EXPANDED METHODS AND DATA SUPPLEMENT

EXPANDED MATERIALS AND METHODS

Experimental animals

Conditional mice bearing floxed GRK2 alleles (GRK2 fl/fl) have previously been described [1]. In this mouse line exons 3 to 6 of GRK2 are targeted by flanking them with loxP recognition sites. Transgenic mice overexpressing Cre-recombinase protein fused to two mutant estrogen receptor ligand-binding domains under the control of the αMHC promoter (αMHC-MerCreMer) [2] were received from The Jackson Laboratory (JAX Mice and Services, Bar Harbor, Maine, USA). Homozygous mice with the floxed GRK2 alleles were crossed with αMHC-MerCreMer mice and resulting αMHC-MerCreMer x GRK2 fl/fl mice and GRK2 fl/fl, as well as αMHC-MerCreMer, were maintained on a C57/Bl6 background. To induce Cre recombination and subsequent deletion of GRK2, adult αMHC-MerCreMer x GRK2 fl/fl transgenic mice were treated with tamoxifen (Tmx) (Sigma-Aldrich, St. Louis, Missouri, USA) by intraperitoneal (ip) injection once a day for 5 consecutive days at a dosage of 40 mg/kg per day [2].

In addition αMHC-Cre mice [3] were bred on to the GRK2 fl/fl background to generate cardiac GRK2-deleted mice, initiated by the activation of the αMHC-promoter. αMHC-Cre x GRK2 fl/fl mice and GRK2 fl/fl were included in the study. All animal procedures and experiments were performed in accordance with the guidelines of the IACUC of Thomas Jefferson University. All animals were bred and maintained on a C57Bl/6 background.

Experimental protocol

The experimental design consisted of both a heart failure (HF) prevention study and a HF rescue study. In the HF rescue study the generated Tmx inducible Cre lines (αMHC-MerCreMer x GRK2 fl/fl and αMHC-MerCreMer) and GRK2 fl/fl mice were subjected to MI at 8

weeks of age; from day 10 to day 14 after MI all groups were treated with Tmx to induce cardiac-myocyte specific Cre-recombinase activity. Animals from 2 groups (αMHC-MerCreMer and αMHC-MerCreMer x GRK2 fl/fl) were also treated with the βAR antagonist metoprolol starting at day 10 after MI until the end of the observation period. All lines in the rescue study were studied until 4 months post-MI.

For the prevention study conditional GRK2 knockout (KO) mice (αMHC-Cre x GRK2 fl/fl and GRK2 fl/fl) at 8 weeks of age were subjected to myocardial infarction (MI) and studied up to 28 days post-MI. All groups were accompanied by sham-operated animals.

Model of LV myocardial infarction

MI was induced by ligation of the left anterior descending coronary artery (LAD) as described previously [4]. Briefly, under general anesthesia with isoflurane (2%) and spontaneous breathing, a small left thoracotomy was performed and the heart was temporary dislocated followed by placing a suture ligation (6.0 silk suture) 2 mm below the origin of the LAD. The heart was replaced immediately into the intrathoracic cavity and the air was evacuated to avoid a pneumothorax. In a similar fashion sham operation (sham) was performed without ligation of the LAD. Following MI the animals remained supervised until fully conscious.

Assessment of infarct size and survival

Size of LV infarction following MI was assessed by the use of 2,3,5-triphenylterazolium chloride (TTC) as described elsewhere [4]. 24 hours and 28 days after surgery, the hearts of randomly chosen mice were removed under general anesthesia, frozen at -80°C and LVs were sliced into 1mm thick sections perpendicular to the long axis of the heart. The slices were stained with 1% TTC and digitally photographed. Infarcted area of the left ventricle (TTC-negative stained tissue) and non-infarcted myocardium (TTC-positive stained tissue) was

measured using Sigma Scan software (Aspire Software International, Ashborn, Virginia, USA) and the MI size was expressed as a percentage of total left ventricular area.

For survival analysis cages were inspected twice a day for animals that had died until the end of the study period.

Echocardiographic and hemodynamic analysis of cardiac function

For transthoracic echocardiography mice were anesthetized with 2.5% Avertin (10 µL/g body weight IP, Sigma-Aldrich, St. Louis, Missouri, USA). Two-dimensional transthoracic echocardiography was acquired with a VeVo 770 imaging system (VisualSonics Inc., Toronto, Ontario, Canada) with a 12-MHz scanhead both in sham-operated animals and mice subjected to myocardial infarction as described previously [4]. Echocardiographic measurements were taken at baseline and after 0.5ug of the selective β_2-AR agonist fenoterol (Sigma-Aldrich, St., Louis, Missouri USA). Measurements were taken during the peak response. LV diameters and contractility (FS% = [(LVEDD -LVESD) ×100/LVEDD]) were assessed by an M-Mode recording in the parasternal short axis view.

For measurements of LV hemodynamics the animals were sedated as described above. A 1.4 French micromanometer-tipped catheter (SPC-320, Millar instruments, Houston, Texas, USA) was inserted into the right carotid artery and then advanced into the LV. All recordings were performed with a closed chest approach at baseline and after increasing doses (0.02 µg and 0.5 µg ip) of the unselective βAR agonist isoproterenol (Sigma-Aldrich, St. Louis, Missouri, USA) as described previously [5]. Furthermore, LV hemodynamics were measured after of a single dose (0.5ug ip) of the selective β_2-AR agonist fenoterol. The peak response, which typically occurred 2 min after the injection of fenoterol, was used as a readout for the intensity of

the β_2-AR signal. The following parameters were analyzed: heart rate (beats/min), LV end-diastolic pressure (LVEDP), LV systolic pressure (LVPsys), maximal (LV $dP/dt_{max.}$), and minimal (LV $dP/dt_{min.}$) first derivative of LV pressure.

Isolation of cardiac myocytes

Adult mouse cardiac myocytes were isolated from sham and infarcted GRK2 fl/fl and αMHC-Cre x GRK2 fl/fl mice as previously described [6]. Briefly, mice were anesthetized using isoflurane, euthanized by excision of the heart and subsequently the ascending aorta was fixed to a perfusion cannula using a 6F silk ligature. The hearts were then perfused retrogradely with tyrodes solution (Hepes 25 mM, 130 mM NaCl, NaH_2PO_4 10 mM, $MgCl_2$ 2.5 mM, KCl 27 mM) supplemented with 0.2% glucose and 10 mM 2, 3-butandione monoxime (BDM) (Sigma-Aldrich, St. Louis, Missouri, USA) at a flow-rate of 3 ml/min for approximately 30 sec before switching to tyrodes solution with BDM containing collagenase type II (Worthington Inc., Lakewood, New Jersey, USA). When digested, hearts were cut into smaller pieces, transferred to a tube containing tyrodes solution supplemented with BDM, 5% fetal calf serum (FCS), and processed to a suspension using a transfer pipette. The suspension was then filtered through a mesh before cardiac myocytes were pelleted and resuspended in tyrodes solution containing BDM and 5% FCS. Cells were then slowly adjusted to increasing concentrations of Ca^{2+} reaching a final concentration of 1 mM. For *in vitro* cell culture assays cells were cultured for 24 hours. For this purpose, cells were pelleted again, resuspended in α-MEM (Gibco, Invitrogen Corporation, Carlsbad, California, USA) supplemented with Hanks' salt, plated onto laminin (Invitrogen Corporation, Carlsbad, California, USA) pre-coated culture dishes, and maintained in α-MEM with Hank's salt solution supplemented with L-Glutamine, BDM and Insulin-Transferrin-Selenium (1:1000 dilution) (Gibco, Invitrogen Corporation, Carlsbad, California, USA). The isolated cardiac myocytes were kept in a humidified atmosphere containing 2% CO_2.

Single myocyte contractility studies

For determination of single-myocyte contractility studies cardiac myocytes were isolated as described above. Cells were kept in BDM-free tyrodes solution containing 1mM $CaCl_2$. The cells were stimulated in an electrical field and continuously flushed with tyrodes containing 1mM $CaCl_2$ without (baseline) and with 10^{-8}M isoproterenol (isoproterenol-stimulation). For measurements of single myocyte contractility rod-shaped cells were selected. Single-cell contractions were measured in cells by video edge detection (Fluorescence and Contractility System, IonOptix, Milton, Massachusetts, USA) [7]. The extent of twitch fractional shortening was calculated as follows: (maximum length – minimum length) × 100/maximum length.

Determination of intracellular cAMP generation

Isolated adult cardiac myocytes (see above) from sham GRK2 fl/fl and αMHC-Cre x GRK2 fl/fl were plated and after 24 hours, cAMP levels were measured using a fluorescence assay (Mediomics, LLC, St. Louis, Missouri, USA) according to the manufacturer's instructions. Intracellular cAMP levels were measured at baseline and after 5 min stimulation with increasing concentrations of isoproterenol.

RNA isolation, reverse transcription and quantitative real-time RT-PCR

Isolated LV cardiac myocytes (rescue-study) or myocardial tissue from the remote zone (prevention-study) was snap-frozen. Analysis was carried out 35 days post MI/ 21 days after the last dose of Tmx (rescue-study) or 28 days post MI (prevention study). Total RNA was isolated using the Ultraspec method (Biotecx Laboratories Inc., Houston, Texas, USA) according to the manufacturer's protocol. An aliquot of each sample was run on a denaturing agarose gel (1%) to assess the quality of RNA. cDNA synthesis was performed on 1 µg of total RNA by the use of the iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, California, USA). Finally, 2.5 µl of diluted cDNA (1/100) was added to a 15 µl mixture that contained a 1×

concentration of iQ SYBR Green Supermix (BioRad Laboratories, Hercules, California, USA) and 100 nM of gene-specific oligonucleotides. Quantitative PCR was carried out on a MyiQ Single-Color Real-Time PCR detection system (BioRad Laboratories, Hercules, California, USA) for murine 28S (FWD 5`- TTG AAA ATC CGG GGG AGA G -3`, REV 5`- ACA TTG TTC CAA CAT GCC AG -3`), 18S (FWD 5`- TCA AGA ACG AAA GTC GGA GG -3`, REV 5`- GGA CAT CTA AGG GCA TCA C -3`), GRK2 (FWD 5`- CCC TCT CAC CAT CTC TGA GC -3`, REV 5`- CGG TTG GGG AAC AAG TAG AA -3`), GRK5 (FWD 5`- CCT CCG AAG GAC CAT AGA CA -3`, REV 5`- GAC TGG GGA CTT TGG AGT GA -3`), ANP (FWD 5`-TGC CGG TAG AAG ATG AGG TC -3`, REV 5`-TGC TTT TCA AGA GGG CAG AT -3`), BNP (FWD 5`- CTG AAG GTG CTG TCC CAG AT -3`, REV 5`- CCT TGG TCC TTC AAG AGC TG -3`), β-MHC (FWD 5`-GCC AAC ACC AAC CTG TCC AAG TTC -3`, REV 5`- TGC AAA GGC TCC AGG TCT GAG GGC -3`), Coll-1 (FWD 5`- CCA GTT CGA GTA TGG AAG CGA -3`, REV 5`- AGG TGA TGT TCT GGG -3`), MMP-9 (FWD 5`- CGT CGT GAT CCC CAC TTA CT -3`, REV 5`- AAC ACA CAG GGT TTG CCT TC -3`) expression levels. The annealing temperature was set to 62.3ºC. PRIMER3 software (http://frodo.wi.mit.edu/) was used to generate sequence-specific oligonucleotide primers (Sigma-Aldrich, St. Louis, Missouri, USA) based on cDNA sequences in the National Center for Biotechnology database. 28S or 18S rRNA levels did not differ between groups and were used for normalization. Following each run a melting curve was acquired by heating the product to 95°C, cooling to and maintaining at 55°C for 20 seconds, then slowly (0.5°C/s) heating to 95°C, to determine the specificity of the PCR products.

Western blot analysis

Western blotting was performed as described previously [4]. Cardiac protein levels of GRK2 (sc-562, C-15, Santa Cruz Biotechnology, 1:5,000), α-actin (A7811, Sigma-Aldrich, 1:5,000) and calsequestrin (CSQ) (208915; Calbiochem; 1:10,000) were assessed in cardiac myocyte cellular preparations. Protein content was quantified with the BioRad DC Protein

Assay (BioRad Laboratories, Richmond, California, USA). Protein samples were separated by 4-20% SDS-PAGE (Invitrogen Corporation, Carlsbad, California, USA), and proteins were transferred to PVDF membrane (Millipore Corporation, Billerica, Massachusetts, USA) and probed with the first antibody at 4°C overnight. The proteins were stained with a corresponding Alexa Fluor 680- (Molecular Probes; 1:10.000) or IRDye 800CW-coupled (Rockland Inc.; 1:10.000) secondary antibody, followed by visualization of the proteins with a LI-COR infrared imager (Odyssey, LI-COR, Lincoln, Nebraska, USA), and quantitative densitometric analysis was performed applying Odyssey version 1.2 infrared imaging software.

Statistical analysis

Data are generally expressed as mean ± SEM. An unpaired two tailed t-test, a one-way ANOVA and a two-way repeated measurement ANOVA was performed for between-group comparisons. Survival analysis was performed by the Kaplan-Meier method and between-group differences in survival were tested by the log-rank test. For all tests, a P value < 0.05 was considered significant. The authors had full access to the data and take full responsibility for its integrity.

DATA SUPPLEMENT

Characterization of conditional GRK2 knock–out (KO) mice

Loss of GRK2 in cardiac myocytes using conditional gene-targeting.

Global deletion of the *GRK2* gene leads to early embryonic death [1, 8] precluding analysis of GRK2 function in the adult heart. To overcome this obstacle, we used a Cre/loxP-dependent conditional gene targeting approach to permit specific GRK2 KO in cardiac myocytes at later developmental stages. Conditional GRK2 KO mice bearing *floxed* GRK2 alleles (homozygous GRK2 fl/fl mice; [1]) were mated with αMHC-Cre transgenic mice [3] to generate cardiac GRK2-deleted mice. *α*MHC-Cre x GRK2 fl/fl mice were generated at predicted Mendelian ratios and showed a normal life expectancy with normal cardiac function. PCR-genotyping for GRK2 in DNA extracts from left ventricle (LV), lungs and livers, revealed a deletion band for GRK2 (350bp) restricted to the heart in αMHC-Cre x GRK2 (fl/fl) mice (supplemental Fig. 1A). Importantly, reduction of GRK2 levels did not cause compensatory upregulation of GRK3 or GRK5 (supplemental Fig. 1B). Cell specificity with respect to GRK2 KO was demonstrated by the finding of similar mRNA levels for GRK2 in the cardiac non-myocyte fraction (fibroblasts and endothelial cells predominantly) in both groups of mice (supplemental Fig. 1C). Further, no differences were found with respect to GRK2 mRNA levels in other organs (lungs and the liver) (supplemental Fig. 1D).

Loss of cardiac GRK2 after MI shows similar favorable effects on LV remodeling and function as chronic βAR blockade.

To examine whether the loss of cardiac GRK2 produced chronic and beneficial effects similar to, or in addition to, current HF therapies, we treated groups of the mice shown in supplemental Fig. 4 chronically with the βAR antagonist, metoprolol, starting at day 10 post-MI (at the onset of Tmx treatment and GRK2 ablation). As shown in supplemental Fig. 4B, metoprolol treatment (green symbols and bar) in control mice with normal levels of GRK2

(αMHC-MerCreMer mice) prevented further adverse remodeling, which was actually similar to the loss of cardiac GRK2 after MI (red symbols and bar). The decline in systolic function (FS [%]) in Tmx-treated αMHC-MerCreMer mice was further prevented by metoprolol treatment with these mice having significantly improved cardiac function at 120 days post-MI compared to non-treated αMHC-MerCreMer mice (supplemental Fig. 4D). The beneficial effect of metoprolol treatment was similar to the loss of GRK2 expression after MI (supplemental Fig. 4). Interestingly, combining the loss of cardiac GRK2 with metoprolol treatment was not superior from a therapeutic stand-point to the loss of GRK2 alone.

Loss of myocyte GRK2 before MI enhances β2-AR signaling

Consistent with the LV hemodynamics data (Fig. 5, main body of the manuscript), echocardiographic measurements revealed a significantly enhanced response to the selective β_2-AR agonist fenoterol in αMHC-Cre x GRK2 fl/fl mice post-MI compared to GRK2 fl/fl mice arguing in favor of facilitated β_2-AR signaling in the GRK2KO mice (supplemental Fig. 6).

REFERENCES

1. Matkovich SJ, Diwan A, Klanke JL, Hammer DJ, Marreez Y, Odley AM, Brunskill EW, Koch WJ, Schwartz RJ, Dorn GW, 2nd. Cardiac-specific ablation of G-protein receptor kinase 2 redefines its roles in heart development and beta-adrenergic signaling. *Circ Res.* 2006;99(9):996-1003.

2. Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, Molkentin JD. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res.* 2001;89(1):20-25.

3. Agah R, Frenkel PA, French BA, Michael LH, Overbeek PA, Schneider MD. Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. *J Clin Invest.* 1997;100(1):169-179.

4. Most P, Seifert H, Gao E, Funakoshi H, Volkers M, Heierhorst J, Remppis A, Pleger ST, DeGeorge BR, Jr., Eckhart AD, Feldman AM, Koch WJ. Cardiac S100A1 protein levels determine contractile performance and propensity toward heart failure after myocardial infarction. *Circulation.* 2006;114(12):1258-1268.

5. Funakoshi H, Kubota T, Kawamura N, Machida Y, Feldman AM, Tsutsui H, Shimokawa H, Takeshita A. Disruption of inducible nitric oxide synthase improves beta-adrenergic inotropic responsiveness but not the survival of mice with cytokine-induced cardiomyopathy. *Circ Res.* 2002;90(9):959-965.

6. 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 Heart Circ Physiol.* 2000;279(1):H429-436.

7. Most P, Pleger ST, Volkers M, Heidt B, Boerries M, Weichenhan D, Loffler E, Janssen PM, Eckhart AD, Martini J, Williams ML, Katus HA, Remppis A, Koch WJ. Cardiac adenoviral S100A1 gene delivery rescues failing myocardium. *J Clin Invest.* 2004;114(11):1550-1563.

8. Jaber M, Koch WJ, Rockman H, Smith B, Bond RA, Sulik KK, Ross J, Jr., Lefkowitz RJ, Caron MG, Giros B. Essential role of beta-adrenergic receptor kinase 1 in cardiac development and function. *Proc Natl Acad Sci U S A.* 1996;93(23):12974-12979.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Cardiac myocyte specific ablation of GRK2 using conditional gene-targeting. A, PCR-genotyping for GRK2 in DNA extracts from LV, lungs and liver. The GRK2 deletion band is present at 350 bp only in LV extracts from αMHC-Cre x GRK2 fl/fl mice, while a non-floxed mouse (GRK2 +/+) shows the wild-type allele at 300bp. B, GRK2, GRK3, and GRK5 mRNA levels from whole heart extracts. C, GRK2 mRNA levels from isolated ventricular cardiac myocytes and cardiac non-myocytes (composed of fibroblasts and endothelial cells). D, GRK2 mRNA levels in lungs and liver. For B, C and D n=3-4 animals/group, **P*<0.05 *α*MHC-Cre x GRK2 fl/fl vs. GRK2 fl/fl, unpaired two-tailed t-test.

Supplemental Figure 2: Loss of GRK2 in cardiac myocytes does not affect infarct size following LAD ligation. Representative slices of the LV 24 hours (A), and 28 days (B), after ligation of the LAD. Viable tissue appears red after staining with TTC. C and D, quantitative analysis of the infarct area normalized to LV area 24 hours (C), and 28 days (D), after LAD ligation. n=7-10 animals/group for C, n=4-5 animals/group for D.

Supplemental Figure 3: Survival of male and female GRK2 fl/fl and αMHC-MHC x GRK2 fl/fl mice throughout the study period. n=18-19 animals/group for sham mice, n=85-91 animals/group for infarcted mice, survival was analyzed by the Kaplan-Meier method and between-group differences in survival were tested by the log-rank test.

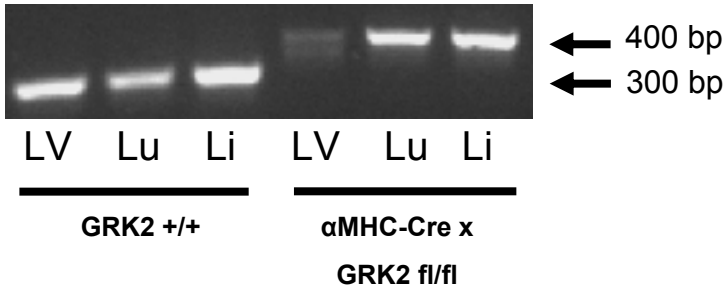
Supplemental Figure 4: Induced loss of GRK2 rescues adverse LV remodeling and cardiac function post-MI - comparison with chronic βAR blockade. A, Echocardiographic assessment of EDD pre-MI, 10d post-MI (prior to Tmx treatment), 42d post-MI (28d post-Tmx treatment) and 120d post-MI. B, EDD as in (A) with addition of metoprolol-treated groups. C,

Echocardiographic assessment of FS pre-MI, 10d post-MI (prior to Tmx treatment), 42d post-MI (28d post-Tmx treatment) and 120d post-MI. D, FS as in (C) with addition of metoprolol-treated groups. n=6-8 animals/group for sham-operated mice, n=12-16 animals/group for MI mice excluding metoprolol treated groups, n=6-7 animals/group for metoprolol treated groups, *P<0.05 vs. MI αMHC-MerCreMer and MI GRK2 fl/fl, †P<0.05 vs. MI αMHC-MerCreMer + metoprolol, #P<0.05 vs. MI αMHC-MerCreMer, two-way ANOVA.

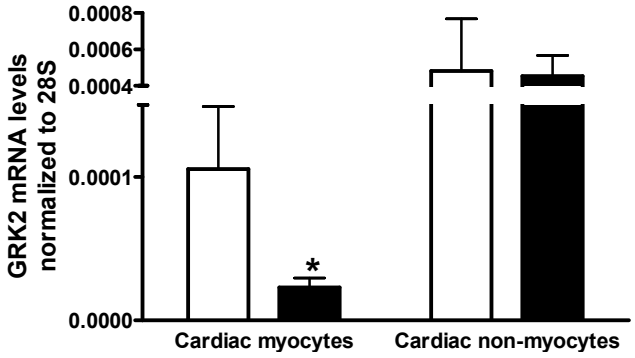
Supplemental Figure 5: Quantitative RT-PCR analysis of GRK5 mRNA levels on cardiac myocytes isolated from the LV 5 weeks post MI (3 weeks after the last dose of Tmx). n=5-6 animals/group for Sham mice, n=7-11 animals/group for MI mice, *P<0.05 MI αMHC-MerCreMer vs. MI αMHC-MerCreMer x GRK2 fl/fl and Sham groups, one-way ANOVA.

Supplemental Figure 6: Echocardiographic measurements of HR (A) and FS (B) 28 days post MI at baseline conditions and after a single intraperitoneal dose of fenoterol. n=10-12 animals/group, *P<0.05 MI αMHC-Cre x GRK2 fl/fl vs. MI GRK2 fl/fl, two-way ANOVA.

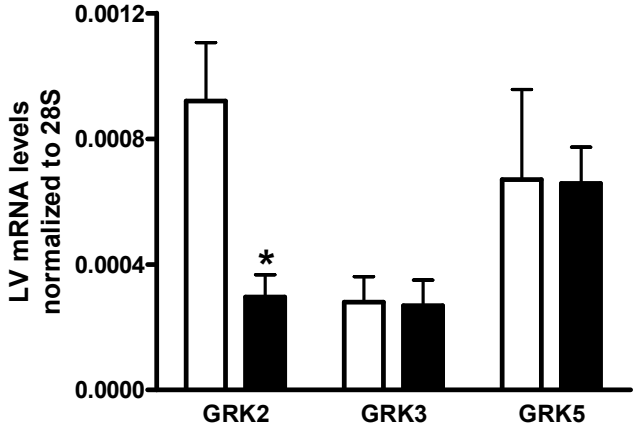
1A



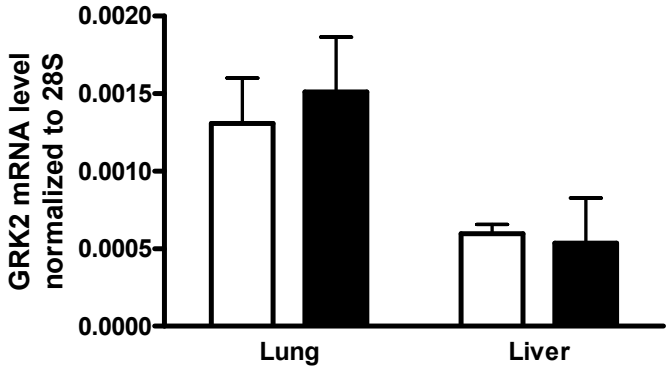
1C

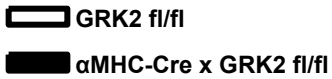


1B



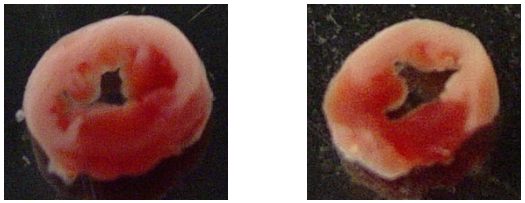
1D



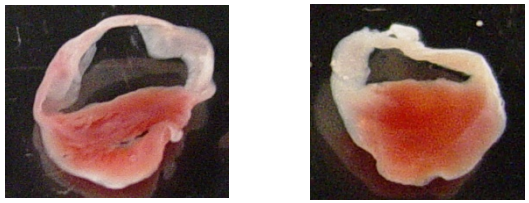


Supplemental Figure 1

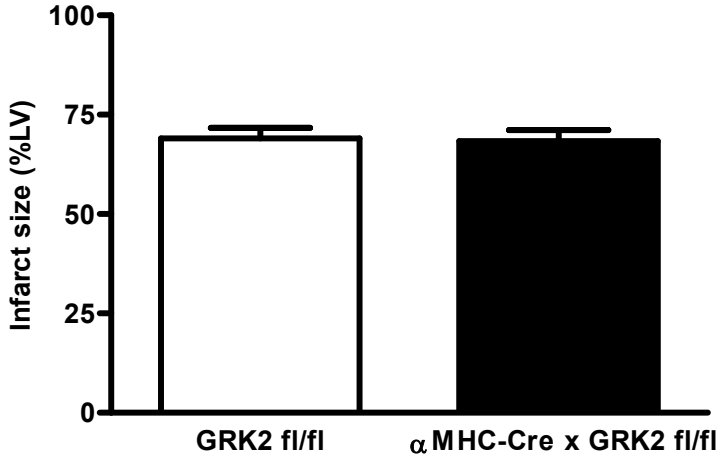
2A



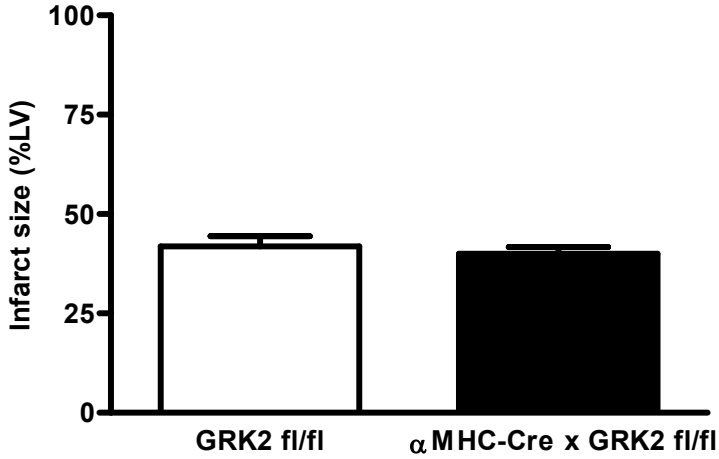
2B



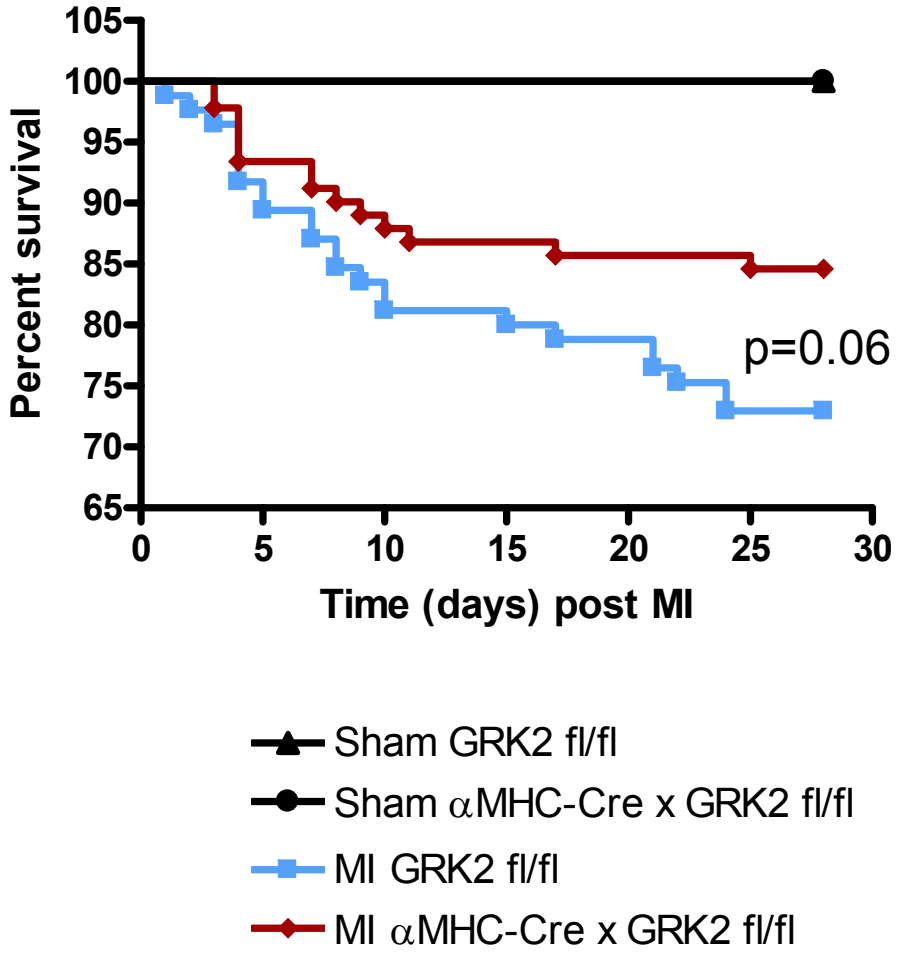
2C



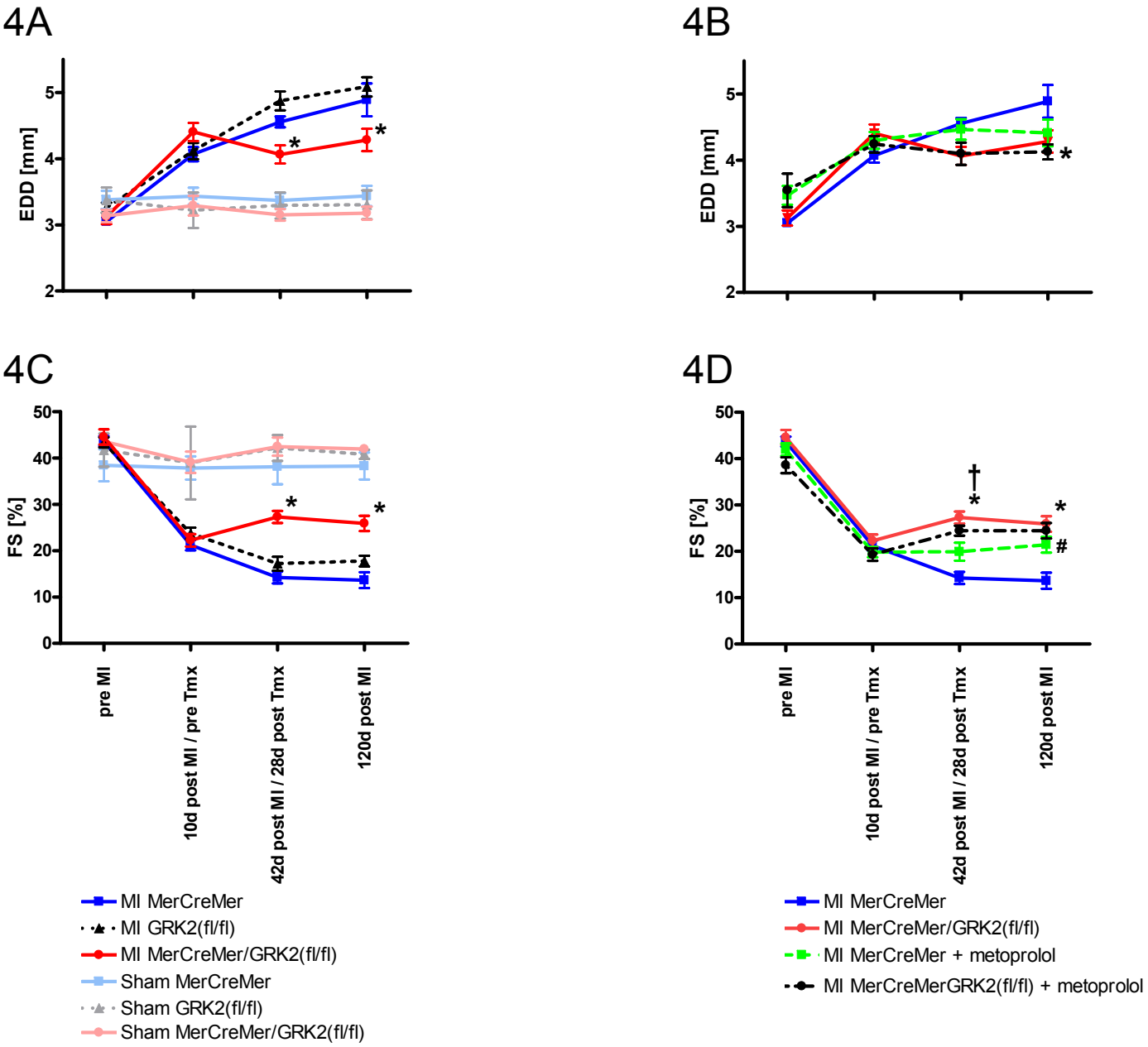
2D



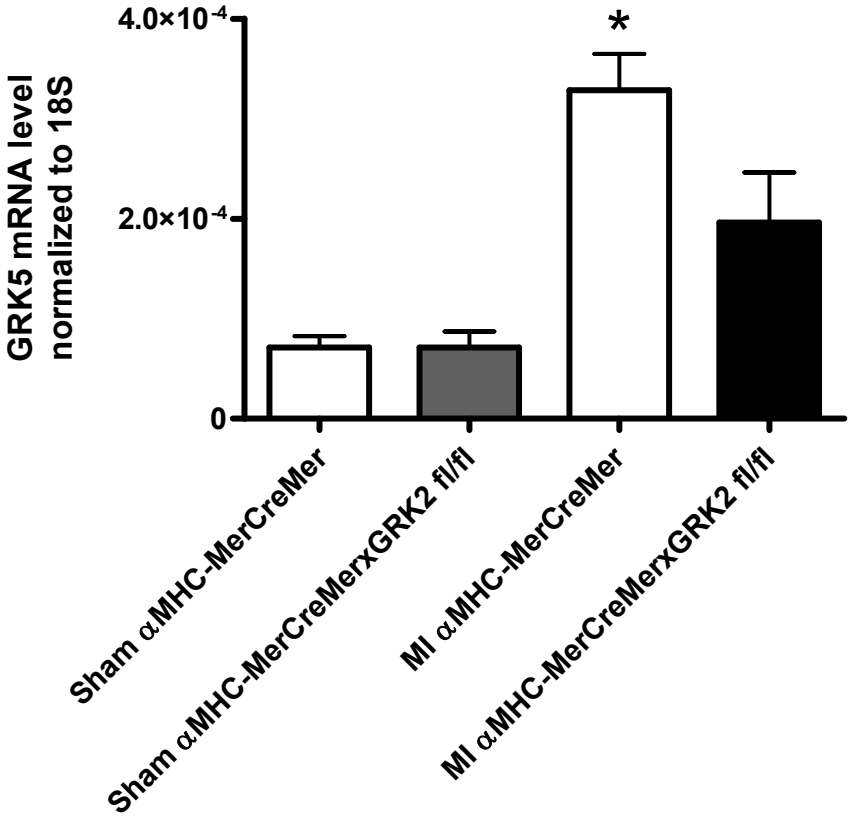
Supplemental Figure 2



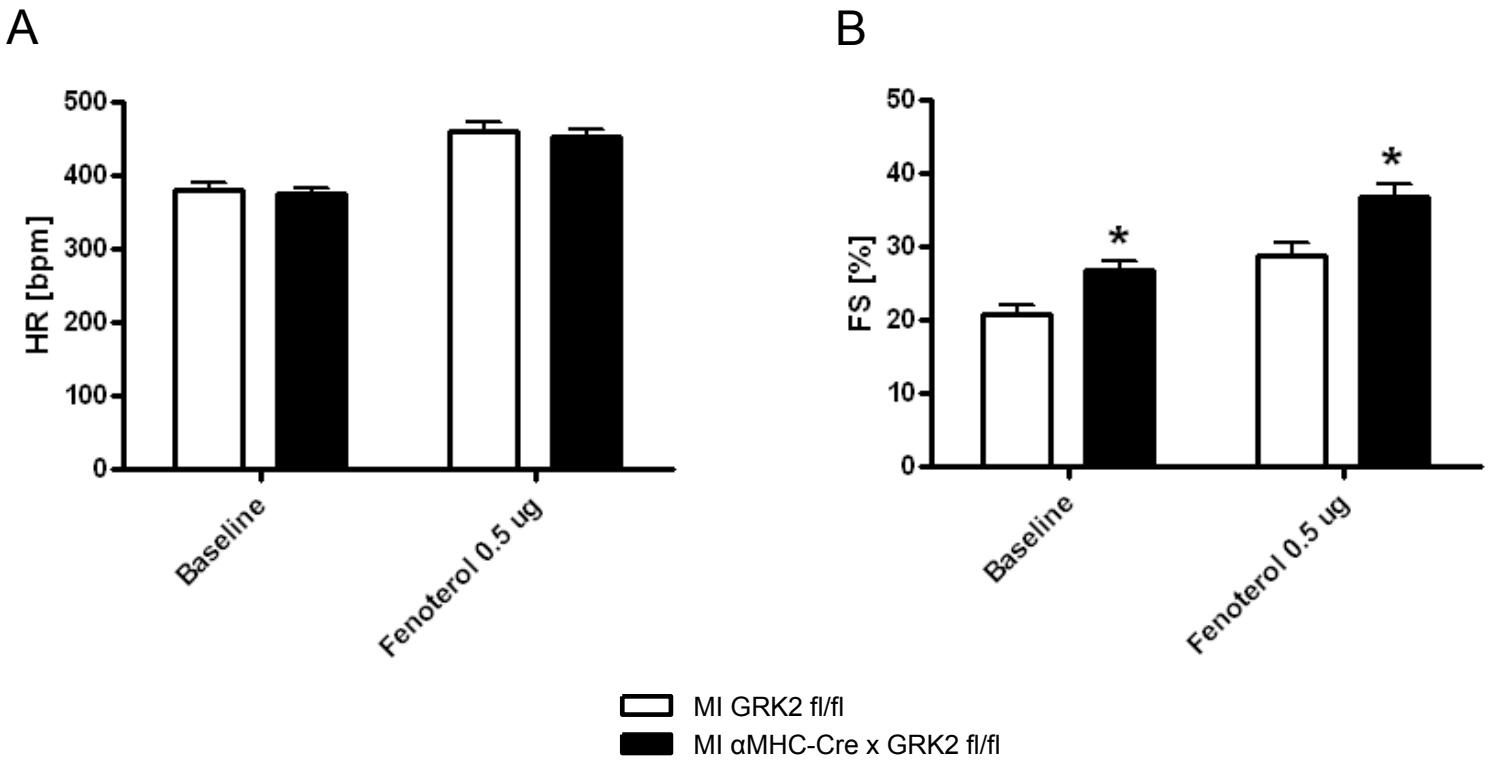
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6