values.

$^{\dagger}P < 0.01$ for WT-AB values after AB.

Table S2. Anatomic and cardiac function data show effects of RGS5 on cardiac hypertrophy induced by AB in $Rgs5^{-/-}$ (KO) and $Rgs5^{+/+}$ (WT) mice

Parameter	WT sham	KO sham	WT AB	KO AB
No.	$n = 8$	$n = 8$	$n = 8$	$n = 8$
BW, g	27.8 ± 0.6	26.2 ± 0.4	27.7 ± 0.6	27.3 ± 0.4
HW/BW, mg/g	4.18 ± 0.11	4.58 ± 0.13	6.69 ± 0.18*	9.03 ± 0.21*†
LW/BW, mg/g	4.14 ± 0.10	4.69 ± 0.09	6.22 ± 0.23*	9.63 ± 0.32*†
CSA, μm^2	188 ± 1.6	188 ± 2.3	379 ± 3.3*	440 ± 3.6*†
SBP, mm Hg	109.0 ± 1.2	105.7 ± 2.6	143.8 ± 1.8*	145.6 ± 2.1*
HR, beats/min	455 ± 4.5	465 ± 6.2	470 ± 5.4	445 ± 6.2
LVEDD, mm	3.71 ± 0.04	3.68 ± 0.06	4.28 ± 0.03*	5.16 ± 0.02*†
LVESD, mm	2.12 ± 0.02	2.26 ± 0.05	3.22 ± 0.01*	4.17 ± 0.08*†
PWT, mm	1.23 ± 0.03	1.23 ± 0.04	2.22 ± 0. 06*	2.72 ± 0.04*†
IVSD, mm	0.67 ± 0.01	0.64 ± 0.02	1.36 ± 0.03*	1.72 ± 0.02*
FS, %	43.0 ± 0.7	45.0 ± 1.5	25.0 ± 0.4*	19.0 ± 1.6*†
LVEDP, mm Hg	9.5 ± 0.2	9.8 ± 0.2	18.5 ± 0.3*	24.4 ± 0.5*†
LV dP/dT_{max}, mm Hg/s	8,790.4 ± 382.8	8,691.0 ± 374.1	6,541.1 ± 205.0*	5,446.9 ± 147.6*†
LV dP/dT_{min}, mm Hg/s	−8,316.5 ± 124.4	−8,943.9 ± 241.8	−6,155.1 ± 58.5*	−4,396.1 ± 106.5*†

FS, fractional shortening.

*$P < 0.01$ for WT sham values.

$^{\dagger}P < 0.01$ for WT-AB values after AB.

Table S3. Anatomic and cardiac function data show effects of inhibition of ERK1/2 signaling by U0126 on cardiac hypertrophy induced by AB in Rgs5$^{-/-}$ mice

Parameter	PBS sham	U0126 sham	PBS AB	U0126 AB
No.	$n = 8$	$n = 8$	$n = 8$	$n = 8$
BW, g	26.4 ± 0.4	26.6 ± 0.5	26.2 ± 0.4	27.1 ± 0.5
HW/BW, mg/g	4.61 ± 0.08	4.45 ± 0.10	6.81 ± 0.14*	5.01 ± 0.11†
LW/BW, mg/g	4.36 ± 0.12	4.25 ± 0.09	6.31 ± 0.21*	4.73 ± 0.18†
CSA, μm^2	188 ± 2.4	191 ± 2.5	376 ± 2.4*	219 ± 3.3†
SBP, mmHg	107.4 ± 2.3	110.5 ± 1.2	141.2 ± 1.7*	142.8 ± 1.6*
HR, beats/min	445 ± 6.7	448 ± 7.4	452 ± 5.7	454 ± 6.5
LVEDD, mm	3.80 ± 0.04	3.65 ± 0.01	4.39 ± 0.06*	3.75 ± 0.04†
LVESD, mm	2.11 ± 0.04	2.13 ± 0.03	3.32 ± 0.07*	2.54 ± 0.02†
PWT, mm	1.21 ± 0.01	1.21 ± 0.03	1.43 ± 0. 02*	1.34 ± 0.04†
IVSD, mm	0.68 ± 0.02	0.67 ± 0.01	1.35 ± 0.01*	0.80 ± 0.01†
FS, %	44 ± 1.1	42 ± 0.9	24 ± 2.0*	32 ± 0.6*†
LVEDP, mm Hg	9.5 ± 0.2	9.2 ± 0.1	19.0 ± 0.5*	12.1 ± 0.4†
LV dP/dT_{max}, mm Hg/s	8,606.4 ± 253.2	8,931.1 ± 123.4	6,203.6 ± 188.0*	8,497.0 ± 196.3†
LV dP/dT_{min}, mm Hg/s	−8,405.4 ± 230.9	−8,162.8 ± 156.0	−5,839.1 ± 81.0*	−7,415.0 ± 119.6†

FS, fractional shortening.
*$P < 0.01$ for PBS sham values.
$^{\dagger}P < 0.01$ for PBS-AB values after AB.