ONLINE SUPPLEMENTARY MATERIAL

For the manuscript entitled: "βarrestin2 improves post-myocardial infarction heart failure via SERCA2a-dependent positive inotropy in cardiomyocytes", by McCrink et al.

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This document contains:

1) The full "Materials and Methods" section of the manuscript

2) Supplementary References

3) Supplementary Figures S1-S6 with associated legends

FULL METHODS

Chemicals. All drugs were from Sigma-Aldrich (St. Louis, MO, USA), except for N106, which was from Xcess Biosciences (San Diego, CA, USA).

H9c2 cell culture, adenoviruses, and transfections. The H9c2 rat cardiomyoblast cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured as previously described[1]. Recombinant adenoviruses encoding for rat WT full-length βarr1 (Adβarr1) or βarr2 (Adβarr2) were constructed as described previously.[2] Briefly, transgenes were cloned into shuttle vector pAdTrack-CMV, which harbors a CMV-driven GFP, to form the viral constructs by using standard cloning protocols. As control adenovirus, empty vector expressing GFP only (AdGFP) was used. The resultant adenoviruses were purified using two sequential rounds of CsCl density gradient ultracentrifugation, as described previously.[2] For the in vivo experiments, premade adenovirus encoding for human WT full-length βarr2, containing C-terminal Flag and His tags was purchased from ViGene Biosciences (Rockville, MD, USA) and amplified in HEK293 cells. Transfection with the K480/585R SERCA2a SUMOylation-deficient mutant was performed with a pReceiver-M01 expression vector encoding for human SERCA2a (Genecopoeia, Rockville, MD, USA), genetically engineered to insert the two point mutations (K480/585R) using the Q5 site-directed mutagenesis kit (New England BioLabs, Ipswich, MA, USA). Proper insertion of the double mutation was confirmed by PCR and cell transfections were performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Co-IP and western blotting. Mouse cardiac or H9c2 cell extracts were prepared, as described previously,[2,3] in a 20 mM Tris pH 7.4 buffer containing 137 mM NaCl, 1% Nonidet P-40, 20% glycerol, 10 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, 2.5 μg/ml aprotinin, and 2.5 μg/ml leupeptin. Protein concentration was determined and equal amounts of protein per sample were used for IP or western blotting. SERCA2a was immunoprecipitated by overnight incubation of extracts with an anti-SERCA2 antibody (#4388; Cell Signaling Technology, Danvers, MA, USA), attached to Protein A/G-Sepharose beads (Sigma-Aldrich). The IPs were then subjected to immunoblotting for βarr1/2 (A1CT, a generous gift from Prof. R.J. Lefkowitz, Duke University, Durham, NC, USA; or sc-28869; Santa Cruz Biotechnology, Santa Cruz, CA, USA), for SUMO1 (#4930; Cell Signaling Technology), for Ubc9 (sc-10759; Santa Cruz Biotechnology), and for SERCA2a, to confirm IP of equal amounts of endogenous SERCA2a among the various treatment conditions/heart samples. Additionally, an anti-Flag antibody (#14793S; Cell Signaling

Technology) was used to detect the transgenic (Flag-tagged) βarr2 protein expressed in mouse hearts in vivo, anti-PAI-1 (sc-8979), anti-TGFβ (sc-1460), anti-pIκBa (sc-8404), and anti-IκBa (sc-371; all from Santa Cruz Biotechnology) antibodies for cardiac PAI-1, TGFβ, pIκBa and total IκBa detection, respectively, and an anti-GAPDH antibody (sc-25778; Santa Cruz Biotechnology) to control for protein loading. Immunoblots were revealed by enhanced chemiluminescence (ECL, Life Technologies, Grand Island, NY, USA) and visualized in the FluorChem E Digital Darkroom (Protein Simple, San Jose, CA, USA), as described previously.[2,3] Densitometry was performed with the AlphaView software (Protein Simple) in the linear range of signal detection (on non-saturated bands). For the immunoblotting in human heart extracts, frozen human heart tissue donated by a deceased (due to stroke) male Hispanic patient was purchased from BioreclamationIVT (Baltimore, MD, USA). Small pieces of healthy left ventricular free wall tissue from that specimen were dissected and homogenized with a Polytron homogenizer to prepare protein extracts for western blotting.

SERCA activity measurements. Cardiac or cellular (maximal) SERCA activity was measured as described.[2,4,5] Briefly, homogenates were prepared and assayed in 10 mM Tris, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 0.1 mM $CaCl_2$, 0.2 mM NADH, 1.5 mM trisodium phosphoenolpyruvate, 15 units/ml pyruvate kinase, and 36 units/ml lactate dehydrogenase. Total ATPase activity was assayed by monitoring the rate of loss of A340 after addition of the membrane preparation to a thermostatically controlled (37 °C) cuvette in a spectrophotometer. Background ATPase activity was determined in the absence of ATP. Ca^{2+}-independent ATPase activity was assayed in the presence of 10 mM EGTA, instead of Ca^{2+}, and subtracted from the total ATPase activity to derive the Ca^{2+}-dependent ATPase (SERCA) activity. SERCA activity was calculated as nmol of inorganic phosphate (P_i) produced per min per mg of protein.

GST pulldown. The glutathione S-transfer (GST) pull-down assay was performed with the Pierce® GST protein interaction pull-down Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Recombinant human βarr2 ("bait") and cell lysates overexpressing human SERCA2a ("prey") were purchased from Novus Biologicals (Littleton, CO, USA). The N-terminal-truncated human SERCA2a coding sequence (GenBank Accession #: 161377445) was PCR-amplified and cloned into a pcDNA3.1 plasmid vector (Invitrogen) for expression in HEK293 cells. Its overexpression was verified with western blotting and lysates from cells transfected with this construct were used as alternative "prey" in the assay.

FRET analysis. FRET efficiency (E_{FRET}) was evaluated based on the acceptor photobleaching method, as described.[6] Briefly, the fluorescence of YFP was photobleached using a mercury lamp and emissions of CFP and YFP were collected using CFP-HQ and YFP-HQ filter cubes (Nikon USA, Melville, NY, USA), respectively. E_{FRET} was calculated as the % increase in CFP emission after YFP photobleaching.

Real-time cAMP accumulation and cardiac cAMP measurements. For real-time cAMP accumulation measurements in H9c2 cardiomyocytes, the mNeon Green cADDis cAMP Assay kit (Montana Molecular, Bozeman, MT, USA) was used, essentially as described.[7] Briefly, the assay is based on a live cell, green fluorescence-emitting sensor "cADDis" (cAMP Difference Detector in situ), whose fluorescence decreases when cAMP levels increase inside the cell due to displacement from Epac2 by cAMP. The changes in emitted fluorescence were detected and measured on an automated fluorescent plate reader (Enspire Multimode Plate Reader; Perkin Elmer, Waltham, MA, USA). Cardiac cAMP levels were measured with the BIOMOL Cyclic AMP PLUS EIA kit (Biomol, Hamburg, Germany), as described previously.[2]

Experimental animals and surgical/gene delivery procedures. The animals in this study were handled according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the IACUC boards of Florida Atlantic and Nova Southeastern Universities. Adult (3-month-old) C57/BL6 male mice were used for the study. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) and MI was performed by ligation of the left anterior descending (LAD) coronary artery, as previously described.[2] While the animal`s chest was open for the LAD ligation, 1.3×10^{10} total particles, diluted in 100 μl phosphate-buffered saline (PBS), of Adβarr2 or AdGFP were rapidly injected (direct injection into the left ventricular cavity) via an apically inserted 31-gauge needle. At the end of the procedure, the chest cavity was closed and the mouse was gradually weaned off the respirator, endotracheal tube removed, and the animal was placed in a cage on a heating pad until regaining consciousness.

Echocardiography. Transthoracic M-mode echocardiography was performed with a linear 30-MHz transducer (VeVo 770 High Resolution Imaging System, VisualSonics, Toronto, ON, Canada), as described.[2,8]

ELISA for cardiac cytokines. Pro-inflammatory cytokines TNFα, IL-6 and IL-1β were measured in serum obtained from left ventricular blood, immediately prior to heart excision and animal

euthanizing, via multiplexed ELISA, as described.[2] The assay was performed with the Mouse Cytokine ELISA Profiling Kit (EA-1091; Signosis, Santa Clara, CA, USA), according to the manufacturer's instructions.

Infarct size measurements. A number of MI-operated mice were sacrificed at 24 hrs post-surgery and their hearts excised to determine their infarct size via triphenyltetrazolium chloride (TTC) staining, as described.[2,8]

In situ TUNEL assay. Deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was performed with an In Situ Cell Death Detection Kit (Sigma Aldrich) according to manufacturer's instructions, as described.[2,8]

βAR density measurements. βAR density was measured in isolated cardiac plasma membranes using ^{125}I-labeled CYP (Iodocyanopindolol), as described previously.[2,8]

Calcium transients. Calcium transient analysis in cardiomyocytes isolated from the left ventricular free wall of three-week post-MI mouse hearts was performed as previously described.[5,9] Briefly, excised hearts were mounted on a steel cannula and retrograde perfused with Ca^{2+}-free HCO_3^- buffer followed by collagenase digestion. Isolated myocytes were plated on laminin-coated coverslips and briefly placed in a series of buffers containing increasing Ca^{2+} concentrations. The final Ca^{2+} buffer was then aspirated and replaced with DMEM (Mediatech, Manassas, VA, USA) containing 1.2 mM Ca^{2+} and 2.5% fetal bovine serum. At 4 hrs after plating, the myocytes were placed in serum-free DMEM containing 0.1 mg/mL BSA at 37 ^oC, loaded with 0.67 μM of the calcium dye Fura 2-AM (ThermoFisher Scientific) and drugs were added. A dual-excitation spectrofluorometer (IonOptix Inc., Westwood, MA, USA) was used to record fluorescence emissions for intracellular calcium concentration determinations.

Statistical analyses. Data are generally expressed as mean ± SEM. Unpaired 2-tailed Student's *t* test and one- or two-way ANOVA with Bonferroni test were generally performed for statistical comparisons, unless otherwise indicated. For most 3-group statistical comparisons, Dunnett's test using SAS version 8.2 software was used, as well. For all tests, a p value of <0.05 was generally considered to be significant.

Supplementary References

1. Yano N, Suzuki D, Endoh M, Tseng A, Stabila JP, McGonnigal BG, Zhao TC, Padbury JF, Tseng YT. Beta-adrenergic receptor mediated protection against doxorubicin-induced apoptosis in cardiomyocytes: the impact of high ambient glucose. *Endocrinology.* 2008; 149:6449-6461.
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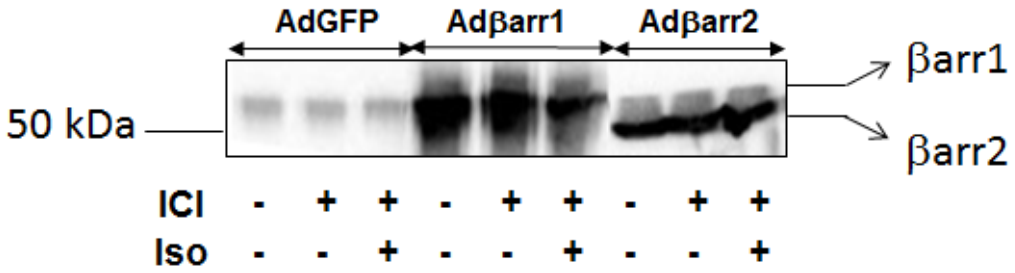

Figure S1. Immunoblotting for βarr1/2 in extracts from H9c2 cardiomyocytes, transfected with AdGFP, Adβarr1, or Adβarr2, and treated with vehicle, 10 μM ICI, or 1 μM Iso in the presence of 10 μM ICI for 20 min, to confirm the respective transgene (βarr1 or βarr2) overexpression. In control (AdGFP-transfected) cells, only βarr1 protein could be detected.

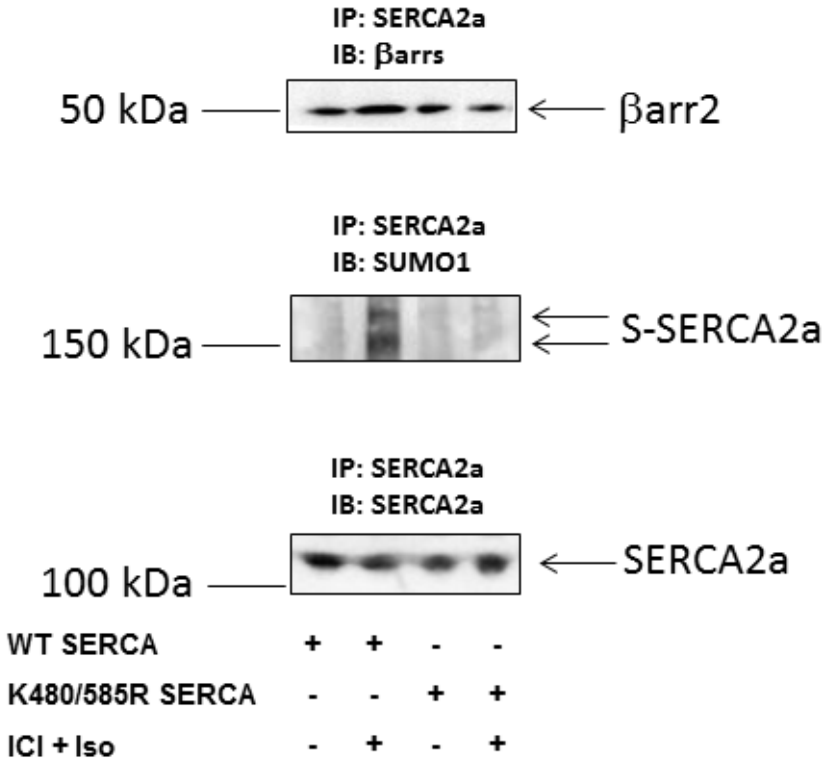

Figure S2. Immunoblotting for βarr1/2, SUMO1 and SERCA2a in SERCA2a IPs prepared from βarr2-overexpressing H9c2 cardiomyocytes, transfected either with empty vector (WT SERCA) or with the K480/585R SERCA2a mutant, and treated with vehicle or 1 μM Iso in the presence of 10 μM ICI (ICI +Iso) for 20 min. ICI alone had no effect (data not shown). IB: Immunoblotting; IP: Immunoprecipitation; S-SERCA2a: SUMOylated SERCA2a.

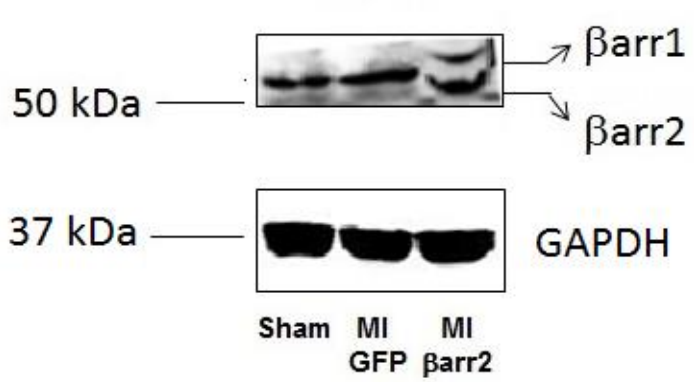

Figure S3. Immunoblotting with the A1CT antibody, which recognizes both βarr1 and βarr2, in C57/Bl6 mouse hearts to confirm the transgenic βarr2 overexpression (i.e. cardiac Adβarr2 transduction). Blots for GAPDH confirming equal protein loading are also included. Only βarr1 (which runs at ~57 kDa) was expressed at appreciable levels in the hearts of sham-operated (Sham) or post-MI mice receiving AdGFP (MI GFP), as expected. βarr2 (representing the exogenous, adenovirus-transduced protein) could be detected only in the hearts of the Adβarr2-treated post-MI (MI βarr2) mice, running right below endogenous βarr1. Note that, since the transduced βarr2 was Flag-tagged, its band ran at a slightly higher than the predicted (50 kDa) molecular weight, shifting also the endogenous βarr1 band higher (MI βarr2 lane).

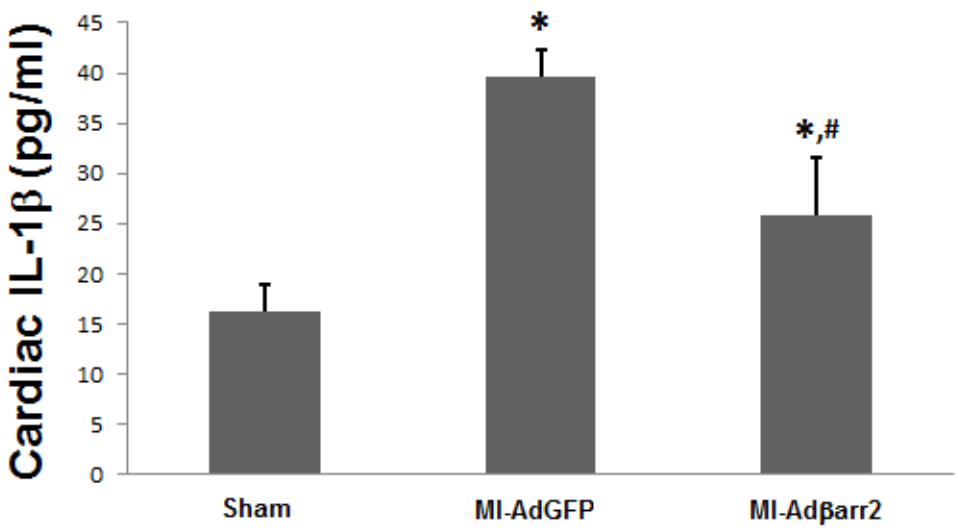

Figure S4. Levels of IL-1β in serum of intra-cardiac blood from these mice at 21 days post-MI/gene delivery. *, $p<0.05$, vs. Sham; $^{\#}$, $p<0.05$, vs. MI-AdGFP; n=3 mice/group.

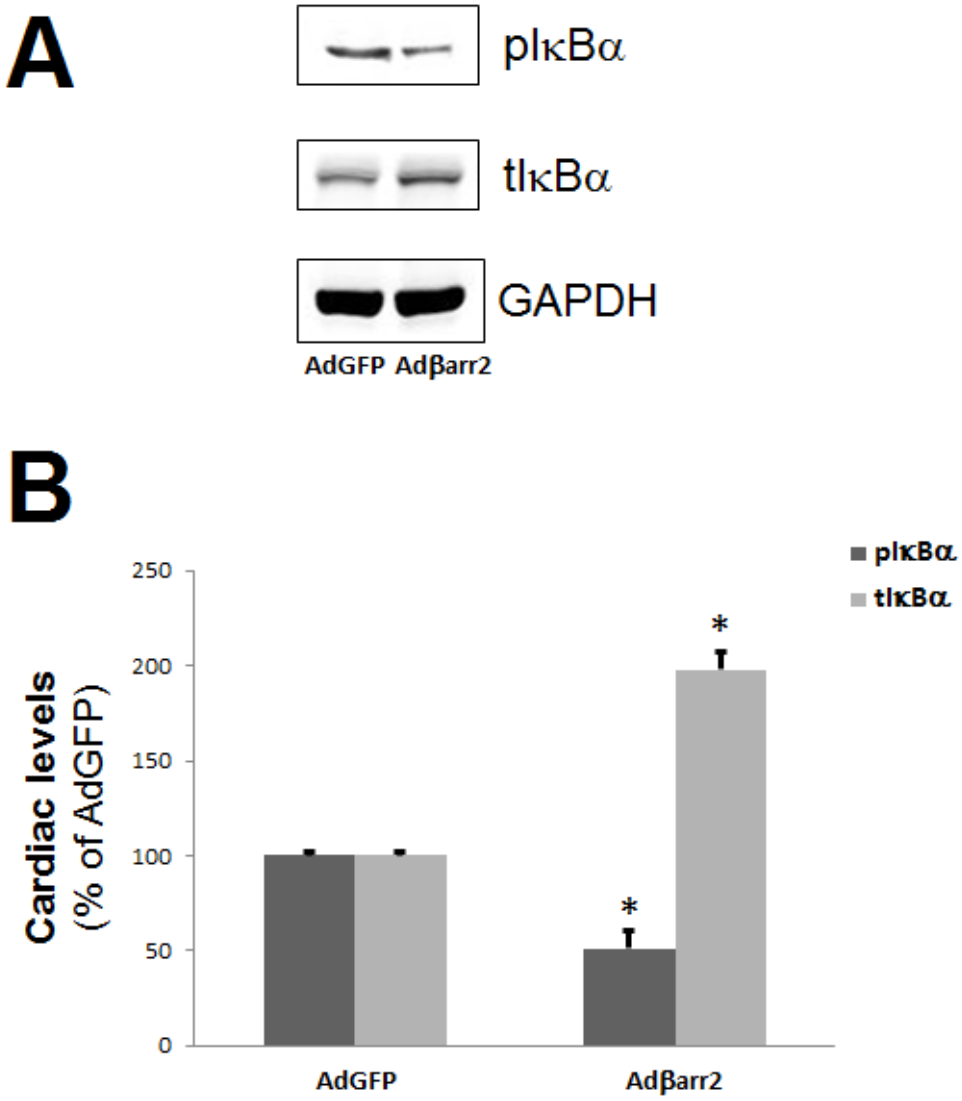

Figure S5. Western blotting for phospho-IκBα (pIκBα) and total IκBα (tIκBα) in total cardiac extracts from 3-week post-MI mice treated with either AdGFP or Adβarr2. Representative blots are shown in (**A**), including blots for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as loading control, and densitometric quantitation, normalized with GAPDH as control, is shown in (**B**). *, $p<0.05$, vs. AdGFP; n=3 hearts/group.

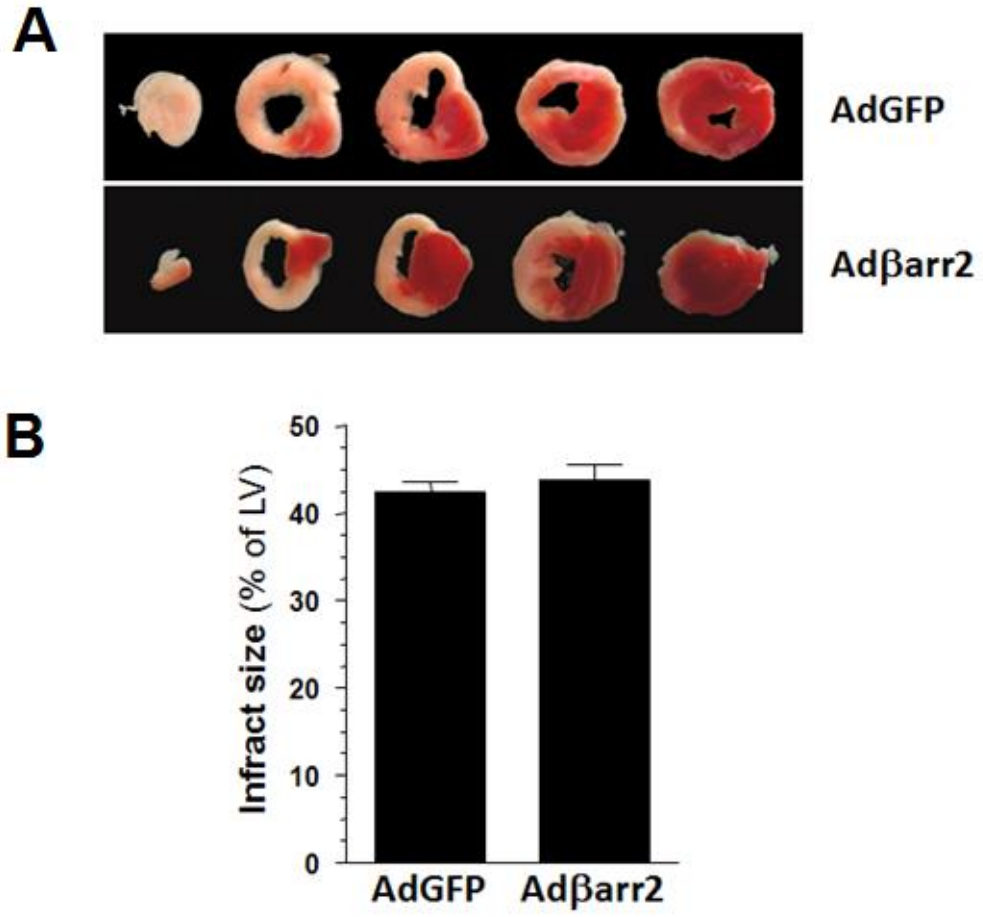

Figure S6. Infarct size of AdGFP- and Adβarr2-treated mice at 24 hrs post-surgery (MI). (**A**) Representative TTC–stained cardiac cross-sections. (**B**) Average left ventricular (LV) infarct size (n=3 hearts/group). No significant difference between the MI groups was observed (p=0.05).