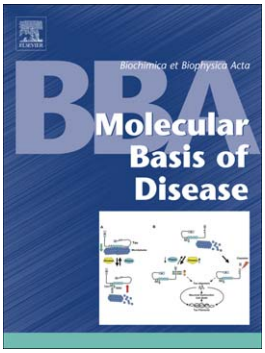

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BAMBI (BMP and activin membrane-bound inhibitor) protects the murine heart from pressure-overload biomechanical stress by restraining TGF-β signaling

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Running title: BAMBI and myocardial remodeling

Abbreviations

AS: Aortic stenosis

α-SMA: α-smooth muscle actin

BAMBI: BMP and activin membrane-bound inhibitor

JNK: c-Jun N-terminal kinase

MAPK: Mitogen-activated protein kinase

MMP: Matrix metalloproteinase

PO: Pressure overload

TAC: Transverse aortic constriction

TAK1: Transforming growth factor-β activated kinase

TGF-β: Transforming growth factor-β

Abstract

Left ventricular (LV) pressure overload is a major cause of heart failure. Transforming growth factors-β (TGF-βs) promote LV remodeling under biomechanical stress. BAMBI (BMP and activin membrane-bound inhibitor) is a pseudoreceptor that negatively modulates TGF-β signaling. The present study tests the hypothesis that BAMBI plays a protective role during the adverse LV remodeling under pressure overload. The subjects of the study were BAMBI knockout mice ($BAMBI^{-/-}$) undergoing transverse aortic constriction (TAC) and patients with severe aortic stenosis (AS). We examined LV gene and protein expression of remodeling-related elements, histological fibrosis, and heart morphology and function. LV expression of BAMBI was increased in AS patients and TAC-mice and correlated directly with TGF-β. BAMBI deletion led to a gain of myocardial TGF-β signaling through canonical (Smads) and non-canonical (TAK1-p38 and TAK1-JNK) pathways. As a consequence, the remodeling response to pressure overload in $BAMBI^{-/-}$ mice was exacerbated in terms of hypertrophy, chamber dilation, deterioration of long-axis LV systolic function and diastolic dysfunction. Functional remodeling associated transcriptional activation of fibrosis-related TGF-β targets, up-regulation of the profibrotic micro-RNA-21, histological fibrosis and increased metalloproteinase-2 activity. Histological remodeling in $BAMBI^{-/-}$ mice involved TGF-βs. BAMBI deletion in primary cardiac fibroblasts exacerbated TGF-β-induced profibrotic responses while BAMBI overexpression in NIH-3T3 fibroblasts attenuated them. Our findings identify BAMBI as a critical negative modulator of myocardial remodeling under pressure overload. We suggest that BAMBI is involved in negative feedback loops that restrain the TGF-β remodeling signals to protect the pressure-overloaded myocardium from uncontrolled extracellular matrix deposition in humans and mice.

Key words: Myocardial remodeling; aortic valve stenosis; pressure overload; TGF-β; BAMBI; miR-21

Introduction

Degenerative aortic valve stenosis (AS) and hypertension have become the most common cardiovascular diseases after coronary disease in developed countries with an increasing worldwide prevalence concomitant with ageing of the populations. The chronic left ventricular (LV) pressure overload condition produced by such pathologies triggers severe LV structural modifications, including cardiomyocyte hypertrophy and interstitial fibrosis [1], which are deleterious over time and result in progressive increases in LV stiffness, diastolic and systolic dysfunction and heart failure [2]. Aortic valve replacement, which is currently the only accepted treatment for AS, frequently fails to reverse the LV maladaptive remodeling [3]. Likewise, regression of the LV remodeling under antihypertensive treatment only seldom correlates with the degree of blood pressure reduction [4]. The persistence of either diffuse myocardial interstitial fibrosis or LV hypertrophy after therapeutic intervention predicts poorer short- and long-term survival and functional postoperative status in AS patients [5] and is a strong independent predictor of adverse cardiovascular outcomes in the hypertensive population [6]. Understanding the mechanisms governing the myocardial response to biomechanical stress is crucial to develop therapeutic strategies aimed at slowing disease progression and favoring the regression of the adverse pressure overload remodeling.

Transforming growth factors-β (TGF-βs) are key players in the LV remodeling response to pressure overload [7] in animal models [8] and in hypertensive and AS patients [9,10,11]. In the stressed myocardium, TGF-β overexpression causes fibroblast proliferation and transdifferentiation into myofibroblasts [12], endothelial-to-mesenchymal transition [13], and hypertrophic growth of cardiomyocytes [14], which collectively contribute to the progression of cardiac fibrosis and hypertrophy. TGF-βs

induce these pleiotropic cellular responses by interacting with the receptor complexes of two types of serine/threonine kinase receptors referred to as types I and II, which trigger the phosphorylation of receptor-regulated Smad (R-Smad) proteins. Phosphorylated R-Smads bind to Smad4, and the complexes move into the nucleus, where they regulate transcription [15]. Several downstream mediators of the TGF-β pathway are involved in the adverse myocardial remodeling induced by pressure overload. The TGF-β profibrogenic signals best analyzed in rodent models are mediated by the canonical transcription factors Smad2 and Smad3 [16,17]. The roles of the non-canonical, mitogen-activated protein kinase (MAPK) pathways, including TGFβ-activated kinase 1 (TAK1) and its downstream kinases p38 and c-Jun N-terminal kinase (JNK) are less well understood [18,19, 20].

MicroRNAs (miRNAs) are small noncoding RNA, which function as post-transcriptional regulators of gene expression. miRNAs play a central role in diverse cellular processes, including proliferation, differentiation, and death. The last decade has revealed microRNA-21 (miR-21) as a new mediator of TGF-β1-induced fibrosis [21]. In rodent models of pressure overload, miR-21 acts downstream of TGF-β to modulate several features of the myocardial fibrotic process [22,23]. A recent report from our group also supports such a relevant role for miR-21 in AS patients [24].

The cellular response elicited by TGF-βs is tightly controlled by multiple mechanisms at each step in their signaling pathway [25]. BAMBI (BMP and activin membrane-bound inhibitor) is a transmembrane glycoprotein structurally related to the TGF-β type I receptors, but it lacks the intracellular kinase domain. BAMBI functions as a decoy type I receptor that antagonizes TGF-β-family signals by preventing the formation of active receptor complexes upon ligand binding [26]. Little is known about the physiological functions regulated by BAMBI or the pathological

consequences of an imbalance between BAMBI and TGF-β signaling. Recent reports indicate that aberrant BAMBI expression plays a critical role in the pathophysiology of inflammatory [27] and fibrotic processes [28] and in cancer development [29,30]. Here, we demonstrate that BAMBI plays a modulator role in TGF-β-induced profibrogenic signals in the myocardium under biomechanical stress in mice and AS patients. We propose that BAMBI protects the pressure overloaded myocardium against uncontrolled extracellular matrix deposition through a negative feedback loop that restrains TGF-β-mediated deleterious remodeling signals. Alterations in BAMBI may play a role in the pathophysiology of myocardial fibrosis, and manipulation of BAMBI might confer disease-specific therapeutic benefits.

Methods

1. Pressure overload studies in humans

During surgery, myocardial tru-cut needle biopsies (4–10 mg) were obtained from the lateral LV wall from 45 individuals undergoing cardiac surgery, including 30 patients with isolated severe AS and a control group of 15 patients with pathologies producing no significant LV pressure or volume overload (atrial septal defect, aortic aneurysm, papillary fibroelastoma). Patients with aortic or mitral regurgitation greater than mild, major coronary stenosis greater than 50%, previous cardiac operations, malignancies or poor renal or hepatic function were ineligible for the study. The demographic and clinical characteristics of the patients are shown in supplemental table S1. The study followed the Declaration of Helsinki guidelines for investigation on human subjects. The institutional ethics and clinical research committee approved the study, and all patients gave written informed consent.

2. Pressure overload studies in mice

Adult (16-20 weeks old) female BAMBI-deficient mice ($BAMBI^{-/-}$) in a C57BL/6 genetic background [31] were kindly provided by Dr. J.C. Izpisua-Belmonte (The Salk Institute, La Jolla, CA). Wild type female mice (C57BL/6) were used as controls. Mice were housed in a room kept at 22°C with 12:12 h light/dark cycle and provided with standard food and water ad libitum. The study was approved by the University of Cantabria Institutional Laboratory Animal Care and Use Committee (approval ID 2008/05) and conducted in accordance with the "European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (European Communities Council Directive 86/606/EEC). All efforts were made to minimize animal suffering.

2.1. Transverse aortic constriction: LV pressure overload was induced by calibrated banding of the aorta at the mid-transverse arch level (TAC), as described [32]. Briefly, the mice were anesthetized by intraperitoneal injection of ketamine (10 mg/kg) and xylazine (15 mg/kg). The surgery was performed under spontaneous ventilation. The aorta was approached extrapleurally and constricted at the mid-arch level with a 7/0 polypropylene ligature using a blunted 27-gauge (0.41-mm OD) needle as a calibrator. This constriction induced a degree of geometric stenosis of approximately 65 % in diameter. The mice were sham operated or subjected to TAC for 1 or 4 weeks (n=5 to 9 per group). After completion of the follow-up, the mice were euthanized, and the cardiac mass was measured gravimetrically and indexed to the animal's body weight. The LV samples were snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde for histology.

For neutralizing antibody studies, a series of $BAMBI^{+/+}$ and $BAMBI^{-/-}$ mice were treated during the post-constriction follow-up period with a pan-neutralizing monoclonal (1D11.16.8 clone) anti-TGF-β antibody (TGF-β Ab) specific for all

isoforms of murine TGF-β or with a control isotype-matched IgG. The mice (n=4 per group) received i.p. injections of TGF-β Ab or IgG1 at the dose of 0.5 mg every other day.

2.2. Echocardiographic measurements: Transthoracic echocardiography was performed with ultrasound equipment [Agilent Sonos 5500 (Philips/Hewlett Packard) using 15-MHz linear and 12-MHz sectorial scanheads and Vevo-770 (VisualSonics, Toronto, ON, Canada) using a high-resolution transducer centered at 30 MHz]. Two dimensionally guided, short axis, M-mode recordings of the LV were recorded by an operator blinded to the study groups. Transcoarctation pressure gradients were measured using continuous wave Doppler analysis at the distal arch. LV dimensions and wall thicknesses were measured following the recommendations of the American Society of Echocardiography. The mitral annular plane systolic excursion (MAPSE) measurements were obtained from four-chamber views using M-mode imaging. The parameters of diastolic LV function were obtained by pulsed-wave mitral inflow analysis and tissue Doppler imaging to obtain the ratio of passive inflow by pulsed-wave to tissue Doppler (E/E'). The following parameters were derived from the recordings: heart rate, pressure gradient across the arch constriction, LV end-diastolic (LVEDd) and end-systolic (LVESd) internal diameters, interventricular septum (IVST) and posterior wall (PBW) thicknesses. The LV ejection fraction (LVEF) and mitral annular plane systolic excursion (MAPSE) were used as indices of radial and longitudinal systolic functions, respectively. The ratio of peak early transmitral flow velocity (E) to peak early myocardial tissue velocity (E′) was used as an index of LV filling pressure. Cardiac mass, estimated echocardiographically or measured gravimetrically, was indexed to the animal´s body weight and expressed in mg/g.

3. Determination of remodeling-related mRNA and microRNA elements in the LV by real-time quantitative PCR

Total RNA from the LV myocardium and cultured cells was obtained by TRIzol extraction (Invitrogen). MirRNA was isolated using a miRNA isolation kit (mirVana, Ambion). The mRNA was reverse transcribed using random primers with an RT-PCR kit (Fermentas). MiRNAs from tissue and cells were reversed transcribed using specific primers for miR-21 and RNU6-2 (Applied Biosystems). The cDNA products were amplified by quantitative PCR (q-PCR) in a MX-3000P Stratagene thermocycler.

The sequences of primers used for SYBR Green real-time PCR were as follows: collagen I (forward primer: 5´-tcctgctggtgagaaaggat-3´; reverse primer: 5´-tccagcaataccctgaggtc-3´; and the housekeeping gene, ribosomal 18S (forward primer: 5´-gtaacccgttgaaccccatt-3´; reverse primer: 5´ccatccaatcggtagtaggg-3´). The specific TaqMan assays (Applied Biosystems) used were miR-21, RNU6-2, TGF-β1, TGF-β2, TGF-β3,Smad2, Smad3, Smad4, TAK1, BAMBI, collagen III α1 (Col III), fibronectin-1 (FN), α-smooth muscle actin (α-SMA) and 18S. The expression levels of the myocardial genes were normalized to the housekeeping gene, ribosomal 18S. Myocardial miR-21 expression was normalized to the endogenous control, RNU6-2. Duplicate transcript levels were determined in a minimum of three independent experiments.

4. Immunodetection of LV remodeling-related proteins by Western Blot.

Thirty micrograms of total or nuclear protein extracts were resolved on a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Lab., California, USA) using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Lab., California, USA). The

primary antibodies were against BAMBI (Abnova), fibronectin-1 (Santa Cruz Biotechnology), p38 (Santa Cruz Biotechnology), JNK1/3 (Santa Cruz Biotechnology), Smad4 (Cell Signaling), GAPDH (Santa Cruz Biotechnology) and Rb (Santa Cruz Biotechnology). After extensive washings in TBS-T (TBS+0.05% Tween 20), the membranes were incubated with peroxidase-conjugated secondary antibodies. Secondary antibodies were detected using the ECL Advance kit (GE Healthcare Europe GmbH, Munich, Germany). Blot quantification was performed by densitometry using Scion Image Software.

5. Assessment of MMP activity in mouse LV by gelatin zymography.

Detection of active forms of MMPs was assessed by zymography, as described previously [33]. Samples for analysis were diluted into a non-reducing SDS-buffer. Ten µg of proteins per lane were separated by electrophoresis in 10% SDS-page gel containing 1% gelatin. After running, the gel was renatured through incubation in Triton X-100 (2.5% in 50 mM tris pH 7.4, 5 mM $CaCl_2$ and 1 µM $ZnCl_2$) for 30 min with gentle shaking at room temperature. The gel was rinsed and incubated overnight at 37ºC with developing buffer (50 mM tris pH 7.4, 5 mM $CaCl_2$ and 1 µM $ZnCl_2$). The gel was stained with 0.5% Coomassie Blue G250 (in 30% ethanol, 10% acetic acid) for 30 minutes. Staining was stopped with 2% acetic acid. Zymographic bands were scanned and the optical density was measured using the ImageJ software. The problem bands were normalized to the control samples in the same gel and the data were expressed as relative activity in fold increase. Silver staining of a strip obtained from the bottom of the gel (below the 37 kDa marker) was used as loading control.

6. Histological assessment of myocardial fibrosis.

The hearts were fixed in paraformaldehyde (3.7% in PBS, freshly prepared) for 48 h and then embedded in paraffin. Four coronal sections (5 µm) at the level of the

papillary muscles from each animal (n=4) were stained using Masson's trichrome. Digital photographs of the full LV sections were captured using a camera (Axiocam MRc5, Zeiss) attached to a stereo-microscope (Zeiss Axiomat). In each complete LV section, the proportion of the total fibrosis area was calculated by densitometric analysis (ImageJ software) as the blue-stained areas divided by the total LV area. The operator was blinded to the experimental group during the analysis.

7. BAMBI immunostaining

7.1. Immunofluorescence: After deep anesthesia with a mixture of xylazine and ketamine, mice were perfused with heparinized saline for 1 min and fixative solution containing 4% paraformaldehyde in PBS for 15 min. After perfusion, the heart was dissected out and post-fixed for 12 h in the same solution at 4ºC. The tissue blocks were washed in PBS, cryoprotected with 30% sucrose at 4°C until they sank, and stored at -80ºC. Seven µm LV sections were cut on a cryostat and mounted on gelatin-subbed slides.

Mouse LV squash preparations were prepared from small LV fragments incubated in paraformaldehyde (4% in PBS, freshly prepared) for 2 h and washed in PBS. Each tissue fragment was transferred to a drop of PBS on a siliconized slide and squash preparations of dissociated cardiomyocytes and fibroblasts were performed as previously reported [34]. Then, the samples were sequentially treated with 0.25% collagenase in PBS for 30 min at 37ºC and 0.5% Triton X-100 in PBS for 30 min.

The primary cultures of cardiac fibroblasts were fixed with paraformaldehyde for 15 min at room temperature. After extensive washing in PBS, the cells were permeabilized using 0.2% Triton X-100.

In all preparation types, nonspecific sites were blocked by incubation with PBS containing 1% BSA and 0.3 M glycine for 60 min at room temperature. After extensive washing, the samples were incubated with the primary antibodies (1/50 in PBS) overnight, at 4°C. After washing in PBS, the samples were incubated with secondary fluorochrome-conjugated antibodies for 45 min at room temperature and washed and mounted in VectaShield (Vector Laboratories, Burlingame, CA). We used primary antibodies against BAMBI (Santa Cruz Biotechnology) and vimentin (Abcam). The specific secondary antibodies were conjugated with FITC (Jackson, USA) or Cy5 (Abcam). Dapi (Sigma) was used as a nuclear counterstain. Omission of the primary or secondary antibodies completely abolished specific staining. Confocal microscopy was performed with an LSM-510 laser scanning microscope (Carl Zeiss Inc., Germany) using a 40x objective.

7.2. Avidin biotin complex (ABC) immunohistochemistry: BAMBI immunostaining was performed in 5 µm sections from human LV samples fixed in 4% paraformaldehide and included in paraffin. Heat-induced antigen retrieval was performed in 10 mM citric acid monohydrate (pH 6.0). Endogenous peroxidase was blocked with 0.3% H_2O_2 for 30 min. Sections were incubated in the anti BAMBI primary antibody (1/50 in PBS) overnight at 4ºC. After extensive washing, the sections were incubated with the biotinylated secondary antibody in PBS for 30 minutes followed by the complex of avidin-biotin peroxidase (Vector ABC kit) and the chromogen diaminobenzidine. Before mounting, the sections were counterstained with Harris hematoxylin. Negative controls were performed using PBS instead of the primary antibody.

8. Transcriptional activation of collagen I and BAMBI by recombinant TGF-β1 in cultured NIH-3T3 fibroblasts as measured by luciferase reporter assays

The changes in collagen I and BAMBI transcription induced by recombinant TGF-β1 were assessed in NIH-3T3 fibroblasts transiently transfected with the collagen Iα1 or BAMBI promoter regions cloned into the luciferase reporter pGL3 basic vector (pBAMBI-Luc was kindly provided by Dr. Tetsu Akiyama, Institute of Molecular an d Cellular Bioscience, Tokyo). The signaling effectors were determined by silencing either Smad2/3 or TAK-1 with specific siRNAs (Santa Cruz Biotechnology). The consequence of BAMBI overexpression on TGF-β1-induced col I transcriptional activation was assessed in cells co-transfected with the BAMBI open reading frame (BAMBI-ORF) (BAMBI-pFLCI; ImaGenes, Berlin, Germany) and pCol-Luc.

The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C, in 5% CO2. The cells were seeded onto 96 well plates ($2x10^4$/well) and cultured for 12 h. For the collagen I promoter luciferase reporter assays, the cells were transiently transfected with the following: a) pCol-Luc (50 ng); b) pCol-Luc (50 ng) plus Smad2/3 siRNA (10 nM) or control scrambled siRNA; c) pCol-Luc (50 ng) plus TAK1 siRNA (10 nM) or control siRNA; and d) pCol-Luc (50 ng) plus BAMBI-ORF-pFLCI (0.4 µg/ml) using FuGENE®6 transfection reagent (Roche Molecular Biochemicals). After 8 h of incubation, increasing concentrations (0, 0.3, 0.6 and 1.5 ng/ml) of recombinant TGF-β1 (R&D Systems) were added to the medium.

For the BAMBI promoter luciferase reporter assays, the cells were transiently transfected with the following: a) pBAMBI-Luc promoter (100 ng); b) pBAMBI-Luc (100 ng) plus Smad2/3 siRNA (10 nM) or control scrambled siRNA and c) pBAMBI-Luc (100 ng) plus TAK1 siRNA (10 nM) or control scrambled siRNA using

FuGENE®6 transfection reagent (Roche Molecular Biochemicals). After 8 h of incubation, recombinant TGF-β1 (0, 0.3 and 0.6 ng/ml) was added to the medium.

Twenty-four hours later, luciferase assays were performed using the Luciferase® Reporter Assay System (Promega) following the manufacturer`s protocol. Luciferase activities were expressed in relation to the background activity of the empty pGL3-Basic or pFLCI vectors.

9. Effects of recombinant TGF-β1 on mature miR-21 expression in cultured NIH-3T3 fibroblasts

NIH-3T3 cells ($1x10^6$) were plated in 25 cm^2 flasks. Twenty-four hours later, the cells were transiently transfected with 10 nM pre-miR-21 or control miRNA using FuGENE®6 in the presence of increasing concentrations of BAMBI-pFLCI (0, 0.2, 0.4 and 0.6 µg/ml) or the empty pFLCI vector control. In a series of experiments, siSmad2/3, siTAK-1 or scrambled siRNA were transfected. Four hours later, the cells were incubated with recombinant TGF-β1 (0.3 ng/ml) for 24h. The cells were harvested, and the mRNA and micro-RNAs were purified and retrotranscribed to perform q-PCR as described above.

10. Transcriptional changes induced by recombinant TGF-β1 in primary cultures of mouse cardiac fibroblasts

Neonatal cardiac fibroblasts were isolated from C57BL/6 and $BAMBI^{-/-}$ mice by enzymatic digestion. Briefly, the ventricles were excised, washed and minced in ice-cold PBS. The minced tissue was then digested at 37ºC with an enzyme cocktail (10 mg trypsine, 5,285 units collagenase, and 85,000 units DNAse) for 30 min. The supernatants from these incubations were collected, centrifuged, and resuspended in DMEM supplemented with 10% FBS, 10% donor bovine serum (DBS), 100 units/ml penicillin and 100 mg/ml streptomycin. For the experiments, low passage cells (p2)

were seeded onto 35-mm cell culture dishes and incubated at 37ºC and 5% CO2 for 15-20 hours. The unattached cells were discarded, and the attached cells were cultured until approximately 70% confluent. The cells were then growth arrested by incubation in DMEM containing 1% FBS for 12 h. The fibroblasts were treated for 6 h with recombinant TGF-β1 (0.6 ng/ml) and harvested for further analysis.

11. Statistics

All the assays in mice were performed in a minimum of five individuals per group (n=5 to 9). The experiments in cultured cells were performed in triplicate and repeated on three separate occasions. Values are reported as means ± S.E.M. The GraphPad Prism 5.01 and PASW Statistics 18 (SPSS Inc, Chicago, IL) packages were used. Student's t-test was used to assess differences between means of continuous variables. The influence of mice genotype and pressure overload on myocardial gene expression was assessed by a two-way ANOVA and, on echocardiographic parameters, by repeated-measures two-way ANOVA. Bonferroni post hoc test was used when appropriate. Linear regression analysis was used to detect correlations between gene expression levels. A p value <0.05 was considered statistically significant.

Results

Myocardial expression of BAMBI in mice and AS patients.

Immunofluorescence studies performed in preparations of dissociated mouse cardiac cells reveal that BAMBI immunoreactivity was present in cardiomyocytes (Fig. 1 A) and fibroblasts (Fig. 1 B). Fibroblasts were identified based on their positive staining for the mesenchymal marker vimentin (Fig. 1 C). Immunohistochemical staining performed in LV sections disclosed the presence of BAMBI immunoreactivity

in the myocardium from TAC mice (Fig. 1 D) and AS patients (Fig. 1 E). BAMBI immunosignals were detected in cardiomyocytes, in the vascular wall (Fig. 1D, insert) including the endothelium, and in fibrotic areas. It is interesting to note that BAMBI immunoreactivity disclosed a peripheral expression pattern in cardiomyocytes, consistent with its transmembrane localization.

Parallel LV up-regulation of BAMBI and TGF-β under pressure overload in humans and mice

We assessed whether cardiac expression of BAMBI was regulated in response to pressure (Fig. 2). We found that the myocardial BAMBI mRNA and protein levels of were significantly up-regulated in AS patients and mice subjected to TAC. BAMBI mRNA levels correlated, significantly and positively, with the expression of genes encoding TGF-β1 in AS patients and in mice with TGF-β1, TGF-β2 ($r=0.56$, $p<0.001$; $n=32$) and TGF-β3 ($r=0.69$, $p<0.001$; $n=32$). Moreover, BAMBI also correlated with the canonical (Smad2) and non-canonical (TAK1) TGF-β transducers (Fig. 2). Pressure overload is a hemodynamic condition that is associated with intense myocardial activation of TGF-β signals in experimental models and patients [7]. Our results demonstrate a coordinated transcription of genes encoding agonists and antagonists of the TGF-β pathway during pressure overload. They also suggest that BAMBI could be a transcriptional target of TGF-βs.

Transcription of BAMBI was activated by TGF-β1 in cultured fibroblasts through Smad and TAK1 pathways

We assessed in cultured fibroblasts whether BAMBI transcription was under the control of TGF-β signaling (Fig. 3). In primary cardiac fibroblasts and NIH-3T3 fibroblasts, the addition of recombinant TGF-β1 to the medium led to a significant increase in endogenous BAMBI mRNA expression. Cell transfection with specific

siRNAs directed against Smad2/3 or TAK1 attenuated significantly the up-regulation of BAMBI mRNA induced by recombinant TGF-β1 in NIH-3T3·fibroblasts. The effectiveness of siRNA transfection into the cells was confirmed by western blot experiments showing diminished protein levels of Smad2/3 and TAK1 (supplemental Fig. S1). Silencing TAK1 also inhibited the nuclear accumulation of the TAK1-downstream kinases p38 and JNK.

We further evaluated whether TGF-β signaling is directly involved in the transcriptional activation of BAMBI in NIH-3T3 fibroblasts transfected with the BAMBI-Luc promoter and treated with recombinant TGF-β1 (0.3 and 0.6 ng/ml). As shown in Fig. 3, upon administration of TGF-β1, the transcriptional activity of BAMBI, reflected by the luciferase signal, increased significantly. Co-transfection of the cells with siRNAs (10 nM) against Smad2/3 or TAK1 significantly reduced the luciferase activity induced by TGF-β1. Overall, these results establish a regulatory role for TGF-β signaling, through canonical and non-canonical pathways, on BAMBI transcriptional activity in fibroblasts.

BAMBI deletion increased the vulnerability of the LV to biomechanical stress

To determine the functional role of BAMBI in pressure overload-induced myocardial disease, we studied $BAMBI^{-/-}$ and $BAMBI^{+/+}$ mice subjected to transverse aortic constriction (TAC). The baseline and 4-week post-TAC values of echocardiographic parameters are depicted in supplemental Table S2. There were no differences between the two genotypes for the transcoarctational gradients at 1 to 4 weeks following TAC, indicating similar degrees of constriction in both groups. The heart rate was not significantly different between the $BAMBI^{+/+}$ and $BAMBI^{-/-}$ groups. The hypertrophy developed after TAC reached greater values and developed at a more accelerated pace in $BAMBI^{-/-}$ mice compared with $BAMBI^{+/+}$, as indicated by the

echocardiographic LV mass (Fig. 4, supplemental Table S2) and the gravimetric heart mass indexed to the body weight ($BAMBI^{+/+}$: 6.20±1.5 mg/g vs $BAMBI^{-/-}$: 7.5±1.5 mg/g; $p<0.001$). Additionally, during the 4-week follow up after TAC, significant differences in the LV geometry and function were apparent between the $BAMBI^{-/-}$ and $BAMBI^{+/+}$ mice. The hearts from the $BAMBI^{-/-}$ mice exhibited more severe LV dilation, reaching significantly higher LVEDds and LVESds than the $BAMBI^{+/+}$ mice (Fig. 4 and supplemental Table S2). The LV PWTs and IVSTs at baseline and their increases after TAC were similar in both genotypes (supplemental Table S2).The LVEF, which reflects the LV short-axis systolic function, was negligibly, although significantly reduced by TAC in the $BAMBI^{-/-}$ compared with the $BAMBI^{+/+}$ mice (Fig 4). The LV long-axis systolic function, reflected by MAPSE, was also significantly lower in the KO than in the wild type group, either at baseline or at any time after TAC (Fig. 4). The LV filling pressure, reflected by the ratio E/E′ (Fig. 4), increased after TAC in both genotypes, although the rise was significantly higher in the $BAMBI^{-/-}$ than in the $BAMBI^{+/+}$ mice (Fig. 4).

Overall, these results indicate that BAMBI was up-regulated during the pressure overload, and that deletion of the gene encoding BAMBI aggravated pressure overload-induced hemodynamic deterioration.

BAMBI deletion promoted increased TGF-β signaling in the myocardium

In the next steps, we focused on the mechanisms involved in the detrimental consequences of BAMBI deletion on heart function under biomechanical stress. Given that BAMBI functions as a negative regulator of TGF-βs, we initially assessed whether the release of such inhibition in $BAMBI^{-/-}$ mice resulted in increased TGF-β signaling. The nuclear amounts of canonical and non-canonical TGF-β signaling mediators were significantly higher in the $BAMBI^{-/-}$ mice compared to the $BAMBI^{+/+}$

mice at baseline and following TAC. As shown in Fig. 5, the nuclear protein levels of Smad4 and the downstream MAP kinases, TAK1, JNK and p38, were significantly higher in the myocardium from the sham BAMBI$^{-/-}$ mice compared to the sham BAMBI$^{+/+}$ mice. Furthermore, when subjected to pressure overload, the myocardium from the TAC-mice by the 4-week follow up developed a significant up-regulation of genes encoding TGF-β1, TGF-β2, and TGF-β3, with no significant differences between the two genotypes (Fig. 5). However, the nuclear accumulations of Smad4, JNK and p38 were higher in the TAC-BAMBI$^{-/-}$ mice compared to their TAC-BAMBI$^{+/+}$ littermates (Fig. 5). These data indicate that BAMBI deletion prompts a gain of TGF-β canonical and non-canonical signaling with pathophysiological consequences on LV remodeling under biomechanical stress.

BAMBI deletion exacerbated the development of myocardial remodeling in response to pressure overload

The pressure overload induced by TAC caused the up-regulation of TGF-β target genes that encode extracellular matrix proteins related to myocardial fibrosis in mice from both genotypes (Fig. 6). Collagen I, collagen III and fibronectin mRNA expression were significantly higher, and their up-regulation was more accelerated during the 4-week follow-up period in the BAMBI$^{-/-}$ mice compared to the BAMBI$^{+/+}$ mice. Histological examination of the hearts with Masson's trichrome staining confirmed that the degree of fibrosis was more severe in the BAMBI$^{-/-}$ mice compared to the BAMBI$^{+/+}$ mice (Fig. 6 and supplemental Fig. S2). Densitometric analysis indicated that the fraction of the LV surface occupied by fibrosis was 3.5 ± 0.9-fold greater in the BAMBI$^{-/-}$ mice compared to the BAMBI$^{+/+}$ mice (supplemental Fig. S2). Western blot analysis confirmed that, following CAT, fibronectin also reached higher expression in the myocardium from BAMBI$^{-/-}$ mice (Fig. 6).

Under basal conditions, the myocardial expression of collagen III mRNA was significantly higher in the sham-BAMBI$^{-/-}$ mice compared to the sham-BAMBI$^{+/+}$ group (Fig. 6). However, the percentage of the LV fibrosis area stained by Masson trichrome was negligible (supplemental Fig. S2) and had no consequences on the systolic function of the sham-BAMBI$^{-/-}$ animals (Fig. 4).

BAMBI deletion increased myocardial metalloproteinase-2 (MMP-2) expression and activity in response to pressure overload

The LV transcript levels of MMP-2 increased following TAC, reaching significantly higher values in BAMBI$^{-/-}$ than in BAMBI$^{+/+}$ mice (Fig. 7). Also, gel zymography revealed in both TAC and sham operated mice a band exhibiting gelatin-degrading activity at 64 kDa molecular weight, consistent with active MMP-2 (Fig. 7). Following TAC, the myocardium from BAMBI$^{-/-}$ mice displayed significantly higher gelatinase activity compared with their wild type littermates (Fig.7). MMP-2 mRNA levels correlated, significantly and positively with the expression of genes encoding TGF-β1 ($r=0.43$, $p<0.01$; $n=32$), TGF-β2 ($r=0.69$, $p<0.001$; $n=32$) and TGF-β3 ($r=0.63$, $p<0.001$; $n=32$). MMP-2 also correlated with the non-canonical effector TAK1 ($r=0.53$, $p<0.001$; $n=32$) but it was unrelated to the canonical (Smads) TGF-β transducers (data not shown). Overall, these results suggest that MMP-2 may be a transcriptional target of TGF-βs.

The mice subjected to TAC displayed, regardless of genotype, a positive linear correlation between the myocardial transcript levels of MMP-2 and the LVEDd ($r=0.56$, $p=0.02$, $n=26$), supporting the notion that, in this scenario, chamber dilation and MMP activity are linked.

Treatment with TGF-β neutralizing antibody attenuated pressure overload-induced cardiac fibrosis in the BAMBI$^{-/-}$ mice

The involvement of TGF-β in the myocardial fibrotic process under pressure overload has been previously demonstrated by our group [35] and others [20] using neutralizing monoclonal antibodies against TGF-β in C56BL6 wild-type mice. Here, we assessed whether TGF-βs were also responsible for the severe fibrosis displayed by the BAMBI$^{-/-}$ mice. For this purpose, a series of mice were treated during the post-constriction follow-up period with the neutralizing antibody anti-TGF-β (0.5 mg every other day, starting on the day of surgery) or with an IgG1 control. Both the TAC-BAMBI$^{+/+}$ and TAC-BAMBI$^{-/-}$ mice treated with the TGF-β-Ab displayed markedly reduced fibrosis compared to the control mice of each genotype treated with IgG1, as indicated by Masson's trichrome staining of the heart sections (supplemental Fig. S2). These results are consistent with a relevant participation of TGF-βs in the fibrotic process induced by pressure overload in both genotypes.

The fibrogenic responses induced by TGF-β1 were dependent on canonical- and non-canonical effectors

We analyzed the signaling effectors involved in TGF-β1-induced transcriptional changes in NIH-3T3 fibroblasts by silencing either Smad2/3 or TAK-1 with specific siRNAs. Cells transiently transfected with pCol-Luc reporter responded upon stimulation with recombinant TGF-β1 with a significant, concentration-dependent increase in the luciferase activity relative to the vector control, which was antagonized by either siSmad2/3 or siTAK1 (Fig. 8).

In cultured cells, the fibrogenic effects of recombinant TGF-β1 were enhanced by BAMBI deletion and attenuated by BAMBI overexpression

To further confirm that TGF-β induces stronger profibrotic responses in the absence of BAMBI, primary cardiac fibroblasts from BAMBI$^{-/-}$ and BAMBI$^{+/+}$ mice were cultured in the presence of recombinant TGF-β1. Upon TGF-β1 stimulation, the mRNA expression levels of fibrosis-related TGF-β-target genes, such as collagen I, collagen III, fibronectin and the myofibroblast marker α-SMA (Fig. 6), and miR-21 (Fig. 9), reached significantly higher values in fibroblasts lacking BAMBI compared to those from BAMBI$^{+/+}$ mice.

We next studied whether BAMBI overexpression has opposite effects on TGF-β1-induced transcription in NIH-3T3 fibroblasts. For this purpose, cells were co-transfected with BAMBI-pFLC1 and a Col-Luc reporter. The overexpression of BAMBI produced by BAMBI-pFLC1 transfection resulted in a significant reduction in collagen I transcription, as reflected by the luciferase activity (Fig. 8), at baseline and following the addition of TGF-β1 to the medium.

BAMBI modulates TGF-β-induced miR-21 overexpression

Recent studies have strongly suggested that overexpression of miR-21 in cardiac fibroblasts is crucially involved in the pro-fibrotic effects of TGF-β in mice subjected to pressure overload [22] and in AS patients [24] . Accordingly, we found that miR-21 was up-regulated in the myocardium from mice of either BAMBI genotype when subjected to TAC, and that its expression reached significantly higher values in BAMBI$^{-/-}$ compared to BAMBI$^{+/+}$ mice (Fig. 9). Moreover, primary cardiac fibroblasts obtained from mice lacking BAMBI responded upon TGF-β1 stimulation with a significantly higher miR-21 overexpression compared to those obtained from the BAMBI$^{+/+}$ (Fig 9). Conversely, transfection of NIH-3T3 fibroblasts with BAMBI induced BAMBI overexpression that prevented the up-regulation of mature miR-21 induced by TGF-β1 (Fig. 9).

Silencing of either Smad2/3 or TAK1 with specific siRNAs prevented TGF-β1-induced up-regulation of mature miR-21 in NIH-3T3 fibroblasts (Fig. 9), indicating the involvement of canonical and non-canonical TGF-β transducers.

Discussion

This study for the first time demonstrates that the TGF-β pseudoreceptor BAMBI is expressed in cardiac fibroblasts and cardiomyocytes. Our central finding is that BAMBI plays a pathophysiological role in the modulation of the adverse cardiac remodeling by restraining TGF-β signals in the myocardium, and BAMBI is a potentially important target for limiting TGF-β activity in the heart under biomechanical stress. Our characterization of BAMBI functions *in vivo* and in cultured cells adds new insights to the complex regulation of TGF-β signaling.

In the last few years, the decoy receptor BAMBI has emerged as a crucial negative regulator of the TGF-β superfamily signals [26], with a relevant role for their fine tuning during embryogenesis [36]. Although BAMBI is dispensable for mouse embryo development and postnatal survival [31], several reports have underlined the importance of a reliable negative regulation of TGF-β by BAMBI for tissue homeostasis during adulthood. Aberrant BAMBI expression conveys TGF-β signaling alterations in different organs and tissues, with relevant pathophysiological implications in processes such as neuropathic pain [31,37], hepatic fibrosis [28], tumor growth and metastasis [29,30,38]. In our study, we identified that myocardial expression of BAMBI mRNA is increased in patients with severe AS and mice subjected to aortic arch constriction. These results support that myocardial BAMBI overexpression is a conserved feature of the pressure overload in the clinic and in experiments. Moreover, in the stressed myocardium from AS patients and TAC-mice,

the transcript levels of BAMBI maintained a direct correlation with TGF-β1 and with the major effectors of its canonical (Smads) and non-canonical (TAK1) signaling pathways. Such a coordinated spatio-temporal expression pattern is typically depicted by BAMBI and agonists belonging to the TGF-β family (BMPs) and their effectors during embryogenesis in a gene organization termed synexpression, which reflects a cooperative function in the regulation of biological processes. In this context, a key feature of the BMP synexpression group is the existence of negative feed-back loops in which the agonists induce the transcriptional activation of the antagonists [36,39]. In a similar way, in the adult myocardium, TGF-β could promote the expression of its inhibitory counterpart, BAMBI, setting up an auto-regulatory negative-feedback loop. Our data in cultured cells support that BAMBI is a direct transcriptional target of TGF-β signaling and that the mechanism involves canonical Smad2/3 and non-canonical TAK1 pathways.

In patients with AS, the increased parietal systolic stress induced by the LV outflow obstruction triggers the activation of various signaling cascades [40] that initiate a progressive process of structural and functional abnormalities, which ultimately result in heart failure [41]. TGF-β has been repeatedly reported to exert a myriad of pleiotropic effects on cardiac cells, which underlie the LV remodeling in AS patients [7,9,10,11] and animal models [8]. Cardiac tissue fibrosis is a major feature among the remodeling phenomena and one that carries with it a bleak clinical prognosis in terms of survival and functional status [42]. Our present results are consistent with the current understanding that augmented TGF-β signaling results in deleterious ECM deposition (mainly collagens I and III and fibronectin) which increases myocardial stiffness and, as a consequence, interferes with LV diastolic and systolic functions [7,42,43]. We propose that the coordinated myocardial

expression pattern of BAMBI with TGF-β aims to restrain the hypertrophic and fibrogenic signals of this potent cytokine. In this way, BAMBI could exert a modulation of the turnover of extracellular matrix and sarcomeric components to reach a “balanced” remodeling response to the current hemodynamic loading conditions. Our results in BAMBI$^{-/-}$ and BAMBI$^{+/+}$ mice subjected to transverse aortic constriction further support this hypothesis. As expected, the absence of the inhibitory influence of BAMBI prompted more effective TGF-β cell signaling, both at baseline and after TAC, involving the canonical (Smad) and non-canonical (TAK1) signaling pathways. Thus, BAMBI$^{-/-}$ mice, compared to BAMBI$^{+/+}$ littermates, showed a higher nuclear expression of Smad4 and MAPKs downstream of TAK1, such as p38-MAPK and JNK-MAPK, at any pressure load condition. Consequently, the progression of LV myocardial fibrosis and hypertrophy were exacerbated and accelerated following CAT in BAMBI$^{-/-}$ mice compared to their BAMBI$^{+/+}$ littermates.

From a functional point of view, TAC induced a significant, albeit small, reduction in the short-axis systolic function (reflected by LVEF) in BAMBI deficient mice in comparison with the wild type group. Also, the baseline LV longitudinal systolic function (reflected by MAPSE) was significantly poorer in BAMBI$^{-/-}$ mice than in their wild type littermates and it experienced a significant and similar worsening in both genotypes following TAC. In the clinical scenario, it is repeatedly observed that even severe myocardial fibrosis does not result in a significant deterioration of the LV short-axis systolic function as reflected by LVEF [42,44]. On the other hand, LV longitudinal systolic function deteriorates early under pressure overload, even though short-axis function remains relatively preserved [42], because it is driven by the subendocardial layer of longitudinal muscle fibers which is most vulnerable to the elevated systolic wall stress and subsequent fibrotic reaction [45]. Many clinical

research studies underscore the importance of myocardial fibrosis to diastolic dysfunction [42]. LV properties such as passive stiffness, viscoelasticity, distensibility and relaxation, which play a very relevant role in dictating LV behavior during diastole, are strongly affected by even small changes in the amount and composition of the extracellular matrix [45]. In BAMBI$^{-/-}$ mice, the LV filling pressure (reflected by its surrogate variable E/E′) increased significantly after TAC and reached higher values than in wild type animals, in the absence of relevant LVEF deterioration, which strongly suggests the existence of diastolic dysfunction. These results are consistent with the more severe fibrosis developed by BAMBI$^{-/-}$ mice and mirror the findings in patients with severe AS that feature a stepwise positive correlation between the severity of the LV histological fibrosis and E/E′ values [42].

BAMBI$^{-/-}$ mice trend to dilate more severely the LVEDD after TAC than the wild type reproduces the clinical finding of greater chamber dilation in those AS patients with more severe myocardial fibrosis [42]. Several clinical and experimental studies suggest that an increase in the activity of MMPs underlies LV dilation while MMP inhibition in mice attenuates LV dilation in experimental models of heart failure [46]. Accordingly, in the present study, MMP-2 was transcriptionally activated following TAC in both BAMBI genotypes and its mRNA levels correlated significantly and positively with the diastolic dilation of the chamber. In parallel with the LV dilation, the transcript levels of MMP-2 and its gelatinase activity reached significantly higher values in BAMBI$^{-/-}$ mice than in the wild type group. Since TGF-β is a profibrotic cytokine that can inhibit MMP transcription, through its Smad-mediated canonical signaling, and thereby reduce ECM turnover [46], it could be presumed that BAMBI deletion and subsequent TGF-β signaling hiperactivity would exaggerate such an effect. However, in our study MMP-2 expression correlated positively and

significantly with the expression of the three isoforms of TGF-β and with their non-canonical effector TAK1, which suggests this signaling pathway to activate MMP-2 transcription under our experimental conditions. Else, it has been demonstrated that TGF-β can activate Smad-independent transcriptional complexes, such as the Ets family of transcription factors, which would also induce certain MMP types [47]. Transcriptional activation of MMP-2 by TGF-β may also occur via an indirect mechanism dependent on miR-21 overexpression [48].Thus, as pointed out by Spinale [46], TGF-β might exert dual responses with respect to MMP transcriptional activity depending on cell type, local concentration or temporal factors. We suggest that the absence of BAMBI, by altering the level of TAK1-mediated TGF-β signaling, would favor MMP transcription which is translated into increased MMP-2 collagenase activity.

Collagen I is a major target gene of TGF-β1 and is responsible for more than 85% of the total ECM deposition in fibrotic processes. Consistently, NIH-3T3 fibroblasts transiently transfected with the Col α1-promoter-Luc reporter, upon stimulation with recombinant TGF-β1, responded with a significant increase in luciferase activity, by a mechanism involving Smad2/3 and TAK1. In contrast, when NIH-3T3 cells were transiently transfected with BAMBI, a significant reduction in TGF-β1-induced Col I-luciferase reporter expression was found, which underscores the functional antagonistic effect of BAMBI on TGF-β signaling.

MiRNAs are important regulators of cardiac biology with significant roles in diverse cardiac pathological processes. MiR-21 has been reported as a pro-fibrotic miRNA in many organs, including heart [21]. In the mouse LV subjected to pressure overload, miR-21 is up-regulated to promote fibroblast survival and growth factor secretion, via down-regulation of Spry1 and subsequent increase of ERK-MAP

kinase activity. The resultant cardiac interstitial fibrosis and hemodynamic dysfunction can be attenuated by treatment of mice with an antagomir against miR-21 [22,23]. In AS patients, we provided evidence supporting that both myocardial and plasmatic miR-21, acting synergically with TGF-β signal transducers, play a significant role in the maladaptive remodeling of the extracellular matrix triggered by pressure overload [24]. Previous studies evidenced that miR-21 is critically involved in the changes in cellular phenotype produced by TGF-β profibrotic signals, including fibroblast proliferation, myofibroblast differentiation and endothelial to mesenchymal transition [23]. Herein, TAC-induced up-regulation of myocardial mature miR-21 reached significantly higher values in BAMBI$^{-/-}$ compared to BAMBI$^{+/+}$ mice. Accordingly, the overexpresion of miR-21 elicited by recombinant TGF-β1 in primary cardiac fibroblasts reached higher values when the fibroblasts were obtained from BAMBI$^{-/-}$ mice. In cultured cells, miR-21 is under the transcriptional and post-transcriptional control by canonical TGF-β signaling; Smad3 is crucial for miR-21 transcription [49] and both Smad2 and Smad3 are able to interact with the p68 RNA helicase of the Drosha microprocessor complex, promoting the restriction of pri-miR-21 into the precursor pre-miR-21 [50]. In our study, TGF-β1 induced up-regulation of mature miR-21 in NIH-3T3 fibroblasts was prevented by silencing Smad2/3 or TAK1 with specific siRNAs and by impairing TGF-β signaling via BAMBI overexpression. Overall, our present results further support that miR-21 biogenesis in fibroblasts is dependent on TGF-β1 signaling, through both canonical Smad-dependent and non-canonical TAK1-dependent pathways, and BAMBI functions as a negative regulator of such TGF-β function.

In conclusion, our findings in AS patients, experimental animals and cultured cells constitute the first evidence identifying the TGF-β pseudoreceptor BAMBI as a

new player, and potential target for therapy, in remodeling of the heart under pressure overload. We propose that myocardial expression of BAMBI is regulated in a coordinated manner with TGF-βs to restrain the hypertrophic and fibrogenic signaling of these cytokines through a negative feedback loop. In this way, BAMBI could modulate the turnover of extracellular matrix components to reach a robust remodeling response “balanced” to the current hemodynamic loading condition. Alterations in BAMBI may play a role in the pathophysiology of myocardial fibrosis, and manipulation of BAMBI might confer disease-specific therapeutic benefits. Further experimental and clinical studies are warranted to clarify whether such approaches may offer new therapeutic opportunities for the palliation of pathological myocardial fibrosis.

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Figure legends

Figure 1. Immunohistological localization of BAMBI in the LV myocardium. **A**, **B** and **C**: Representative confocal microscopy images showing immunofluorescence staining for BAMBI (green) in a squash preparation of cardiomyocytes (**A**) and primary cardiac fibroblasts (**B**). Fibroblasts were identified based on their positive staining for the mesenchymal marker vimentin (**C**, red). Dapi was used as a nuclear counterstain (blue). **D** and **E**: Representative immunofluorescence staining of BAMBI in the mouse LV subjected to aortic constriction (**D**) and immunohistochemical (avidin biotin complex) staining of BAMBI in the LV from AS patients (**E**). BAMBI immunosignals were detected in cardiomyocytes, in fibrotic areas and in the vascular wall (insert in D) including the endothelium. Note the marked peripheral staining for BAMBI in cardiomyocytes, consistent with its transmembrane localization. **D**' and **E'**: Negative controls using PBS instead of the primary antibody.

Figure 2. Pressure overload induced LV myocardial up-regulation of BAMBI mRNA relative expression (RE, normalized to 18S) in AS patients (**A**) and mice subjected to transverse aortic constriction (TAC) (**F**). Representative western blots showing myocardial overexpression of BAMBI protein in AS patients (**B**) and TAC mice (**G**). Linear regression analysis, in AS patients and TAC mice, showing the positive correlation between the myocardial mRNA expression of BAMBI and TGF-β1 (**C** and **H**), Smad-2 (**D** and **I**) and TAK1 (**E** and **J**). r = Pearson´s correlation coefficient.

Figure 3. A: Effect of recombinant TGF-β1 on endogenous BAMBI mRNA relative expression (RE, normalized to 18S) in primary cardiac fibroblasts. **B**: Effect of silencing Smad2/3 and TAK1 with specific siRNAs (10 nM) on TGF-β1-induced

BAMBI relative expression in NIH-3T3 fibroblasts. **C**: Effect of recombinant TGF-β1 on luciferase activity and the antagonism by siRNAs (10 nM) against Smad2/3 and TAK1 in NIH-3T3 cells transfected with the promoter region of BAMBI in a luciferase reporter vector. The results are expressed as relative luciferase units (RLU). ***p<0.001 vs TGF-β1+siScr-RNA (ANOVA followed by Bonferroni).

Figure 4. The echocardiographic dimensional and functional changes induced by pressure overload during a 4-week follow-up period in mice subjected to transverse aortic constriction (TAC). IVST: interventricular septum thickness; LVEDd: left ventricular end-diastolic dimension; E/ E′: ratio of peak early transmitral flow velocity (E) to peak early myocardial tissue velocity (E′); LVEF: left ventricular ejection fraction; MAPSE: mitral annular plane systolic excursion. TAC-BAMBI$^{-/-}$ vs TAC-BAMBI$^{+/+}$: *p<0.05; **p<0. 01; ***p<0.001 (Repeated-measures two-way ANOVA and Bonferroni post-hoc test).

Figure 5. Myocardial relative expression (RE) levels of mRNAs (normalized to 18S) encoding TGF-β1 (**A**), TGF-β2 (**B**), and TGF-β3 (**C**) in BAMBI$^{+/+}$ and BAMBI$^{-/-}$ mice, either sham operated or subjected to transverse aortic constriction (TAC) for 1 or 4 weeks. Pressure overload induced a significant up-regulation of TGF-βs, with no significant differences between genotypes (two-way ANOVA). Representative western blot images showing that the nuclear accumulation of canonical and non-canonical TGF-β signaling mediators Smad4 (**D**), p38 (**E**) and JNK (**F**) were higher in the BAMBI$^{-/-}$ compared to the BAMBI$^{+/+}$ mice that were either sham operated or subjected to TAC. Loading controls were performed with the nuclear protein ELK-1.

Figure 6. BAMBI–deficient mice exhibit more severe cardiomyopathy after pressure overload. Relative mRNA expression of genes encoding remodeling-related elements in $BAMBI^{+/+}$ and $BAMBI^{-/-}$ mice, either sham operated or subjected to aortic arch constriction (TAC). **A**: Collagen I (Col I); **B**: Collagen III (Col III); **C**: Fibronectin 1 (FN-1). The mRNA expressions were normalized to 18S. Data are expressed as means ± SEM. *p<0.05, **p<0.01, ***p<0.001, $BAMBI^{-/-}$ vs $BAMBI^{+/+}$ (two-way ANOVA and Bonferroni post-hoc test). The insert in **C** shows representative western blot images of fibronectin protein levels in each experimental group. **D**: Representative transverse cross-sections of Masson's trichrome-stained hearts. **E**: Relative gene expression of extracellular matrix proteins (collagen I, collagen III and fibronectin), the myofibroblast marker α-SMA and the housekeeping protein GAPDH in primary cardiac fibroblasts cultured in the presence of recombinant TGF-β1 (0.6 ng/ml) for 6 hours. The results are expressed as the fold increase in the mRNA levels upon TGF-β1 stimulation in fibroblasts from $BAMBI^{-/-}$ mice compared to $BAMBI^{+/+}$ mice. **p<0.01, *p<0.05 (Student´s t test).

Figure 7. Effects of transverse aortic constriction (TAC) on myocardial relative expression (RE) of mRNA encoding matrix metalloproteinase-2 (MMP-2) (**A**) and zymography determined myocardial MMP-2-gelatinase activity (**B**) in $BAMBI^{+/+}$ and $BAMBI^{-/-}$ mice. The transcript levels of MMP-2 and the gelatinase activity dependent of MMP-2 (64 kDa) reached significantly higher levels in $BAMBI^{-/-}$ compared with $BAMBI^{+/+}$ mice 4 weeks after TAC. **A**: The mRNA expression was normalized to 18S. **B**: Representative gel showing gelatin lysis in the regions comigrating with MMP-2. Silver staining of a strip obtained from the bottom of the gel (below the 37 kDa marker) was used for loading control. The bar diagram represents the gelatinase

activity determined by optical densitometry of the zymographic bands. The problem bands were normalized to the control bands in the same gel. Data are expressed as means ± SEM. *p<0.05, ***p<0.001, BAMBI$^{-/-}$ vs BAMBI$^{+/+}$ (ANOVA and Bonferroni post-hoc test).

Figure 8. **A**: NIH3T3 fibroblasts transiently transfected with the Col α1-luc reporter responded to recombinant TGF-β1 with a significant, concentration-dependent increase in luciferase activity (black circles), which was prevented by silencing Smad2/3 (open circles) and TAK1 (open diamonds) with specific siRNAs (10 nM). **B**: Co-transfection of NIH-3T3 cells with collagen α1-luc reporter plus BAMBI-ORF (0.2 μg/ml) produced a significant reduction in luciferase activity, either basal or induced by TGF-β1. The results are expressed as luciferase activity relative to the signal of cells transfected with empty vectors (PGL3 in A and pFLC1 in B). Data are the mean ± SEM of three independent experiments. *p<0.05; **p<0.01; ***p<0.001

Figure 9. **A**: Myocardial relative expression (RE) of mature miR-21 (normalized to RNU6-2). Up-regulation of myocardial miR-21 after transverse aortic constriction (TAC) was significantly higher in BAMBI$^{-/-}$ mice compared to BAMBI$^{+/+}$ mice (*p<0.05, ***p<0.001,TAC vs sham; $^{\$}$p<0.05, BAMBI$^{+/+}$ vs BAMBI$^{-/-}$; two-way ANOVA followed by Bonferroni). **B**: Recombinant TGF-β1 induces mature miR-21 overexpression in primary cardiac fibroblasts from BAMBI$^{+/+}$ and BAMBI$^{-/-}$ mice. The expression of miR-21 was significantly higher in BAMBI$^{-/-}$ mice compared to BAMBI$^{+/+}$ mice (***p<0.001, ANOVA followed by Bonferroni). **C:** In NIH-3T3 fibroblasts transfected with pre-miR21 (10 nM), TGF-β1 induces the up-regulation of mature miR-21, which is prevented by silencing Smad2/3 and TAK1 with specific siRNAs (10 nM). **D**: In NIH-

3T3 fibroblasts transfected with pre-miR21 (10 nM), co-transfection of the BAMBI open reading frame (BAMBI-ORF) caused increased expression of BAMBI mRNA (right Y axis) and protein (insert). In parallel, the up-regulation of mature miR-21 (left Y axis) induced by TGF-β1 (0.3 ng/ml) was prevented by BAMBI overexpression. Insert: representative western blot showing increased BAMBI protein expression following transfection with BAMBI-ORF.

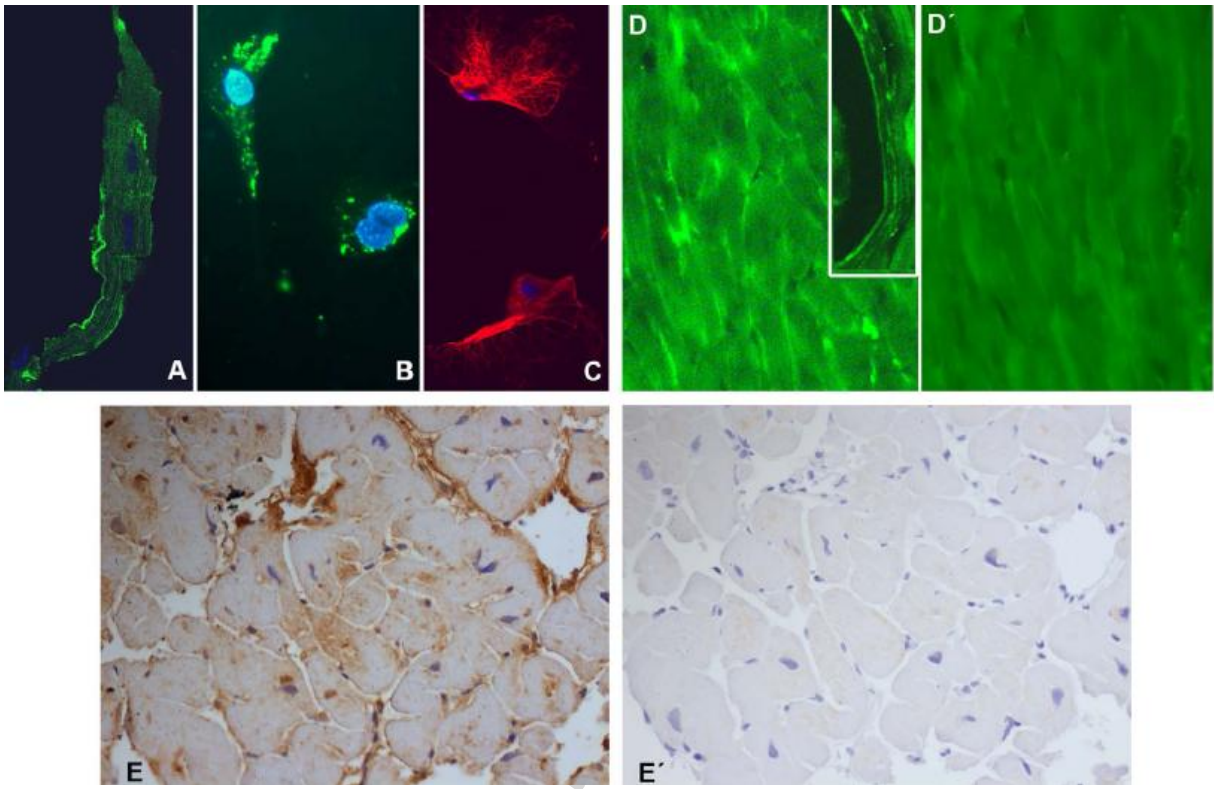


Figure 1

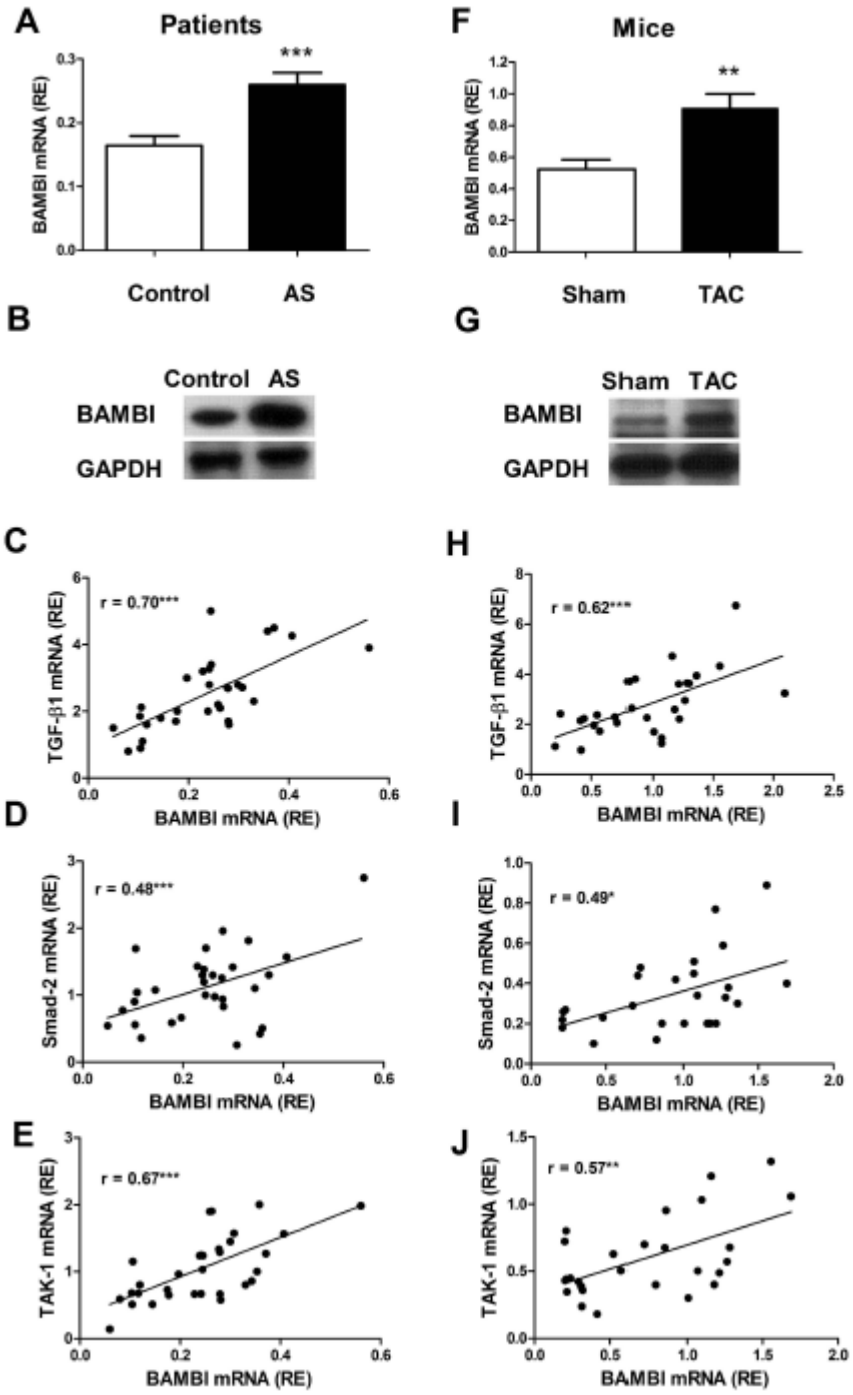
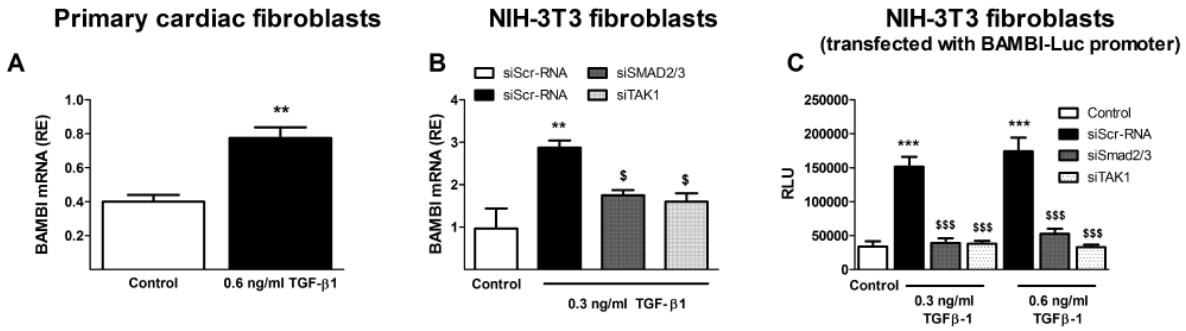


Figure 2



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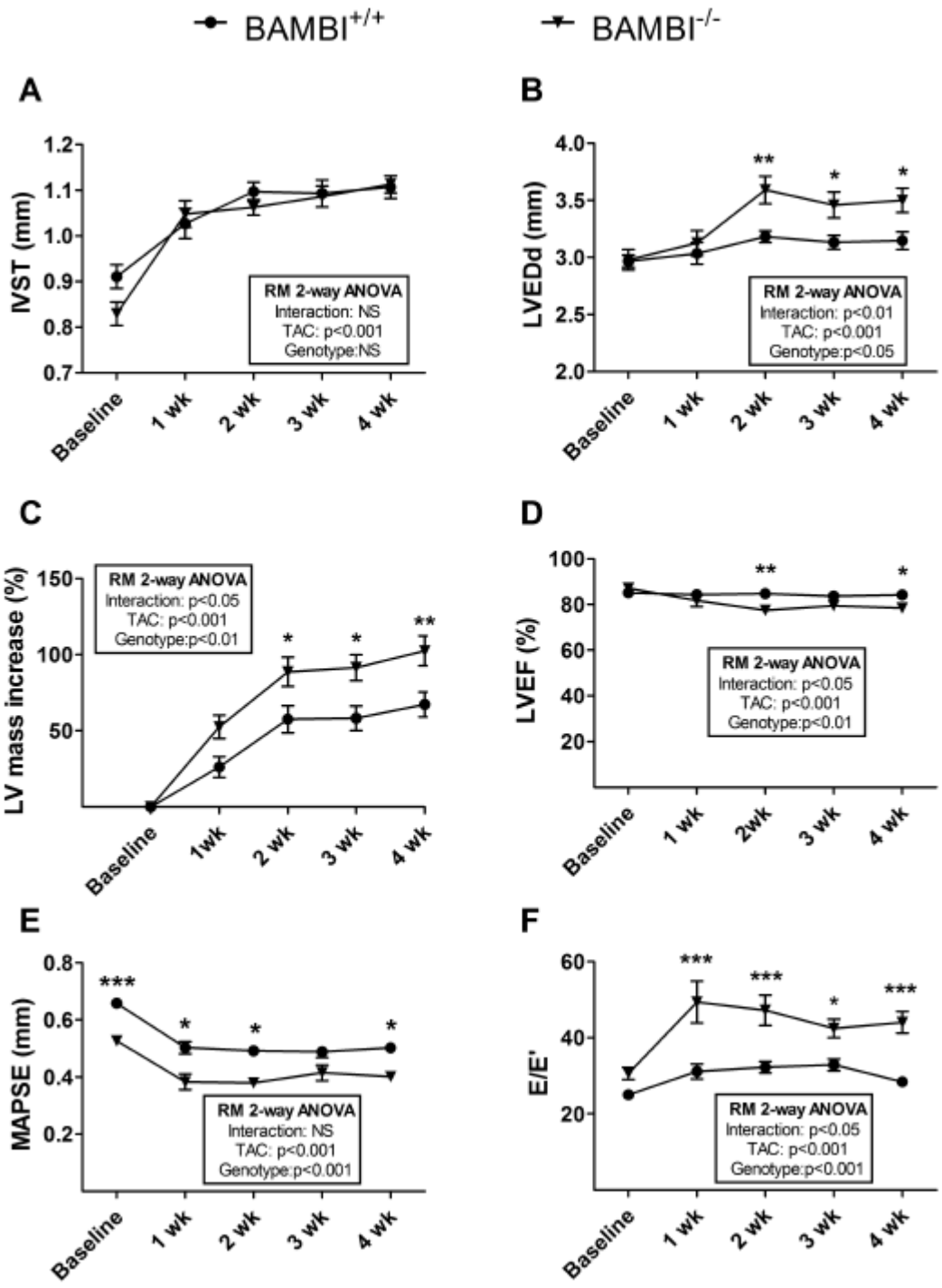


Figure 4

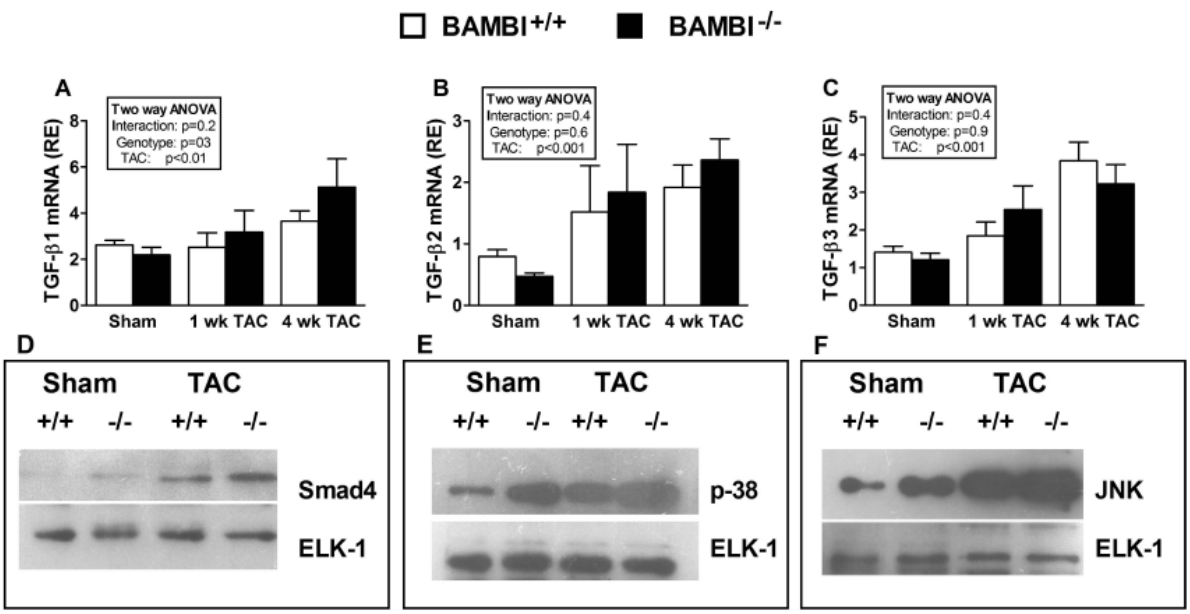
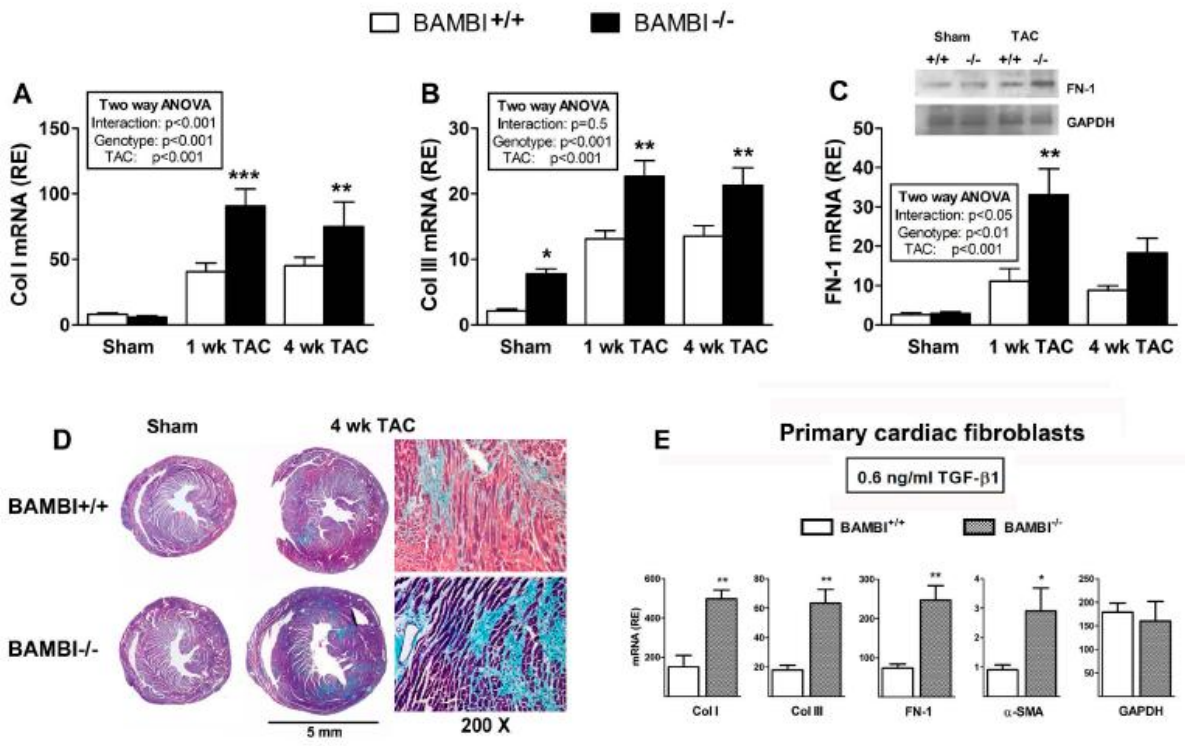


Figure 5



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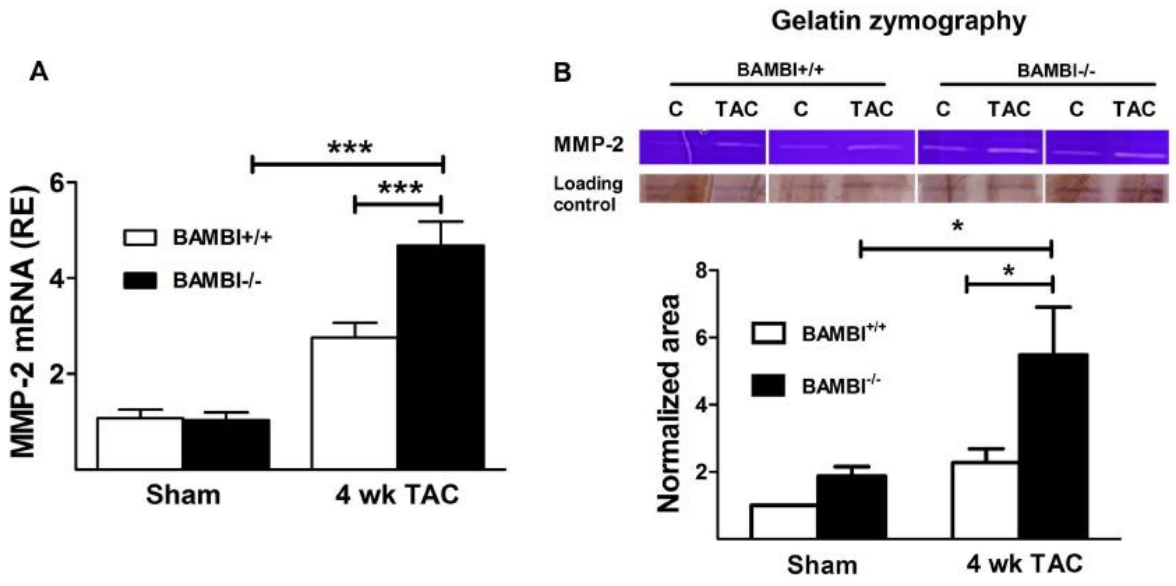

Figure 7

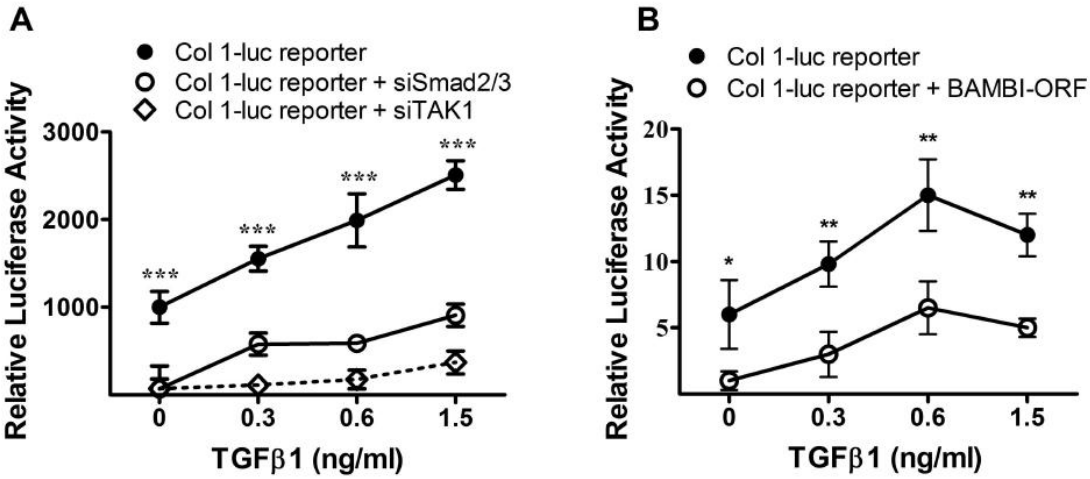

Figure 8

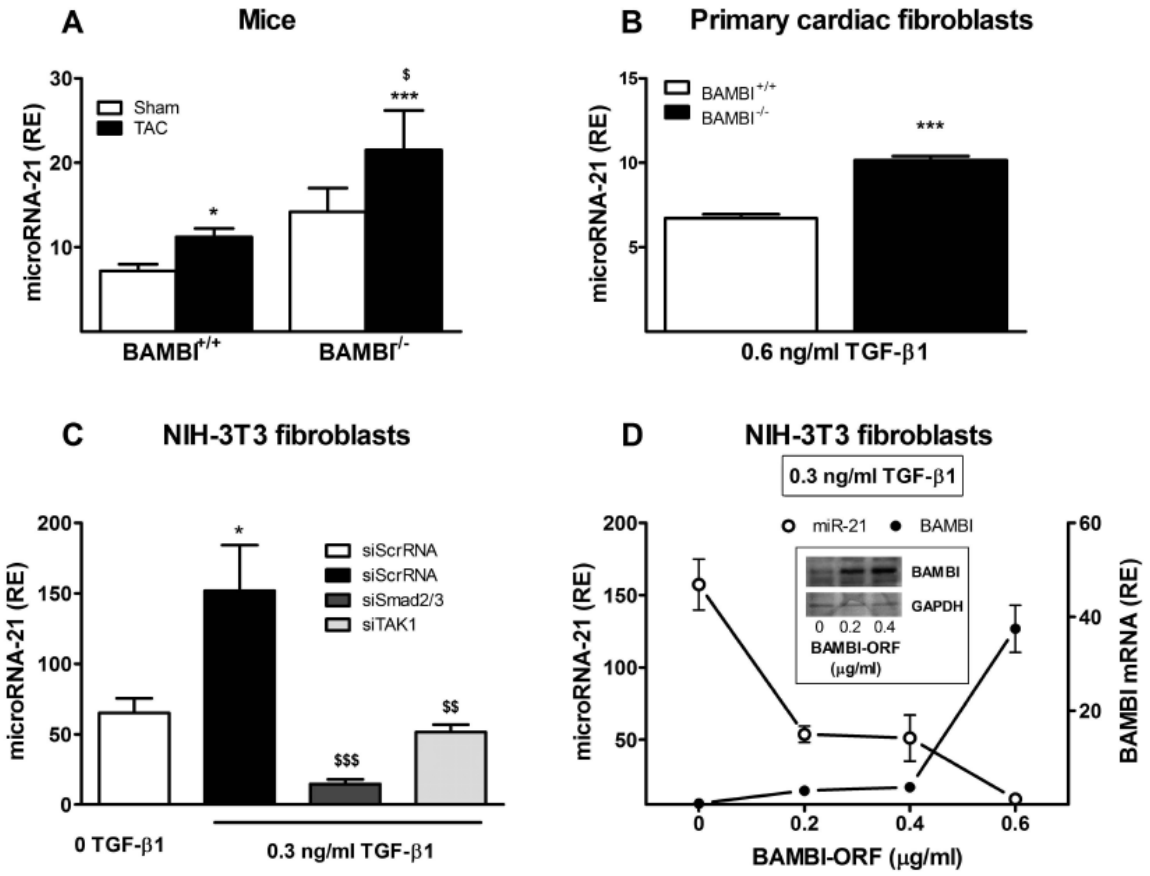

Figure 9

Highlights

Cardiomyocytes and cardiac fibroblasts express BAMBI

BAMBI and TGF-β are up-regulated in the LV under pressure overload in humans and mice

BAMBI restrains TGF-β fibrogenic signaling through a negative feedback loop BAMBI deletion exacerbates LV remodeling induced by TGF-β under pressure overload

BAMBI protects the pressure overloaded myocardium against adverse remodeling