Supplement Material

Materials and Methods

Generation of ERK5 Cardiac Knockout Mice

The *erk5-flox* mice (referred to as $ERK5^{f/f}$) were previously generated[1]. $ERK5^{f/f}$ mice were mated with mice expressing Cre under α myosin heavy chain (αMHC) promoter to generate cardiac-specific ERK5 knockout mice (referred to as $ERK5^{cko}$). The αMHC-Cre line (kindly provided by Dr. MD Schneider, National Heart and Lung Institute, UK) is a well established model that provides efficient Cre recombinase activity in the myocardium. We did not observe any abnormality in cardiac morphology and function in the αMHC-Cre line up to six months of age. All mice used in this study were maintained in a pathogen-free facility at the University of Manchester. The animal studies were performed in accordance with the UK Home Office and institutional guidelines.

Echocardiography

For cardiac morphological and functional analysis, M-mode and Doppler echocardiographic recordings were performed using an Acuson Sequoia C256 system (Siemens) following a protocol described previously[2]. Parameters of intraventricular septal thickness (IVS), left ventricular posterior wall thickness (LVPW), left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD) and fractional shortening (FS %), aorta maximum velocity (Ao Vmax) and ratio of the E/A-wave were obtained.

Histology

Freshly dissected heart tissue was fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. 5-7μm thick sections were cut and stained with hematoxylin & eosin or Masson's trichrome method as described[2]. To calculate the mean cross-sectional area approximately 150 randomly selected cardiomyocytes were measured. 45 randomly chosen frames from Masson's trichrome stained sections were quantified to assess the degree of myocardial fibrosis using Image J software.

Preparation of Lysates and Immunoblot Analysis

Proteins were homogenised and extracted from tissues in Triton lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM ß-glycerophosphate, 10% glycerol, 1 mM orthovanadate, 1 mM phenylsulphfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Protein extracts (30 μg) were subjected to Western blot analysis with antibodies against JNK, p38 MAPK, p53 (Santa Cruz); ERK1/2, PKB, Bcl-2, CREB, phospho-PKB (Ser 473), phospho-ERK1/2, phospho-JNK, phospho-p38 MAPK, phospho-CREB (Ser 133), MEF2C (Cell Signalling); Bad (BD Transduction); ERK5 (Upstate); phospho-ERK5, GFP (Invitrogen) and tubulin (Sigma). Immunecomplexes were detected by enhanced chemiluminescence with anti-mouse, anti-rabbit, or anti-goat immunoglobulin G coupled to horseradish peroxidise as the secondary antibody (Amersham-Pharmacia).

Characterization of Effect of BIX02189 on Neonatal Rat Cardiomyocytes

Primary cultures of neonatal rat cardiomyocytes (NRCMs) were prepared as previously described[2]. NRCMs were incubated with 10μM BIX02189 (Boehringer Ingelheim) for 2h prior to the addition of isoproterenol (10μM, 30min). Cell extracts were lysed for immunoblotting to determine the effect of BIX02189 on inhibition of the activation of ERK5.

siRNA Transfection

NRCMs were transfected with siRNA (100nM) using Lipofectamine LTX and Plus reagents according to the manufacturer's instructions (Invitrogen). Rat ERK5 siRNA (5'-AAAGGGTGCGAGCCTATAT-3') was purchased from Dharmacon, siRNA negative control (Si Neg) was obtained from Eurogenetec. To assess the specific effect of ERK5 siRNA on silencing ERK5 expression, the protein levels of MEK5 and ERK1/2 were detected by immunoblot analysis 72h post-transfection.

Adenoviral Infection

NRCMs were infected with either Ad-MEF2C-R3T (Seven Hills Bioreagents), or Ad-ERK5 (kindly provided by Jun-ichi Abe, Rochester University), or Ad-MEF2C-R3T combined with Ad-ERK5 at 50 MOI in serum-free medium for 24h, followed by additional 24h incubation of ISO, afterwards cells were then subjected to quantitative real-time PCR analyses or [^{3}H] leucine incorporation assay. Ad-GFP was used for a control experiment.

Apoptosis Assays

TUNEL assay to detect apoptosis was performed on paraffin-embedded heart sections using the *in situ* Cell Death Detection kit (Roche). Triple staining with Hoechst, anti-α-actinin antibody (Sigma), and TUNEL was performed to confirm apoptotic morphology in cardiac nuclei. An average of total 10,000 myocyte cells from random fields was analyzed. The data are obtained from three independent experiments. For caspase activity assay, proteins were homogenised and extracted from heart tissues in the lysis buffer (25mM HEPES pH 7.5, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1mM benzamidine, 1mM dithiothreitol [DTT], 1mM phenylsulphfonyl fluoride, 1% Triton X100). Protein extracts (50 μg) were incubated with 200μM DEVD-AMC caspase-3 specific fluorogenic substrate (Alexis Biochemicals) for 1h in the caspase reaction buffer (20mM HEPES, 100mM NaCl, 10mM DTT, 0.1% CHAPS, 10% w/v sucrose). Cleavage of the substrate was measured by spectrofluorometer at 380 nm excitation and 460 nm emission wavelengths. To evaluate the effect of ERK5 knockdown or the inhibition of its kinase activation on apoptosis, BIX02189-treated or siERK5-treat NRCMs were stimulated with sorbitol (Sigma) at 300mM to induce apoptosis[3, 4]. After 4h sorbitol stimulation, cell lysates were prepared for measuring caspase 3 activity.

Quantitative Real-Time PCR

Tissues or NRCMs were extracted to prepare total RNA using Trizol reagent, followed by the synthesis of cDNA. Real-time quantitative PCRs were performed using the SYBR-green I Core Kit (Eurogentec). The primers used for *ANP, BNP, Myh6, Myh7, Acta1, Ctgf, Col1α2, Col3α1* and *GAPDH* were obtained from Qiagen. PCR products were detected in the ABI-PRISM 7700 sequence detection system (Applied Biosystems), and the results were analyzed using the $2\text{-}\Delta\Delta^{CT}$ method[5]. The level of expression of mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA.

Luciferase Reporter Assay

To measure MEF2 luciferase activity after blocking ERK5 activation, NRCMs were infected with recombinant adenovirus encoding the MEF2-luciferase reporter gene (AdMEF2-Luc, Seven Hills Bioreagents) at 25 MOI in serum-free medium for 24h, prior to the incubation of BIX02189. After 2h incubation with the inhibitor, the cells were then treated with 10μM isoproterenol for an additional 18h in the presence of BIX02189. To measure MEF2 luciferase activity in the absence of ERK5 expression, 48h post-transfection of siRNA, NRCMs were infected with AdMEF2-Luc for 24h followed by isoproterenol stimulation. After various treatments, aliquots of NRCM lysates were assayed for the MEF2 luciferase activity using the luciferase assay kit (Promega). To further assess whether MEF2 transcriptional activity is regulated by either ERK5 knockdown or the inhibition of ERK5 kinase activation, NRCMs were transiently transfected with the reporter plasmid pG5E1bLuc together with a construct encoding the fusion proteins Gal4-MEF2A, or Gal4-MEF2D[3], prior to 2h incubation with BIX02189. After BIX02189 incubation, MEF2 transcriptional activity was measured at 24h post ISO stimulation by the dual-luciferase reporter assay system (Promega). Gal4 was used for a control experiment. A pRL-TK plasmid encoding *Renilla* luciferase was employed to monitor transfection efficiency. In parallel, siRNA-treated NRCMs were transfected with various plasmid vectors as described above using Lipofectamine LTX and Plus reagents (Invitrogen). Following 24h ISO stimulation, MEF2 transcriptional activity was measured by the dual-luciferase reporter assay system (Promega).

[^{3}H] Leucine Incorporation

NRCMs were transfected with siRNA (100nM) for 48h, or infected with various adenoviruses (Ad-GFP, Ad-MEF2C-R3T, Ad-ERK5, or Ad-ERK5 plus Ad-MEF2C-R3T) at 50 MOI for 24h following the isoproterenol treatment (10μM, 24h). The cells were then incubated in the same medium with 1.0μCi/ml [^{3}H] Leucine for an additional 24h. After washing in ice-cold PBS, the cells were precipitated with 10% trichloroacetic acid for 30 minutes at 4°C. The precipitates were then solubilized in 0.4 N NaOH for 1h and neutralized with 1N HCl. Radioactivity was measured in a liquid scintillation counter. Each well was normalized to the total DNA content measured at 260nm.

Data Analysis

Data are expressed as mean ± SEM and analyzed using one-way or two-way ANOVA followed by Bonferonni's post-test where appropriate. Comparisons between two groups were performed using Student's *t*-test. P-values <0.05 are considered statistically significant.

Expanded Discussion

The Loss of ERK5 in Cardiomyocytes Does Not Have a Primary Influence on Hypertrophy-Induced Angiogenesis

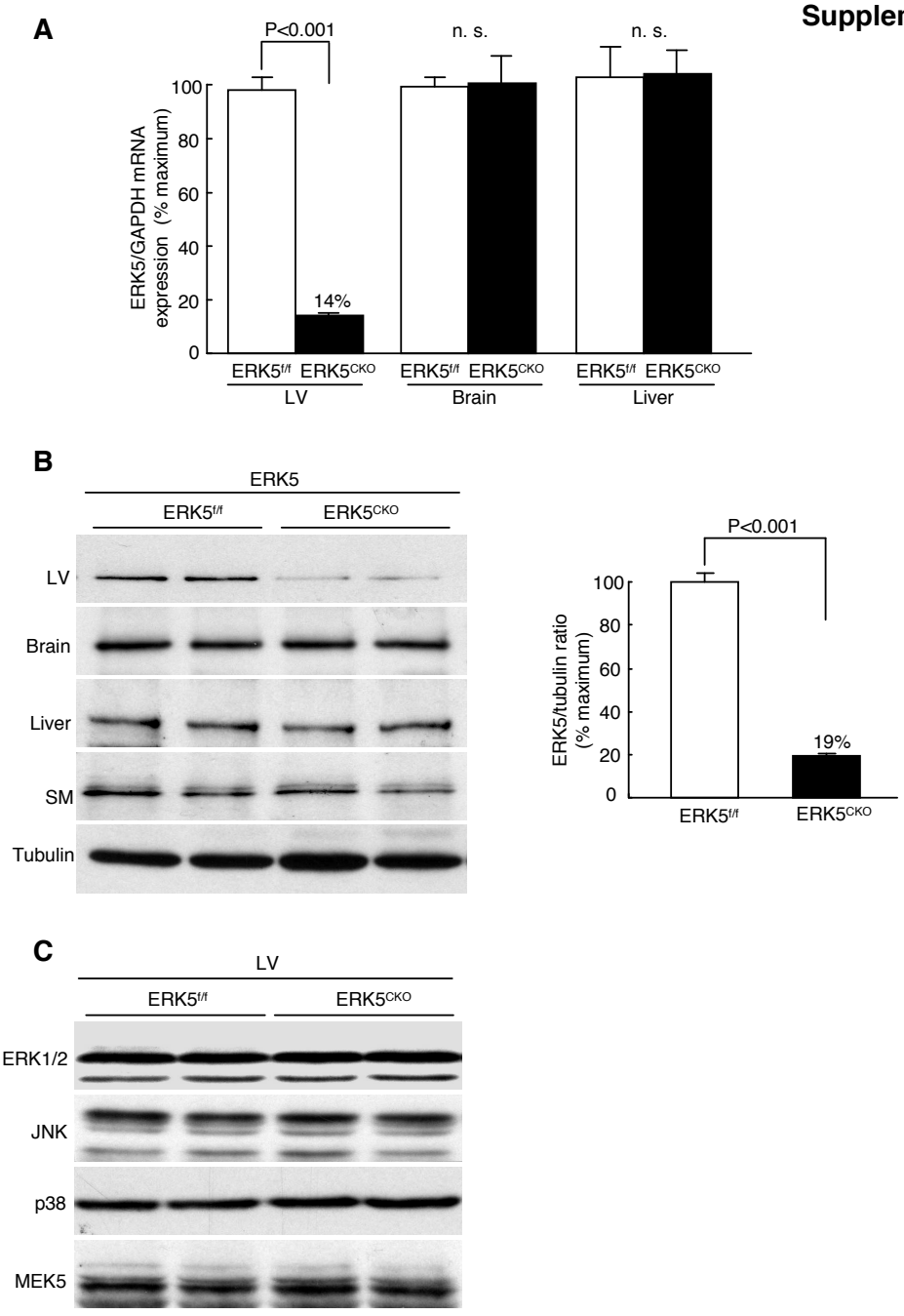
Enhanced angiogenesis under pressure overload is a critical feature of adaptive hypertrophic growth. Sustained stress eventually causes deregulation of microvascularization and insufficient oxygenation which contribute to the pathogenesis of heart failure[6]. ERK5 is reported to be a negative regulator of angiogenesis[7, 8]. During embryonic development, deficiency of ERK5 results in upregulated hypoxia inducible factor 1α (HIF-1α) activity and increased expression of the vascular endothelial growth factor (VEGF) which impedes the angiogenesis process and vascular maturation[8]. Interestingly, a recent study reported that cardiomyocyte-specific deletion of HIF-1α caused significantly less microvessels and impaired cardiac function after TAC[6]. Taking these findings into account, we assessed capillary density in TAC-treated ERK5^{cko} hearts. No difference in the capillary-to-cardiomyocyte ratio was found in the two genotypes (data not shown); suggesting the lack of ERK5 in cardiomyocytes does not have a primary influence on capillary density. However, whether ERK5 expressed in nonmyocytes is involved in regulating capillary density in the process of hypertrophic remodeling needs further study.

References

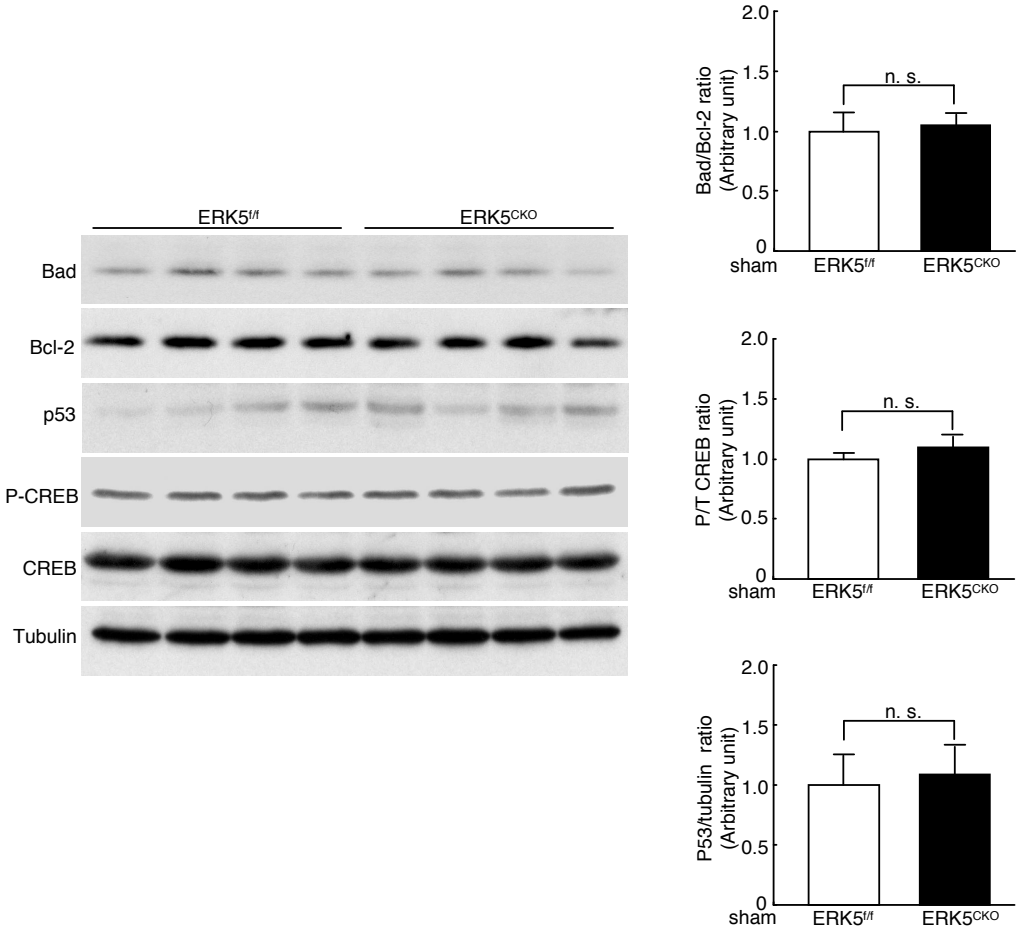
1. Ananieva O, Macdonald A, Wang X, McCoy CE, McIlrath J, Tournier C, Arthur JSC. ERK5 regulation in naïve T-cell activation and survival. *Eur J Immunol*. 2008;38:2534-2547.
2. Liu W, Zi M, Jin JW, Prehar S, Oceandy D, Kimura TE, Lei M, Neyses L, Weston AH, Cartwright EJ, Wang X. Cardiac-Specific Deletion of *Mkk4* Reveals Its Role in Pathological Hypertrophic Remodeling, but Not in Physiological Cardiac Growth. *Circ Res*. 2009;104:905-914.
3. Wang X, Merritt AJ, Seyfried J, Guo C, Papadakis ES, Finegan KG, Kayahara M, Dixon J, Boot-Handford RP, Cartwright EJ, Mayer U, Tournier C. Targeted deletion of *mek5* causes early embryonic death and defects in the extracellular signal-regulated kinase 5/myocyte enhancer factor 2 cell survival pathway. *Mol Cell Biol*. 2005;25:336-345.
4. Wang X, Fiegan KG, Robinson AC, Knowles L, Khosravi-Far R, Hinchliffe KA, Boot-Handford RP, Tournier C. Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress. *Cell Death Differ*. 2006;13:2099-2108.
5. Livak KL, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*. 2001;25:402-408.
6. Sano M, Minamino T, Toko H, Miyauchi H, Orimo M, Qin Y, Akazawa H, Tateno K, Kayama Y, Harada M, Shimizu I, Asahar T, Hamada H, Tomita S, Molkentin JD, Zou Y, Komuro I. P53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. *Nature*. 2007;446:444-448.

7. Pi X, Garin G, Xie L, Zheng Q, Wei H, Abe J, Yan C, Berk BC. BMK1/ERK5 is a novel regulator of angiogenesis by destabilizing hypoxia inducible factor 1α. *Circ Res.* 2005;96:1145-1151.
8. Sohn SJ, Sarvis BK, Cado D, Winoto A. ERK5 MAPK regulates embryonic angiogenesis and acts as a hypoxia-sensitive repressor of vascular endothelial growth factor expression. *J Biol Chem.* 2002;277:43344-43351.

Supplement Fig. I

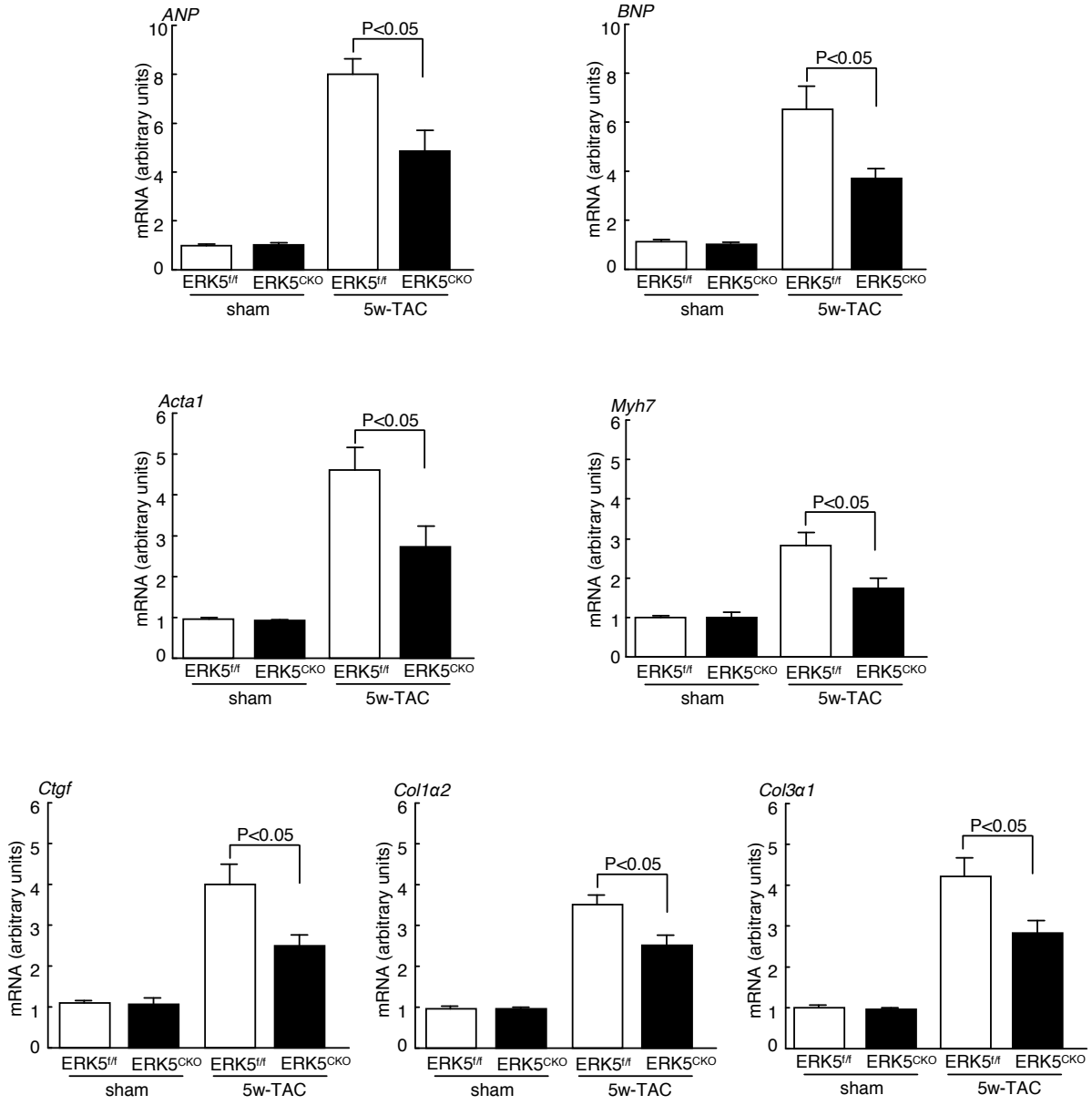


Supplement Figure I. Characterization of cardiac-specific deletion of ERK5. (A) Quantitative RT-PCR analysis of the mRNA levels of ERK5 in the left ventricle (LV), brain and liver, demonstrating a 86% decrease in ERK5 mRNA in LV. (B) Western blot analyses to determine specificity of the deletion of ERK5 in the left ventricle compared to protein extracts from various tissues, tubulin expression served as the protein loading control. The ratio of ERK5 expression to tubulin is shown in the bar graph. Data are presented as mean ± SEM, n=3 for each group. (C) Immunoblot analyses show similar expression levels of ERK1/2, JNK, p38 MAPK and MEK5 in the two genotypes. n.s.: no difference found between two groups.



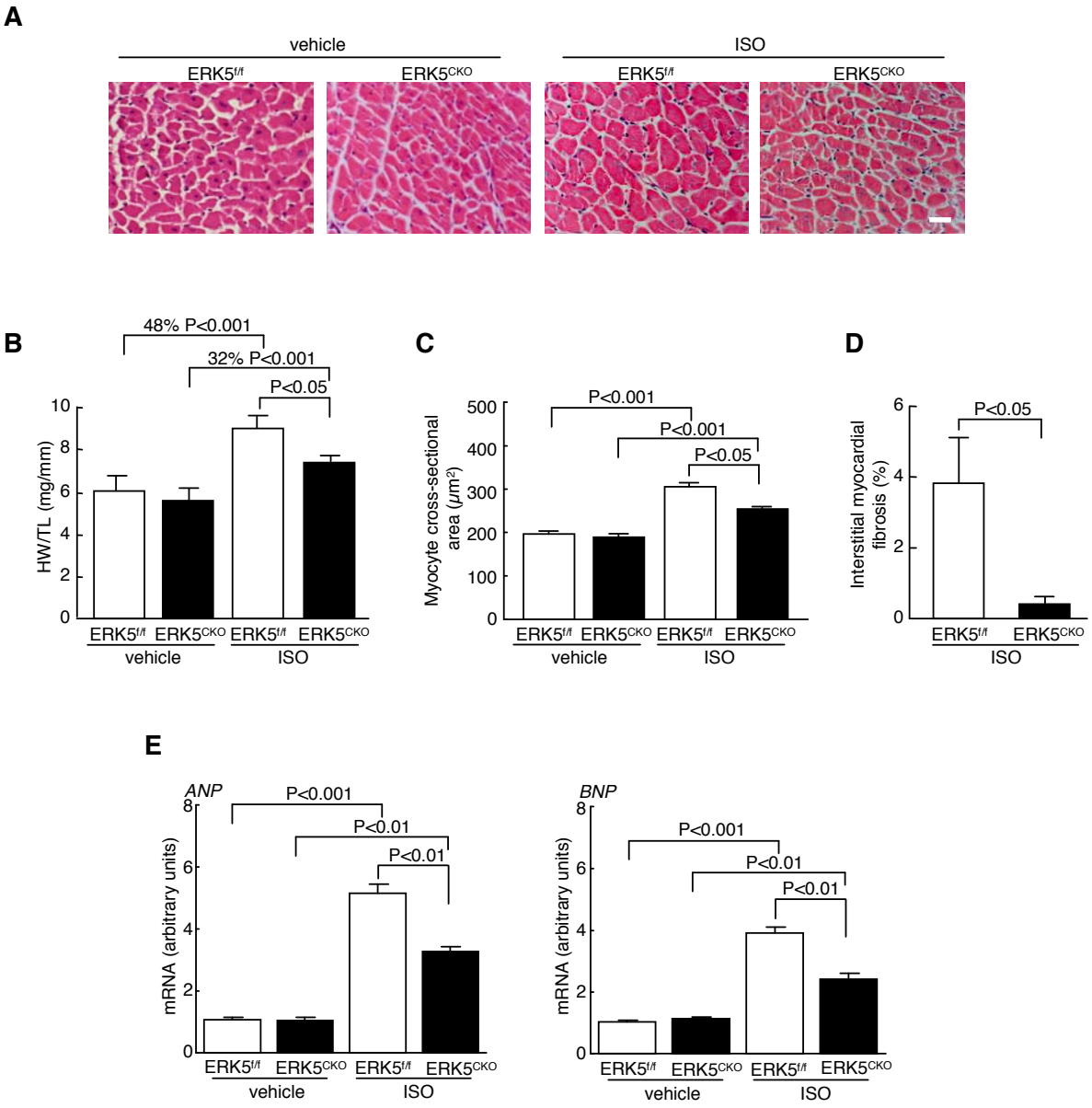
Supplement Figure II. Immunoblot analyses of protein levels of Bad, Bcl-2, p53, CREB, and phosphorylation level of CREB in the two sham groups. Tubulin expression is the protein loading control. The ratios of Bad/Bcl-2, p53/tubulin and P/T CREB are represented by the bar graphs, n=4 per group. Data are presented as mean ± SEM, n.s.: no difference found between two groups

Supplement Fig. III



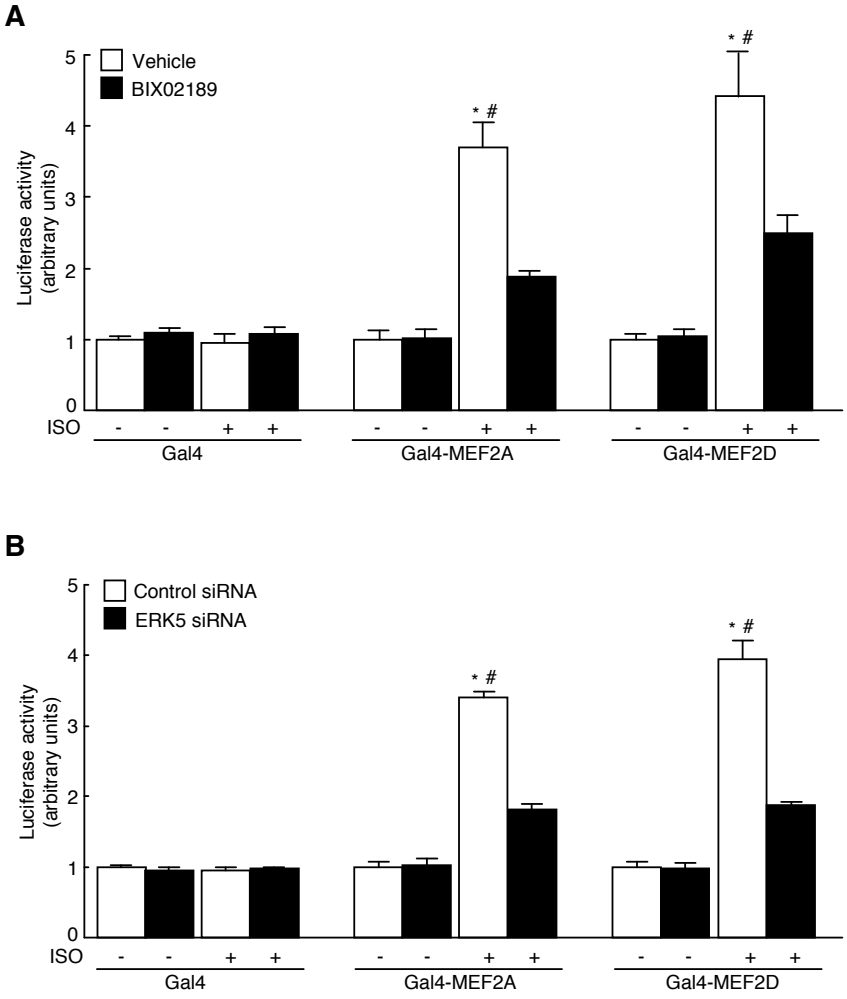
Supplement Figure III. Quantitative real-time PCR analyses of gene markers associated with hypertrophy and fibrosis after 5 weeks of TAC. The data are derived from three independent experiments performed in triplicate and are normalized to the GAPDH content, n= 5 per group. Data are presented as mean ± SEM.

Supplement Fig. IV

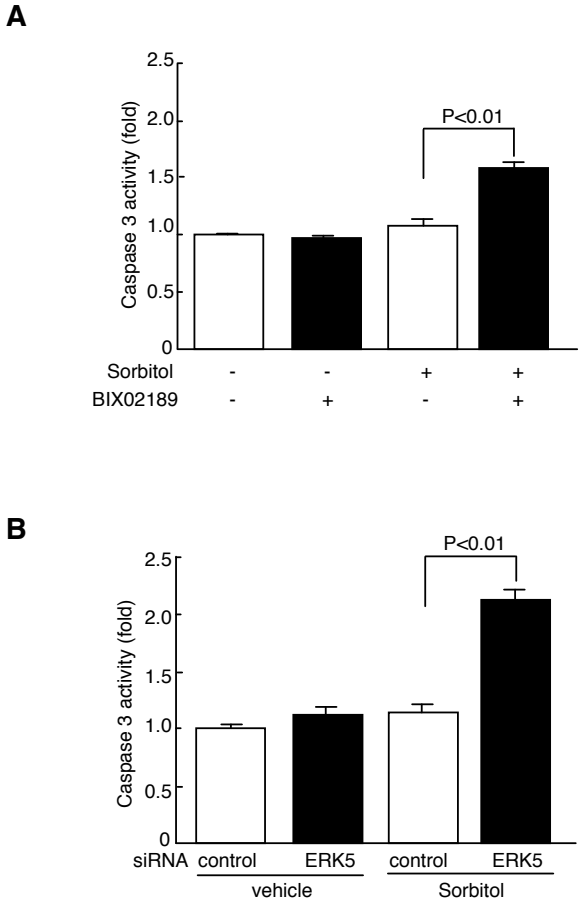


Supplement Figure IV. The loss of ERK5 attenuated isoproterenol induced-cardiac hypertrophy. (A) ERK5^{cko} mice showed blunted cardiac hypertrophy following isoproterenol infusion (scale bar: 20μm). (B) HW/TL ratios of ERK5$^{f/f}$ and ERK5^{cko} mice were evaluated after 7-day isoproterenol treatment, n=4 to 9 per group. (C) Mean cross-sectional areas of cardiomyocytes in ERK5$^{f/f}$ and ERK5^{cko} mice were calculated, n=4. (D) Ventricular interstitial fibrosis was minimal in ERK5^{cko} hearts detected by Masson's trichrome-staining of cross-sections. Quantification of the relative fibrotic area is expressed as percentage of the area of fibrosis in the microscope views, n=4. (E) Quantitative real-time PCR analyses of hypertrophic gene markers, *ANP* and *BNP*. The data are derived from three independent experiments performed in triplicate and are normalized to the GAPDH content, n=3. Data are presented as mean ± SEM.

Supplement Fig. V



Supplement Figure V. The ERK5 knockdown or the inhibition of its kinase activation suppressed MEF2 transcriptional activity in NRCMs. (A) NRCMs were transfected with the reporter plasmid pG5E1bLuc together with constructs encoding Gal4-MEF2A, or Gal4-MEF2D, followed by BIX02189 treatment. After 2h inhibitor treatment, MEF2 transcriptional activity was measured by the dual-luciferase reporter assay system before and after ISO stimulation. A control experiment was performed with pG5E1bLuc and Gal4. As expected, both Gal4-MEF2A and Gal4-MEF2D activities were reduced in BIX02189-treated NRCMs, indicating diminished MEF2 transcriptional activity. (B) siRNA-treated NRCMs were co-transfected with various plasmid vectors as indicated. MEF2 transcriptional activity was measured by the dual-luciferase reporter assay system following ISO stimulation. Firefly luciferase activity was normalized to that of *Renilla* luciferase. The data are derived from three independent experiments performed in duplicate, n=6. Data are presented as mean ± SEM. *P<0.05 versus vehicle alone (A) or control siRNA alone (B); #P<0.05 versus BIX02189 treated + ISO (A) or ERK5 siRNA treated + ISO (B).



Supplement Figure VI. The ERK5 knockdown or the inhibition of its kinase activation sensitizes NRCMs to apoptosis. BIX02189-treated NRCMs (A); or siERK5-treated NRCMs (B) were stimulated with sorbitol for 4h. Following stimulation, caspase 3 activity was measured by caspase activity assay, n=6. Data are presented as mean ± SEM.