Supplemental Data

Materials and Methods

Generation of ERK2 cardiomyocyte-specific knockout mice

To exclusively study ERK2 signaling in hypertrophic remodeling the Cre-loxP system was used to generate a cardiomyocyte-specific ERK2 knockout mouse model. For development of a conditional knockout mouse, two different mouse lines were generated. The first line contained a transgene that led to a cardiomyocyte-specific expression of Cre under the myosin light chain (MLC2v) promoter (kindly provided by Dr KR Chien, Massachusetts General Hospital, USA). The MLC2v-Cre mouse line sustains efficient ventricular-specific Cre activity and is indistinguishable from wild-type mice in cardiac morphology and stress response [1]. The second mouse line ERK2-flox mice (referred to as $ERK2^{f/f}$) carried two loxP sites flanking exons 2 and 3 of ERK2 gene (kindly provided by Dr S Endo, Institute of Science and Technology, Okinawa, Japan). The two loxP sites were inserted in introns or non-transcription regions. Both mouse models were generated by homologous recombination in embryonic cells.

Transverse aortic constriction (TAC)

The 8-10 weeks old mice were anesthetized with a mixture of ketamine (100mg/kg) and xylazine (5mg/kg) by intraperitoneal injection and endotracheal intubated. TAC was performed as previously described [2]. Briefly, the transverse aorta was isolated and constricted by a 7-0 nylon suture ligature, tied to yield a constriction. Sham mice were used as reference control and underwent the same surgery in which the aorta was only visualized but not constricted. After surgery, buprenorphine (0.1mg/kg) and saline (0.1ml/10g) were intraperitoneally injected and mice were allowed to recover. After 1 or 5 weeks of treatment the mice were further analyzed.

Osmotic pump infusion of Isoproterenol

To achieve β-adrenergic-induced hypertrophy 8 to 10 weeks old male mice were anesthetized with 1.5% isoflurane and ALZET minipumps (model 2001) were inserted subcutaneously to deliver Isoproterenol (ISO, Sigma-Aldrich) at 10mg/kg/day or vehicle (Water for Injection, Braun). Following 1 week of treatment, mice were further analyzed.

Swimming exercise

Physiological cardiac hypertrophy was induced by swimming exercise. Eight-week old male $ERK2^{f/f}$ and $ERK2^{cko}$ mice swam twice a day for up to 90 minutes in pre-warmed water, maintained at 34°C. The mice were supervised throughout the exercise to ensure their safety. After a total of 4 weeks of swimming, the cardiac function was determined by echocardiography and mice were sacrificed for further analysis. Sedentary, 8 week old mice of both genotypes were confined to cages for 4 weeks without following the swimming protocol and served as a reference control (rest).

Echocardiography

Mice were anesthetized with Avertin (240mg/kg). The cardiac function was evaluated by echocardiography using an Acuson Sequoia C256 ultrasound machine. For each mouse, measurements of the left ventricle end-systolic (LVESD) and left ventricle end-diastolic (LVEDD) dimensions, intraventricular septum thickness (dIVS) and left ventricular posterior wall thickness (dPW) were obtained. From these parameters LV fraction shortening (FS) was calculated using the following formula: $FS = \frac{LVEDD - LVESD}{LEVDD} \cdot 100$ [3].

Histological analysis and TUNEL assay

Freshly dissected heart tissue was fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. 5µm thick sections were cut and stained with Haematoxylin/eosin staining to measure cross sectional area, and stained using the Masson's trichrome method for examining fibrosis formation as previously described [4]. The mean cross-sectional areas of 200 randomly selected cardiomyocytes were measured by ImageJ software. To calculate the mean interstitial fibrosis 45 randomly chosen frames of each experimental group were assessed using ImageJ software.

TUNEL was performed to detect apoptosis on 5µm thick paraffin embedded heart tissue sections using *in situ* Cell Death Detection Kit (Roche Applied Science). Triple staining with TUNEL, DAPI (1:1000) and anti-α-actinin antibody (1:20, Sigma, A7811) was performed to identify apoptosis exclusively in cardiomyocytes.

Preparation of lysates and immunoblotting

Tissue samples were homogenised in triton lysis buffer (20mM Tris pH 7.4, 137mM NaCl, 2mM EDTA pH 7.4, 1% Triton X-100, 25mM b-glycerophosphate, 1mM Na_3VO_4, 1mM phenylmethanesulphonylfluoride (PMSF), 1.54μM Aprotinin, 21.6μM Leupeptin, 10% Glycerol). The protein concentration was determined by Bradford assay (Bio-Rad). Protein extracts (30µg) were subjected to Western blot analyses with antibodies against ERK1 (Santa Cruz), ERK2 (Santa Cruz), ERK5 (Upstate), Phospho-ERK5 (Invitrogen), p38 (Santa Cruz), Phospho-ERK1/2, MEK1/2, Phospho-MEK1/2, Phospho-p38, JNK1/2, Phospho-JNK1/2, PKB, Phospho-PKB, active caspase 3 (Cell signaling) or α-Tubulin (Sigma-Aldrich). Immunecomplexes were detected by enhanced chemiluminescence with anti-mouse, anti-rabbit or anti-goat immunoglobulin G-coupled to horseradish peroxidase as the secondary antibody (Amersham-Pharmacia).

ERK1/2 kinase assay

Tissue samples were homogenised in triton lysis buffer. Protein extracts were applied for ERK1/2 kinase assay following the manufacturer's instruction (Cell signaling).

siRNA transfection of neonatal rat cardiomyocytes (NRCMs)

Primary cultures of neonatal rat cardiomyocytes (NRCMs) were prepared as previously described [5]. NRCMs were transfected with either control siRNA (Sigma-Aldrich) or rat ERK2 siRNA (gene ID #116590; si genome SMART pool, Dharmacon) using Lipofectamine Plus reagent accourding to the manufacturer's instruction (Invitrogen) for 48 hours.

For detection of apoptosis *in vitro* NRCMs were plated onto laminin-coated coverslips and infected with control or ERK2 siRNA followed by treatment with 100μM H_2O_2 for 8 hours. The cells were fixed with 4% PFA and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate. Apoptosis was detected with *in situ* Cell Death Detection Kit® (Roche). The cells were counterstained with DAPI to detect the nuclei and analysed by using ImageJ software.

Immunocytochemistry

To evaluate the hypertrophic response, NRCMs were plated onto laminin-coated coverslips and transfected with either control or ERK2 siRNA in 1% serum-medium for 24 hours, followed by further 48 hours treatment with PE (30mM, Sigma-Aldrich). Thereafter, NRCMs underwent triple immunostaining using anti-ANP antibody (1:500, Peninsula Laboratories, T4015) and anti-α-actinin antibody (1:100, Sigma-Aldrich, A7811) detected by secondary anti-rabbit antibody conjugated to Alexa Fluor 568 and anti-mouse antibody conjugated to FITC (Invitrogen), in addition, nuclei were stained with DAPI (Invitrogen). With ImageJ software the surface area of 150 cells per group was measured.

Luciferase reporter assay

To analyse whether dominant negative overexpression of ERK2 affects BNP promoter activity, NRCMs transfected with either control or ERK2 siRNA were infected with recombinant adenovirus encoding BNP-luciferase reporter gene (Ad-BNP-Luc) at MOI 25 for 24 hours followed by 24 hours treatment of PE (30μM). BNP luciferase activity was analysed using luciferase assay kit (Promega).

Quantitative real-time PCR

Total RNA from heart ventricle tissues were prepared using TRIzol (TRI reagent, Invitrogen) followed by synthesis of cDNA. Real time quantitative PCRs were performed by using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations. Primers to identify ERK2, ANP, BNP, Collα2, Col3α1, Ctgf and GAPDH were obtained from Qiagen. Thermal cycling was performed in the 7500 Real Time PCR System (Applied Biosystems). The fold change was determined using the comparative threshold method ($2^{-\Delta\Delta CT}$) [6]. The reporter signal was normalised to reactions performed using GAPDH.

References

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[3] Gardin JM, Siri FM, Kitsis RN, Edwards JG, Leinwand LA. Echocardiographic assessment of left ventricular mass and systolic function in mice. Circulation research. 1995;76:907-14.

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Supplement Table I: Echocardiographic assessment of mice following swimming exercise.

	rest		swimming	
	ERK2$^{f/f}$	ERK2^{cko}	ERK2$^{f/f}$	ERK2^{cko}
dPW (mm)	0.76±0.06	0.68±0.07	0.71±0.07	0.75±0.06
dIVS (mm)	0.82±0.09	0.91±0.09	0.85±0.03	0.83±0.03
LVEDD (mm)	3.84±0.23	3.77±0.23	3.49±0.13	3.61±0.11
LVESD (mm)	2.67±0.27	2.64±0.22	2.24±0.13	2.41±0.08
FS (%)	31.53±3.92	30.77±2.55	30.77±2.55	33.15±1.69

dPW, end-diastolic left ventricular posterior wall thickness; dIVS, end-diastolic interventricular wall thickness; LVEDD, diastolic left ventricular dimensions; LVESD, systolic left ventricular dimensions; FS%, fraction shortening. Data presented as mean±SEM (n= 6 to 8 per group).

Supplement Figure I

A

rest

swimming

ERK2$^{f/f}$ ERK2^{cko} ERK2$^{f/f}$ ERK2^{cko}

B

p<0.05 n.s.

Cardiomyocyte cross-sectional area (μm^2)

300 250 200 150 100 50 0

ERK2$^{f/f}$ ERK2^{cko} ERK2$^{f/f}$ ERK2^{cko}

rest swimming

C

ANP

n.s.

mRNA (arbibary units)

1.2 1.0 0.8 0.6 0.4 0.2 0

ERK2$^{f/f}$ ERK2^{cko} ERK2$^{f/f}$ ERK2^{cko}

rest swimming

BNP

n.s.

mRNA (abritrary units)

1.4 1.2 1.0 0.8 0.6 0.4 0.2 0

ERK2$^{f/f}$ ERK2^{cko} ERK2$^{f/f}$ ERK2^{cko}

rest swimming

D