SUPPLEMENTAL MATERIAL

Tumor Necrosis Factor Receptor Associated Factor 2 Signaling Provokes Adverse Cardiac Remodeling in the Adult Mammalian Heart

Methods/Results

Characterization of Founder Lines of MHC-TRAF2 Mice

The TRAF2 cDNA used for to generate the MHC-TRAF2construct was the generous gift of Dr. Yongwon Choi.[1] To facilitate separation of endogenousTRAF2 from the TRAF2 that was driven by the alpha-myosin heavy chain promoter, we FLAG-tagged the TRAF-2 construct using an EcoRV cleavage site with the primer sequence: CTAGGA TAT CCG TTT AGT GAA CCG TCA GAA TTG ATC TAC CAT GGA CTA CAA AGACGA TGA CGA CAA GGC TGC AGC CAG TGT GAC. The FLAG-tag was inserted at theN-terminus using a NotI cleavage site with the primer sequence: ACG TGC GGC CGC CTAGAG TCC TGT TAG GTC. The transgene construct was injected into single cell embryos of FVB mice at the Baylor College of Medicine transgenic core facility. Founder lines of MHC-TRAF2 transgenic mice (FVB background) were identified byPCR and confirmed by Southern blotting using a 286 bp probe targeted to exon 8 of the TRAF2 gene (supplemental Figure 1A). Western blotting was performed to confirm the expression of TRAF2 protein in the heartsof 12 week MHC-$TRAF_{HC}$ mouse hearts (supplemental Figure 1B) using an anti-TRAF2 antibody (sc-877, Santa Cruz Biotechnology, Santa Cruz, CA). Immunohistochemical staining of founder lines showed that TRAF2 protein was localized diffusely throughout the cytoplasm in MHC-TRAF2 mouse hearts compared to littermate control mice (supplemental Figure 1C). We obtained three founder lines: 329W (7 copies of TRAF2 transgene); 330W (10 copies of transgene); 335W (24 copies of transgene).

Supplemental Table 1 displays the morphometrics of the MHC-TRAF2 founder lines of mice. The 329W line, with the lowest copy of the transgene, referred to as MHC-$TRAF2_{LC,}$ has been characterized in detail and reported previously.[2] Mice with 10 and 24 copies of the TRAF2 transgene developed an increased heart-weight-to-body weight ratio and a dilated cardiac phenotype by 12 weeks of age. Lines of mice that

expressed 24 copies of the transgene (referred to here as MHC-TRAF2$_{HC}$ mice) were found to express ~ two-fold higher levels of TRAF2 protein compared to mice that expressed 7 or 10 copies by Western blot analysis (supplemental Figure 1B). Mice expressing 24 copies of the TRAF2 transgene (referred to here as MHC-TRAF2$_{HC}$) were selected for further study.

LV Structure and Function. LV structure and function were assessed using 2D directed M-mode echocardiography as described previously.[3] Anesthesia was induced using 1% isoflurane, and was maintained using 0.5% isoflurane. The heart rate was maintained at > 400 beats per minute by adjusting the level of anesthesia. All echocardiographic measurements were performed by an investigator who was blinded to the experimental group. LV contractility of 12 week MHC-TRAF2$_{HC}$ and LM mouse hearts was measured ex-vivo using a buffer perfused Langendorff apparatus, as described.[4] All hearts were paced at 420 beats per minute with pacing electrodes placed on the right atrium. Functional data were recorded at 1 KHz on a data acquisition system (Power Lab, AD Instruments, Colorado Spring, CO), and left ventricular +dP/d*t* and –dP/d*t* were determined.

Extracellular Matrix. Deparaffinized sections of perfusion fixed hearts from 4, 8 and 12 week old MHC-TRAF2$_{HC}$ and littermate control mice were stained using the picrosirius red technique, as described (see data supplement for details).[5] The sections were imaged using polarized light microscopy, and the percent area of extracellular picrosirius red staining was computed using commercially available software (Metavue, Visitron systems, Germany) by examining 8 random fields within the mid-myocardium in order to exclude large epicardial arteries and/or veins, as well as any cutting and/or compression artifacts. MMP activity from 4, 8 and 12 week old MHC-TRAF2$_{HC}$ and LM control mouse hearts were obtained using gelatin zymography, as previously described (see data supplement for details).[6] The intensity of the zymographic bands was quantified by image analysis software (Image-J, NIH). Levels of TIMP-1 were measured in myocardial extracts from MHC-TRAF2$_{HC}$ mice and littermate controls at 4, 8 and 12 weeks of by ELISA (Amersham RPN 2611), according to manufacturer's recommendations. [5]

References

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Supplemental Table 1
Characterization of founder lines of mice with targeted overexpression of TRAF2

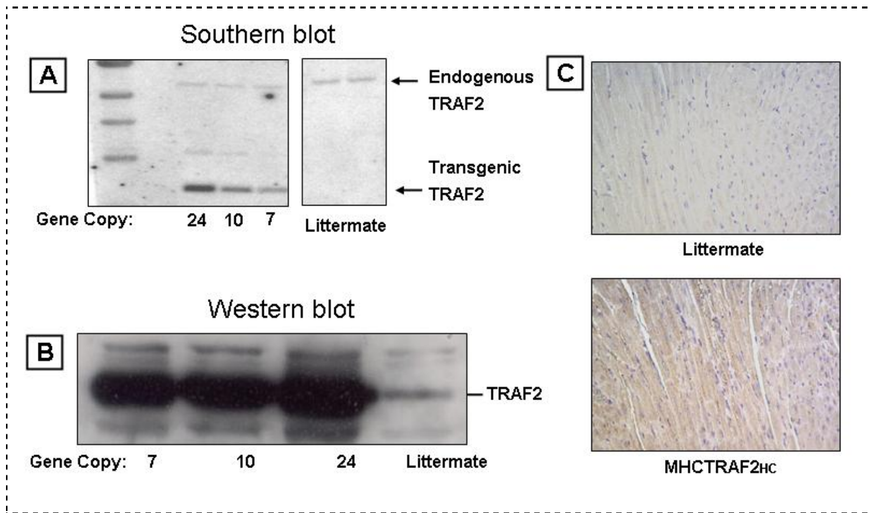
Mice	Copy #	HW (mg)	BW (g)	HW/BW (mg/g)	Cardiac Phenotype
MHC-TRAF2$_{LC}$	7	132.0±7.3*	28.9±1.6	4.6±0.2*	Hypertrophy
LM	-	120.7±4.4	30.1±1.5	4.0±0.1	
MHC-TRAF2$_{IC}$	10	184.3±0.01*	25.7±1.2	7.2±0.5*	Dilated
LM	-	117.7±0.01	27.9±2.6	4.2±0.1	
MHC-TRAF2$_{HC}$	24	130.5±5.5*	25.5±0.8	5.3±0.1*	Dilated
LM	-	112.9±5.4	24.1±0.8	4.5±0.1	

(Data are expressed as mean ± SEM. Necropsy data for heart weight (HW), body weight (BW), heart-weight-to body-weight- ratio (HW/BW) and phenotype of 12 week old mice with varying copies of the TRAF2 transgene and 12 week littermate (LM) controls. ($n \geq 5$ mice per group; * = $p < = 0.05$ vs. littermate control)

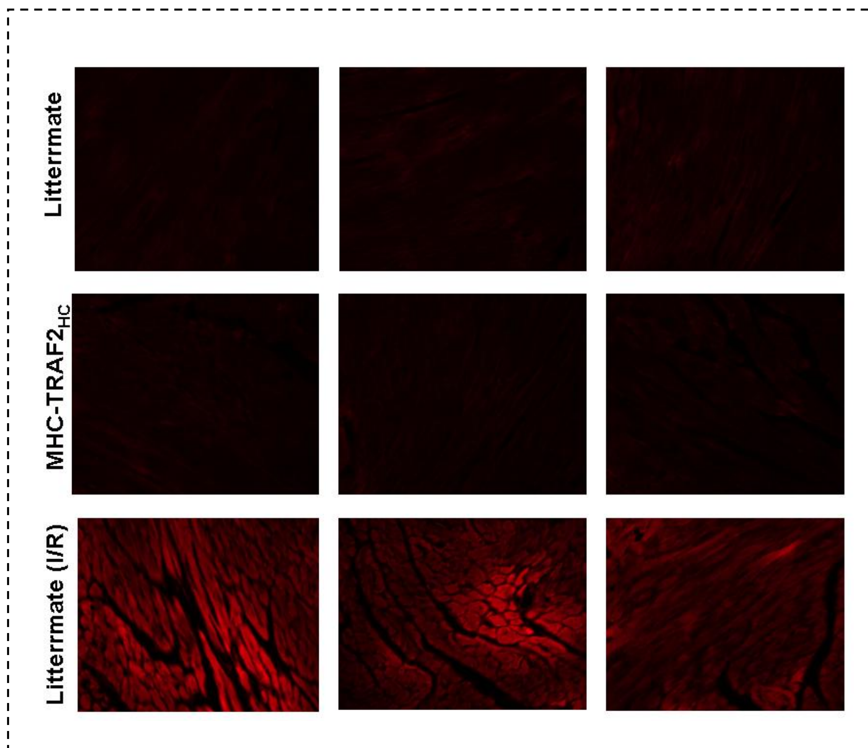
Supplemental Table 2: Changes in Gene Expression in MHC-TRAF2$_{HC}$ Mice

Symbol	Gene Name	Expression level (relative to LM)	p value
		Increased	
MYH7	myosin, heavy chain 7, cardiac muscle, beta	14.92	$7.61x10^{-05}$
TGFB3	transforming growth factor, beta 3	4.00	$9.82x10^{-06}$
TIMP1	Tissue inhibitor of metalloproteinase 1	3.39	$2.82x10^{-3}$
MMP23	Matrix metallopeptidase 23	2.88	$5.252x10^{-3}$
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	2.60	$8.49x10^{-04}$
TGFB2	transforming growth factor, beta 2	2.29	$3.88x10^{-02}$
DES	Desmin	2.22	$9.84x10^{-05}$
ITGA11	integrin, alpha 11	2.05	$2.53x10^{-02}$
PRKB2	protein kinase, AMP-activated, beta 2 non-catalytic subunit	1.81	$7.88x10^{-3}$
COL1A1	collagen, type I, alpha 1	1.77	$1.70x10^{-2}$
COL3A1	collagen, type III, alpha 1	1.77	$3.24x10^{-2}$
MMP14	matrix metallopeptidase 14	1.65	$2.96x10^{-2}$
MMO2	matrix metallopeptidase 2	1.60	$2.66x10^{-2}$
ITGB5	integrin, beta 5	1.51	$4.16x10^{-02}$
LMNA	lamin A/C	1.50	$9.95x10^{-04}$
ACTB	actin, beta	1.48	$7.37x10^{-03}$
TIMP2	Tissue inhibitor of metalloproteinase 2	1.38	$1.44x10^{-2}$
IGF1	insulin-like growth factor 1 (somatomedin C)	1.37	$1.77x10^{-03}$
MMP7	Matrix metallopeptidase 7	1.34	$6.34x10^{-3}$
MYBPC3	myosin binding protein C, cardiac	1.29	$2.93x10^{-02}$
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	1.28	$3.42x10^{-02}$
		Decreased	
SGCA	sarcoglycan, alpha (50kDa dystrophin-associated glycoprotein)	-1.94	$8.27x10^{-04}$
TNNI3	troponin I type 3 (cardiac)	-1.66	$2.39x10^{-03}$
ITGA7	integrin, alpha 7	-1.65	$6.78x10^{-03}$
SGCG	sarcoglycan, gamma (35kDa dystrophin-associated glycoprotein)	-1.62	$1.11x10^{-03}$
ADCY6	adenylate cyclase 6	-1.59	$2.05x10^{-03}$
MMP15	matrix metallopeptidase 15	-1.57	$1.43x10^{-3}$
PRKAB1	protein kinase, AMP-activated, beta 1 non-catalytic subunit	-1.54	$1.18x10^{-2}$
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	-1.47	$1.37x10^{-02}$
ITGB6	integrin, beta 6	-1.41	$3.48x10^{-03}$
TGFB1	transforming growth factor, beta 1	-1.33	$2.41x10^{-02}$
ADCY4	adenylate cyclase 4	-1.31	$3.09x10^{-03}$
MYH6	myosin, heavy chain 6, cardiac muscle, alpha	-1.26	$2.40x10^{-02}$
RYR2	ryanodine receptor 2 (cardiac)	-1.25	$2.84x10^{-02}$
CACNG4	calcium channel, voltage-dependent, gamma subunit 4	-1.24	$4.40x10^{-02}$

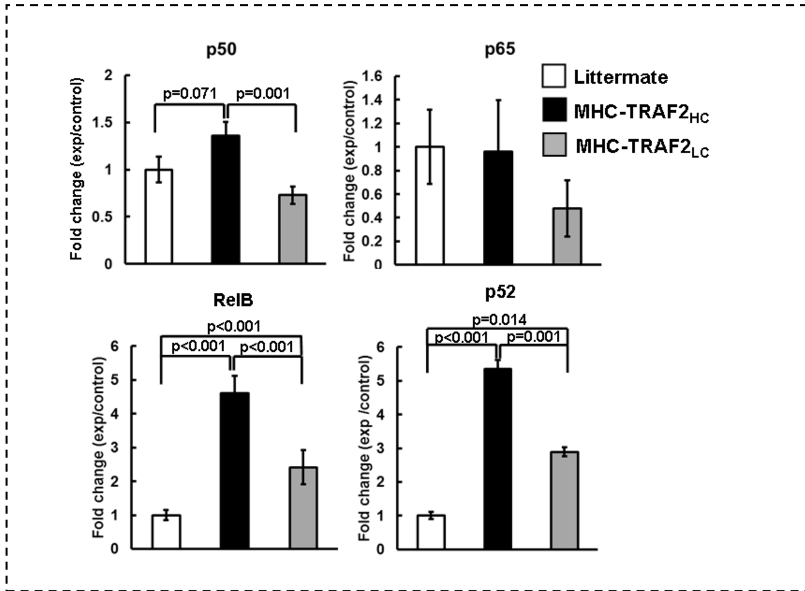
Changes in gene expression in MHC-TRAF2$_{HC}$ mice vs. LM control mice at 12 weeks of age identified by the KEGG functional pathway for cardiac hypertrophy/dilated cardiomyopathy were modified to include changes in extracellular matrix gene expression that have been linked to the development of dilated cardiomyopathy. Changes in gene expressed are displayed visually in Figure 6 in the manuscript.



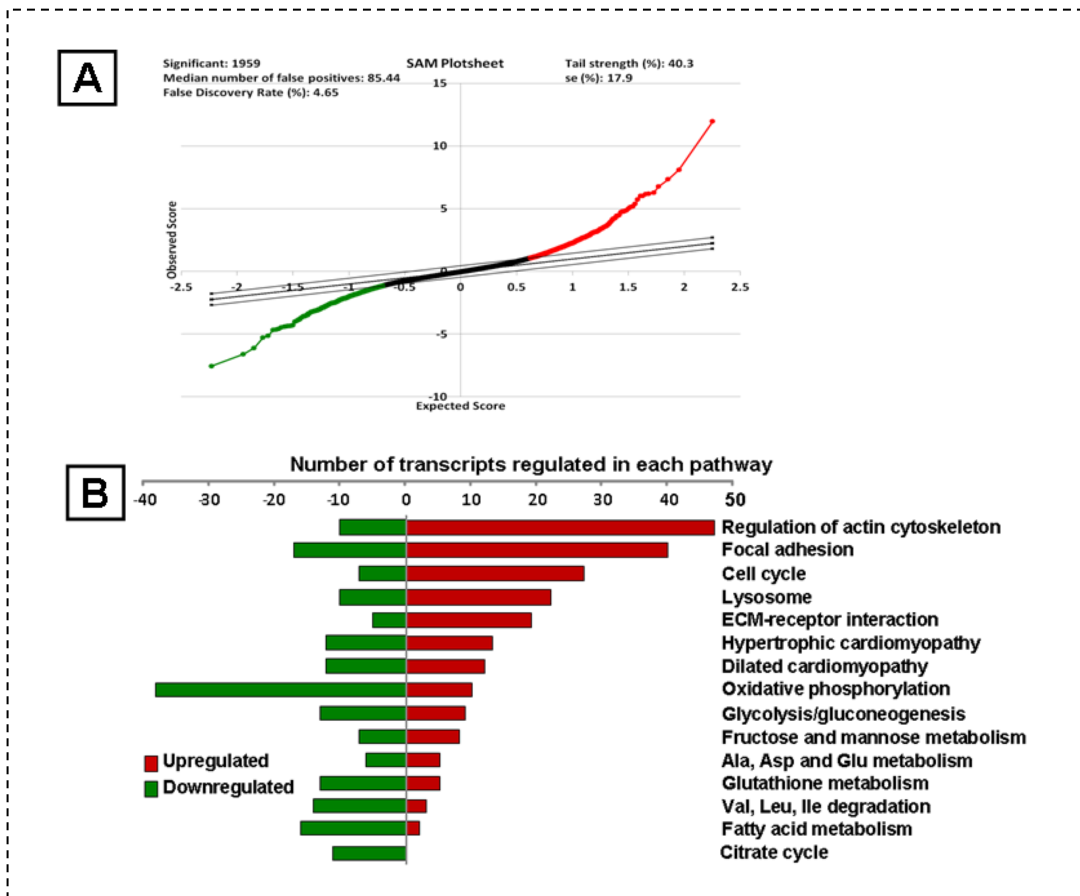
Supplemental Figure 1: Generation of transgenic mice overexpressing TRAF2 in the heart. (A) Representative southern blot showing three lines of mice overexpressing 7, 10 and 24 copies of the TRAF2 transgene in comparison to controls. (B) Western blot of TRAF2 protein expression in hearts of MHC-TRAF2 mice expressing 7, 10 and 24 copies of the TRAF2 transgene. (C) Representative immunohistochemical staining of littermate and MHC-TRAF2$_{HC}$ mouse hearts (100 x).



Supplemental Figure 2: Evans Blue dye uptake in littermate control and MHC-TRAF2$_{HC}$ mouse hearts. To determine the presence of absence of myocyte cell necrosis, 4 week littermate control and MHC-TRAF2$_{HC}$ mice were injected intraperitoneally with Evans Blue dye, the hearts excised, fixed and examined by fluorescence microscopy (200 x). As a positive control, littermate control mouse hearts were subjected to ischemia reperfusion injury ex vivo, and stained with Evan Blue dye as described.[2]



Supplemental Figure 3: NF-κB subunits. An NF-κB ELISA was performed on nuclear extracts from obtained from 12 week littermate control and MHC-TRAF2$_{HC}$ mouse hearts (n = 6-9 hearts/group).



Supplemental Figure 4: Transcriptional profiling. (A) SAM plot of changes in gene expression in the 12 week MHC-TRAF2$_{HC}$ mouse hearts compared to LM controls (n = 4 hearts/group). Genes that were significantly increased are denoted by red circles; genes that were significantly decreased are denoted by green circles. A false discovery rate (FDR) less than 5% was used for the SAM plots. (B) KEGG analysis of functional pathways that were significantly different in the MHC-TRAF2$_{HC}$ compared to LM control hearts at 12 weeks of age.