DATA SUPPLEMENT FOR "THE DEVELOPMENT OF MYOCARDIAL FIBROSIS IN TRANSGENIC MICE WITH TARGETED OVEREXPRESSION OF TUMOR NECROSIS FACTOR REQUIRES MAST CELL-FIBROBLAST INTERACTIONS"

METHODS

Cell Culture

Cardiac fibroblasts. Primary cultures of mouse cardiac fibroblasts were prepared from 12 week old LM and MHCsTNF mice using a modification of previously published methods.[1] Briefly, hearts were removed under aseptic conditions and the ventricles of the hearts minced and digested at 37°C in Hanks' Balanced Salt Solution (HBSS with Ca and Mg) containing 10μmol/L HEPES, 2.78% Liberase Blendzyme 4 (Roche, Indianapolis, IN), and 0.1% trypsin (without EDTA). At the end of each cycle, the supernatant was collected and stored on ice after adding Krebs-Henseleit Buffer Modified (KHB, Sigma). The dissociated cells were collected and resuspended in fibroblast growth medium comprised of Dulbecco's Modified Eagle's Medium (DMEM high glucose 4.5g/L) supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin. The cells were plated into tissue culture flasks and incubated at 37°C in a humidified atmosphere (5% CO_2) for 3- to 4-hours in order to allow the fibroblasts to attach to the flasks. Subsequently, the medium, containing the cardiac myocytes, was aspirated and discarded. The highly enriched cultures of fibroblasts were split after confluence. Because fibroblast phenotype is influenced by growth conditions such as cell passage and cell density,[2, 3] only cells from passage 2 were used for these studies.

Mast cell and cardiac fibroblast co-culture. For the 3-D gel contraction assays,[4] cardiac fibroblasts from LM or MHCsTNF hearts were harvested and resuspended in DMEM. Rat tail type I collagen was then added to a final gel concentration of 1.5mg/ml. The number of fibroblasts in the final mixture was held constant at 1×10^5 cells per milliliter. In parallel studies, mast cells were added to the collagen gels alone, or along with the fibroblasts at a ratio

(fibroblasts: mast cells) of 1:0, 1:1 or 1:10. The resulting admixture of cells and rat tail collagen (total volume 1/ml) was incubated at 37°C for 1h in order to allow the collagen to polymerize. After collagen polymerization, the circumferential edge of the collagen gel was gently freed from the sides of the culture well to permit the gels to float freely in the culture media, and 1 ml of mast cell growth medium was added to each well. The co-cultures were treated with diluent, NP-40208 (1μM), losartan (100μM), EGTA (5mM), verapamil (50μM), and cromolyn (100μM). The collagen gels were incubated at 37°C for 24 hours. Images of the collagen gels were obtained at 24 hours using an Alpha Innotech gel capture system, and the surface area of the collagen gel was determined as a measure of the degree of gel contraction. Final results were expressed as a percent of gel area at baseline.

Mast cell conditioned medium. Mast cell conditioned medium was also used to stimulate fibroblasts from LM and MHCsTNF mouse hearts. Briefly, mast cells were maintained in mast cell growth medium for 24 hours, and the cells were removed through centrifugation and the mast cell conditional medium was used for fibroblast stimulation. Fibroblasts were stimulated with mast cell conditioned medium for 48 or 72 hours and then examined for α-SMA, proliferation and collagen gene expression.

Characterization of Isolated Cardiac Fibroblasts

Fibroblast activation was measured as: (1) the mean fluorescence intensity (MFI) of fibroblasts with α-SMA staining, (2) fibroblast proliferation, measured as BrdU incorporation, and (3) fibroblast production of extracellular matrix components, measured as collagen 1A1, 1A2 and 3A1 mRNA levels.[5]

a-SMA expression. Flow cytometry was used to determine the degree of α-SMA staining in cardiac fibroblasts isolated from LM and MHCsTNF mouse hearts. Briefly, fibroblasts were seeded at 50-60 percentage of confluence in 100mm cell culture dishes in cardiac fibroblast growth medium for 24 hours. Cardiac fibroblasts were treated with diluent, TNF (200U/ml) or

TGF-β1 (2ng/ml) for an additional 48 hours, and removed from the culture dishes. The cells were fixed in 2% paraformaldehyde for 10 min and permeabilized with 0.02% Triton-X for 5 min on ice. The cells were washed in phosphate buffered saline (PBS) and resuspended in 300 µl 0.02% Triton-X containing FITC conjugate anti-mouse α-SMA (final antibody concentration of 10µg/ml, Sigma-Aldrich, St. Louis, MO) and were incubated on ice for 60 min. An isotype-matched IgG2a mouse FITC conjugate was used as the appropriate control (Sigma-Aldrich, St. Louis, MO). After being washed, cell preparations were resuspended in 2% paraformaldehyde (500 µl) and were immediately analyzed by a flow cytometry (FACScalibur, Becton-Dickinson). Each measurement was conducted on 20,000 events using CellQuest software (Becton Dickinson). The extent α-SMA staining in the fibroblast cultures was expressed as MFI.

Fibroblast proliferation. Cardiac fibroblast proliferation was determined using the colorimetric Cell Proliferation ELISA, BrdU kit (Roche, Indianapolis, IN), as described.[6] We have shown previously that there is a linear correlation between BrdU incorporation and cell number obtained by direct manual counting of cells.[7] Cardiac fibroblasts from LM or MHCsTNF mice were seeded at a final concentration of 5000 cells/well in 96 well plates in fibroblast growth medium for 24 hours and replaced with serum–free media for an additional 24 hours. The cell cultures were stimulated with TNF (200U/ml) and bFGF (25ng/ml) for additional 48 hours in serum–free media, followed by the addition of BrdU for 24 hours. The cultures were harvested and BrdU incorporation was determined according to the manufacturer's instructions via a microplate reader (BioRad Model 680). All experiments were performed in triplicate.

Collagen gene expression. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad CA), in collagen gene expression, as described.[8] One microgram of RNA was

converted to cDNA using a high capacity cDNA reverse transcription kit with random primers (Applied Biosytems, Foster City, CA). Collagen I (collagen 1A1 and 1A2) and collagen III (collagen 3A1) mRNA levels were quantified by real-time PCR using an ABI 7300 real-time PCR system (A&B Applied Biosystems). 18S ribosomal RNA was used as an internal control and expression levels of target genes in samples were normalized to a control sample using the comparative Ct ($\Delta\Delta$Ct) method as described before.[9]

Cardiac Histology, Morphometry and Function

Cardiac mast cells. Toluidine Blue staining was performed to identify the presence of mast cells in the LM and MHCsTNF mouse hearts at 4, 8 and 12 weeks of age. Briefly, hearts from MHCsTNF and LM mice were perfusion fixed with 4% paraformaldehyde as described, [10] and sectioned into 4-µm serial sections. Deparaffinized sections were soaked in a solution containing 0.5% Toluidine Blue, 0.1 M citric acid and 0.2 M dibasic sodium phosphate for 20 min at room temperature. Positively stained cells in at least 20 fields of the entire heart per section (× 200 magnifications) were enumerated. Each section was counted by two observers who were blinded with respect to genotype.

Myocardial fibrosis. Myocardial collagen content was determined in the hearts from LM/c-kit$^{+/-}$, MHCsTNF/c-kit$^{+/-}$, and MHCsTNF/c-kit$^{-/-}$ mice at 4, 8 and 12 weeks of age using the picrosirius red technique, as described. [11] To calculate the extent of myocardial fibrosis, the percent area of extracellular picrosirius red staining was computed from 20 random images obtained within the mid-wall of the left ventricle. Large epicardial arteries and/or veins were excluded, as well as any cutting and/or compression artifacts.

Cardiac hypertrophy. Cardiac mass was assessed by determining the heart-weight to body-weight ratios of LM/c-kit$^{+/-}$, MHCsTNF/c-kit$^{+/-}$, and MHCsTNF/c-kit$^{-/-}$ mice at 4, 8 and 12 weeks of age.[11]

Left ventricular diastolic filling. Hearts from LM, c-kit$^{-/-}$, LM/c-kit$^{+/-}$, MHCsTNF/c-kit$^{+/-}$, and MHCsTNF/c-kit$^{-/-}$ mice were isolated and perfused in the Langendorff mode, as described.[12] The hearts were paced at 420 bpm, and functional data were recorded at 1 KHz on a data acquisition system (Power Lab, AD Instruments, Colorado Spring, CO). Left ventricular developed pressure (LVDP) was calculated as the difference between peak-systolic pressure and LV end-diastolic pressure (LVedp). The data for LVedp were plotted as a function of increasing balloon volumes, via a best-fit exponential curve, as described.[13] The volumes at a given pressure were averaged for animals in each group.

Myocardial Gene Expression

Total RNA was extracted from the hearts of LM/c-kit$^{+/-}$, MHCsTNF/c-kit$^{+/-}$ and MHCsTNF/c-kit$^{-/-}$ mice using TRIzol reagent (Invitrogen, Carlsbad CA), in order to assess TGF-β signaling and collagen gene expression, as described.[8] TGF-β1, TGF-β2, TβR I and TβR II mRNA, Collagen 1A1, 1A2 and 3A1 mRNA levels were quantified by real-time PCR using an ABI 7300 real-time PCR system (A&B Applied Biosystems).

References

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RESULTS

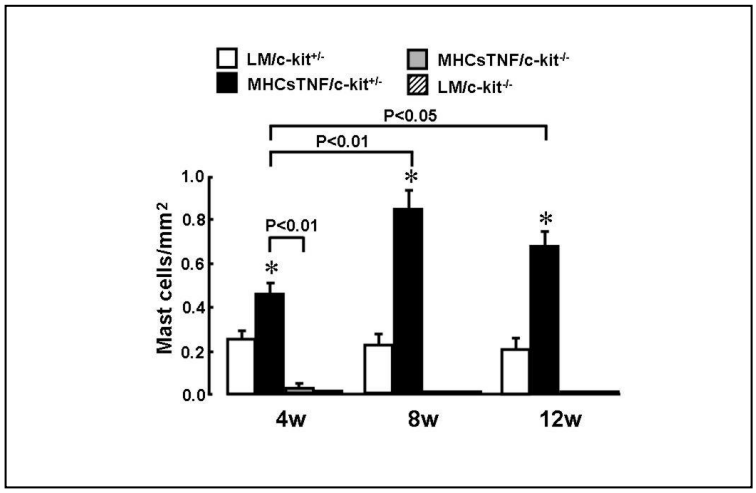

Figure S-1: Mast cell density in LM/c-kit$^{+/-}$, MHCsTNF/c-kit$^{+/-}$, MHCsTNF/c-kit$^{-/-}$, and LM/c-kit$^{-/-}$ mouse hearts at 4, 8, and 12 weeks of age. Mast cells were identified by Touidine Blue staining as described in the methods (n= 4-6 hearts/group/time). (* = $p < 0.01$ compared to LM/c-kit$^{+/-}$)

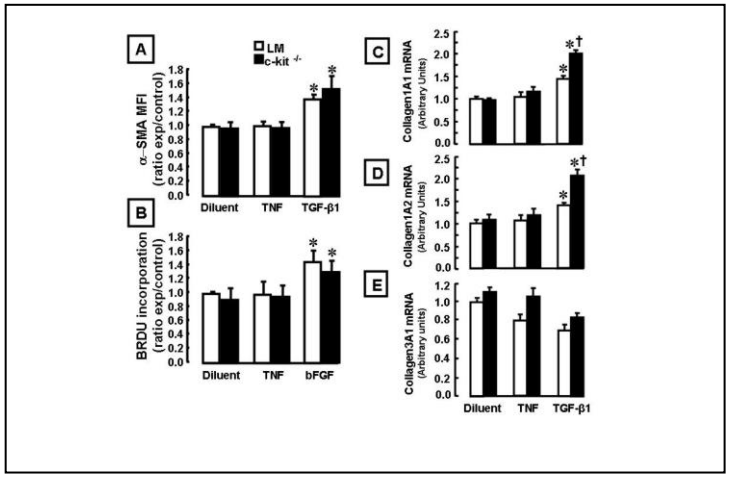

Figure S-2: (A) Group data for fold change of mean fluorescence intensity (MFI) of α-SMA staining in cardiac fibroblasts isolated from LM and c-kit$^{-/-}$ mouse hearts. (B) BrdU incorporation in fibroblasts isolated from LM and c-kit$^{-/-}$ mouse hearts treated with diluent, TNF, and bFGF. (C-E) Collagen 1A1, 1A2 and 3A1 gene expression in fibroblasts isolated from LM and c-kit$^{-/-}$ mouse hearts treated with diluent, TNF, and TGF-β1. (Fibroblast isolations were obtained from 6 hearts/group) (* = $p < 0.05$ compared to diluent, † = $p < 0.05$ compared to LM, by post-hoc ANOVA Fisher's PLSD).

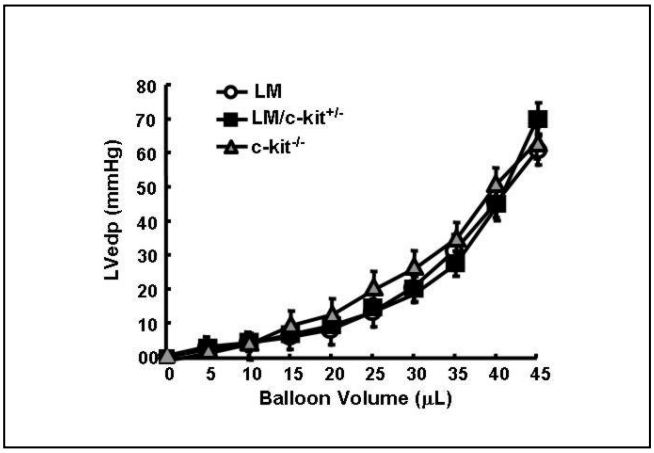

Figure S-3. Langendorff buffer perfused LV pressure-volume curves for LM (n = 7) mice, LM/c-kit$^{+/-}$ (n = 6), and c-kit$^{-/-}$ (n = 5) mice at 12 weeks of age. ANOVA showed that there was no significant difference ($p < 0.05$) in left ventricular end-diastolic pressure (LVedp) between LM, LM/c-kit$^{+/-}$, and c-kit$^{-/-}$ mice.

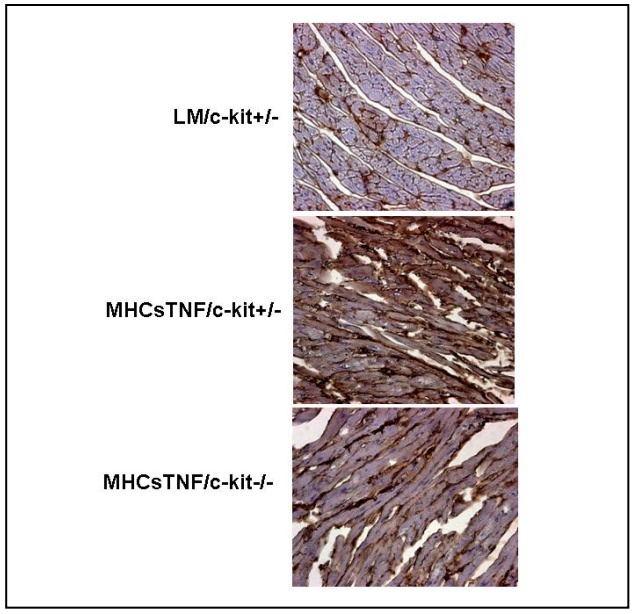

Figure S-4: TGF-β Immunolocalization. Representative TGF-β immunostaining in hearts from LM/c-kit$^{+/-}$, MHCsTNF/c-kit$^{+/-}$, and MHCsTNF/c-kit$^{-/-}$ mice at 12 weeks of age were fixed and immunostained with TGF-β1, 2, 3 Ab (1:20 Dilution, R&D systems) and a secondary anti-mouse-IgG-biotin Ab (1:200 Dilution) following IHC protocol of R&D systems. Histological specimens were examined at 200x magnification.

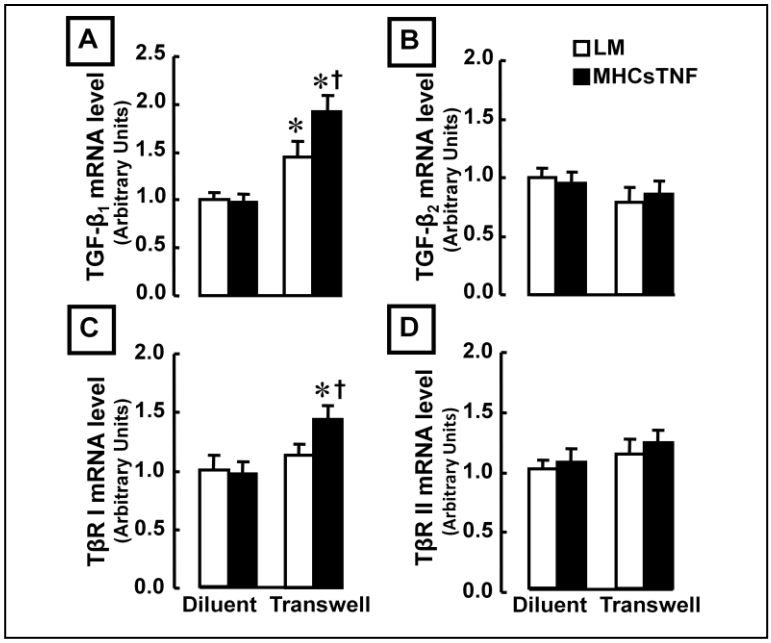

Figure S-5. TGF-β1, TGF-β2, TβR I and TβR II mRNA expression in LM and MHCsTNF cardiac fibroblasts in the absence and presence of mast cells. (A) TGF-β1, (B) TGF-β2, (C) TβR I and (D) TβR II mRNA levels. All experiments were performed in triplicate. (*= $p < 0.05$ compared to the respective diluent treated LM and MHCsTNF fibroblasts , †$p < 0.05$ compared to the respective LM mast cell co-cultured fibroblast by post-hoc ANOVA Fisher's PLSD) .

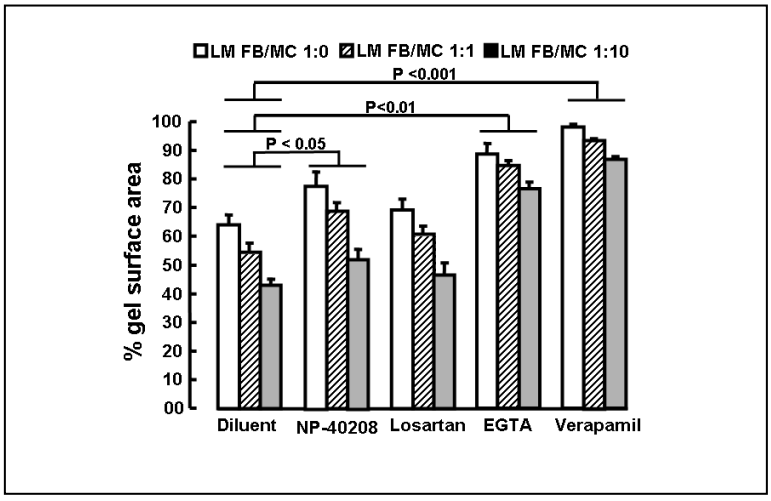

Figure S-6. Effect of NP-40208, losartan, EGTA and Verapamil on 3-dimensional collagen gel contraction in LM cardiac fibroblasts. All experiments were performed in triplicate. (n = 6 cultures per culture condition). Two-way ANOVA testing revealed significant differences in gel contraction between groups for effect of NP-40208, EGTA and verapamil ($p< 0.05$, $p< 0.001$, and $p< 0.001$, respectively), but not for losartan.

Table

Effect of Cromolyn on 3-D Collagen Gel Contraction

Groups	LM		MHCsTNF		p value
	Diluent	Cromolyn	Diluent	Cromolyn	
FB:MC=1:0	52.81±1.87	58.17±1.94	46.81±0.33	46.96±0.32	NS
FB:MC=1:1	50.67±1.18	51.89±0.76	42.59±1.31	46.06±2.31	NS
FB:MC=1:10	46.81±0.32	47.93±1.35	38.56±0.35	39.63±2.20	NS

Data are expressed as the percent of the collagen gel surface area at baseline (n = 6 cultures per culture condition).