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Cardiac myocyte β3-adrenergic receptors prevent myocardial fibrosis by modulating oxidant stress-dependent paracrine signaling

Hermida et al. Paracrine anti-fibrotic role of beta3-adrenoceptor

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ABSTRACT

**Aims** - Human and mouse cardiac beta3-adrenergic receptors (beta3AR) exert antipathetic effects to those of beta1-2AR stimulation. We examined their role in modulating myocardial remodeling, particularly fibrosis in response to hemodynamic stress.

**Methods and Results** – Mice with cardiac myocyte-specific expression of beta3AR (ADRB3-tg) or tamoxifen-inducible homozygous deletion (c-Adrb3-ko, with loxP-targeted Adrb3) were submitted to transaortic constriction. A superfusion assay was used for proteomic analysis of paracrine mediators between beta3AR-expressing cardiac myocytes and cardiac fibroblasts cultured separately. We show that cardiac beta3AR attenuate myocardial fibrosis in response to hemodynamic stress. Interstitial fibrosis and collagen content were reduced in ADRB3-tg, but augmented in c-Adrb3-ko. ADRB3 and collagen (COL1A1) expression were also inversely related in ventricular biopsies of patients with valve disease. Incubation of cardiac fibroblasts with media conditioned by hypertrophic myocytes induced fibroblast proliferation, myo-differentiation and collagen production. These effects were abrogated upon ADRB3 expression in myocytes. Comparative shotgun proteomic analysis of the myocyte secretomes revealed a number of factors differentially regulated by beta3AR, among which CTGF (CCN2) was prominently reduced. CTGF was similarly reduced in stressed hearts from ADRB3-tg, but increased in hearts from c-Adrb3-ko mice. CTGF expression was mediated by reactive oxygen species production which was reduced by ADRB3 expression *in vitro* and *in vivo*. This antioxidant and anti-fibrotic effect involved beta3AR coupling to the neuronal isoform of nitric oxide synthase (nNOS) in cardiac myocytes, as both were abrogated upon nNOS inhibition or Nos1 homozygous deletion.

**Conclusion** – Cardiac beta3AR protect from fibrosis in response to hemodynamic stress by modulating nitric oxide and oxidant stress-dependent paracrine signaling to fibroblasts. Specific agonism at beta3AR may offer a new therapeutic modality to prevent cardiac fibrosis.

Keywords: myocardial remodeling; fibrosis; catecholamines; beta3 adrenergic receptor; nitric oxide; oxidant stress
Translational perspective

Cardiac fibrosis leads to progressive systolic and diastolic heart failure. Interstitial fibrosis is driven by hemodynamic overload and neurohormones, including catecholamines acting on adrenergic receptors (AR).

Cardiac beta3 AR exerts effects that are antipathetic to those of to beta1 and beta2 AR isotypes and are uniquely resistant to homologous desensitization. We show that expression of human beta3 AR in cardiac myocytes protects from the development of fibrosis after transaortic constriction (TAC) in mice by modulating paracrine signaling to fibroblasts, with downregulation of pro-fibrotic cytokines and matricellular proteins. This is mediated by beta3AR coupling to nitric oxide synthase and antioxidant effects in cardiac myocytes. In biopsies from patients with valve disease, collagen I expression is inversely related with beta3 AR abundance, extending our findings in the clinical setting.

Our study provides further insight into the protective effect of cardiac beta3AR and suggests the possibility to prevent cardiac fibrosis by using new specific agonists of beta3AR, such as mirabegron, currently tested in clinical trials.
INTRODUCTION

Fibrosis is a common consequence of injury leading to failure in many organs. Cardiac fibrosis is an integral part of adverse remodelling leading to alterations in diastolic distensibility, arrhythmias and systolic failure (1). In humans, the absence of recovery of LV function after aortic valve replacement correlates with the degree of fibrosis (2, 3). Although myocardial fibrosis can be reversed in some cases (4), targeted therapies remain largely elusive.

Recent lineage tracing experiments have identified specific populations of fibroblasts that contribute myocardial fibrosis (5, 6). They concluded to a major participation of resident cardiac fibroblasts developmentally deriving from epicardium or endocardial endothelium (6). Upon pressure overload, local proliferation and subsequent myodifferentiation of these resident fibroblasts produce myocardial fibrosis, emphasizing the importance of signalling within “fibrogenic niches” (7). This involves reciprocal paracrine signalling between parenchymal and neighboring cells. Although much attention has been focused on the influence of fibroblasts in regulating cardiac myocytes hypertrophy, little attention has been devoted so far to signals sent from “stressed” myocytes to fibroblasts.

Beta3AR are expressed in human atrial and ventricular myocytes (8), in which their contractile effects are antipathetic to those of beta1-2AR (9). Although prolonged activation or high heterologous expression of beta1AR exacerbates adverse myocardial remodelling (10), this opposing effect of beta3AR suggests unique protective properties for this isotype (11). However, the underlying mechanisms, particularly through paracrine signalling, remain undefined. In the present work, we use genetic mouse models with cardiac myocyte-specific expression of ADRB3 or conditional deletion of Adrb3 to study the receptor’s specific role in myocardial protection from fibrosis in response to transaortic constriction (TAC). Using a model of superfusion of cardiac fibroblasts incubated with media conditioned by cardiac myocytes adenovirally-expressing ADRB3, combined with unbiased proteomic analysis of their secretome, we identify a number of beta3AR-regulated signalling or matricellular proteins that modulate the activation of fibroblasts.
Among these, we found that CTGF/CCN2 expression was downregulated by beta3AR through its coupling to neuronal nitric oxide synthase (nNOS) and anti-oxidant effects in cardiac myocytes.

**METHODS**

Expanded methods are available in the Supplementary material online.

**RESULTS**

1. Expression of the beta3AR in cardiac myocytes protects from myocardial fibrosis under hemodynamic overload

Heterozygous adult mice harbouring a transgene coding the human ADRB3 under the Myh6 (alpha-Myosin Heavy Chain, alpha-MHC) promoter (ADRB3-tg) were subjected to TAC and their phenotype analysed at 9 weeks post-TAC. Morphometric data are illustrated in Fig 1A. Note that, in our model, the abundance of transgenic human beta3AR proteins was comparable to that observed in human ventricular extracts (Suppl Fig 1 G) and that transcripts levels of Adrb1 and Adrb2 were unchanged between ADRB3-tg and WT, both at baseline and after TAC (Suppl Fig1 A-B). All mice included in the study (ADRB3-tg and WT) developed a trans-stenotic gradient with maximum velocity (by Doppler echo) of at least 3 m/s, and gradients were comparable between genotypes (Suppl. Fig 2). ADRB3-tg mice developed a moderate hypertrophic response which was significantly milder than WT (Fig 1A). Histological analysis showed that myocardial fibrosis was strikingly attenuated in ADRB3-tg (Fig 1B). This was confirmed by quantification of collagen volume fraction, as well as of collagen type 1 both by immunohistochemical analysis and western blotting (Figs 1 C-D). Capillary density and inflammatory cells (CD45+) infiltration were comparable between genotypes. No differences in apoptosis were observed between genotypes 9 weeks post-TAC (Suppl. Fig 3).

To further confirm the causal role of the cardiac Adrb3, we analyzed the phenotype of mice with cardiac myocyte-specific deletion of the mouse Adrb3 using an inducible Cre-lox
system. Mice with exon 2 of Aдрb3 flanked by 2 lox-P sites were generated and back-crossed in the C57Bl/6J background for at least 9 generations, then cross-bred with mice (in C57Bl/6J background) expressing a tamoxifen-inducible Cre recombinase under the alpha-MHC promoter (alpha-MHC-MerCreMer) (Fig 2A). Double-transgenic mice were then treated with tamoxifen and underwent TAC (or sham operation) and their cardiac remodelling analysed. We previously tested several treatment schemes with tamoxifen to ensure efficient recombination while avoiding any independent effect of tamoxifen on hypertrophic or fibrotic remodelling. Tamoxifen alone at 30 μg/g body weight/day injected on three consecutive days produced an efficient recombination in double-transgenic mice but did not per se induce fibrosis in all controls tested (Suppl. Fig 4).

Heterozygous alpha-MHC-MerCreMer^{0/+}, homozygous Aдрb3^{flx/flx} and double-transgenic mice without tamoxifen were submitted to TAC (or sham) in parallel as controls. Note that transcripts levels of Aдрb1 and Aдрb2 were unchanged between single or double transgenics with tamoxifen, both at baseline and after TAC (Suppl Fig 1 C-D). As expected, single transgenic mice and double transgenics without tamoxifen developed TAC-induced hypertrophy (Fig 2B) and fibrosis (Fig 2 C-D-E) to a comparable extent. However, after tamoxifen treatment, double transgenics developed similar hypertrophy (Fig 2B) but a higher degree of fibrosis compared to all other controls (including double transgenics without tamoxifen) (Fig C-D-E). This included higher collagen volume fraction (fig 2C), increased collagen type I (Fig 2D) and type III (Fig 2E).

2. Expression of beta3AR regulates paracrine signalling from cardiac myocytes to fibroblasts

As the expression of the beta3AR in our transgenic models was either upregulated or deleted specifically in cardiac myocytes (CM), and in absence of changes in apoptosis, we reasoned that the fibrotic phenotype may have resulted from altered paracrine signalling between these cells and neighboring fibroblasts. To examine this hypothesis, we developed an in vitro superfusion model (Fig 3A) in which cardiac fibroblasts were incubated in culture media conditioned by CM expressing (or not) the human ADRB3 after infection with a recombinant adenovirus. Conditioned media from non-infected CM or CM infected with GFP were used as controls. Note that heterologous expression of ADRB3 did not alter the expression of endogenous
Adrb1 or Adrb2 in CM (Suppl Fig 1 E-F). When control CM (non-infected or GFP-expressing) were pre-stimulated with the alpha-adrenergic agonist, phenylephrine (PE), their conditioned media induced proliferation (Fig 3B) and myofibroblasts differentiation (detected as alpha-smooth muscle actin expression, Fig 3D-E), but no significant effect on migration (Fig 3C) in superfused cardiac fibroblasts. Further analysis of superfused fibroblasts showed that conditioned media from PE-stimulated myocytes activated ERK1/2 phosphorylation, procollagen type 1 mRNA and collagen type 1 protein expression (Suppl Fig 5).

By contrast with control PE-treated cardiac myocytes, the above effects on proliferation, differentiation, ERK1/2 phosphorylation and collagen type 1 expression were not observed in fibroblasts superfused with culture media conditioned by PE-treated cardiac myocytes after adenoviral expression of the human ADRB3.

Proteomic analysis of the cardiac myocytes "secretome"

As these effects of the media conditioned by PE-stimulated CM were abrogated after heating inactivation (Suppl Fig 5D,E), they most probably involved peptide (or peptide-associated) factors in the secretome of CM. In order to identify putative paracrine factors, the conditioned media from GFP- or ADRB3-expressing cardiac myocytes treated (or not) with PE were submitted to shotgun proteomic analysis by liquid chromatography tandem mass spectrometry. Several filters were used to retain only candidate proteins for which solid sequence identification was obtained based on a minimum of two peptides fragments. Principal component analysis allowed to segregate a limited number of candidates. By comparing the variation of candidate proteins that were statistically different between the secretomes of PE-stimulated myocytes expressing GFP versus ADRB3, a number of up- or down-regulated proteins were listed (Table), starting with the most strongly divergent ones. High in the list was CTGF/CCN2, which appeared to be strongly downregulated in the secretome of ADRB3-expressing myocytes. Direct measurement of CTGF by ELISA in the secretomes (Suppl Fig 6A) confirmed significantly lower CTGF content in the media from ADRB3-expressing cardiac myocytes (compared with GFP) after
PE stimulation. Moreover, analysis of extracts of PE-treated cardiac myocytes showed lower Ctgf transcripts and CTGF proteins abundance upon ADRB3 (vs GFP) expression (Suppl Fig 6B-C). Likewise, activation of endogenous beta3AR (i.e. in non-transfected cardiac myocytes) with the beta3AR-specific agonist, CL316243, resulted in significantly reduced Ctgf transcripts levels upon PE treatment (Suppl Fig 6D).

To validate the functional importance of CTGF as paracrine mediator for the effect of PE, CTGF expression was downregulated by siRNA in cardiac myocytes and their conditioned media tested on fibroblasts (Suppl Fig 6E-G). Conditioned media from PE- and siRNA-treated myocytes reduced procollagen 1 expression (Suppl Fig 6 F) (without effect on proliferation, Fig 6E) in superfused fibroblasts (compared with controls). Next, we examined the effect of cardiac ADRB3 on CTGF expression in our transgenic models in vivo. As shown in Fig 4A-B, upon TAC, the expression of CTGF was decreased in cardiac extracts from ADRB3-tg (Fig 4A), but significantly increased in hearts from TAM-treated alpha-MHC-MerCreMer0/+, Adrb3flox/flox (Fig 4B), compared with controls.

3. ROS-dependent production of CTGF in cardiac myocytes: role of beta3AR

The expression of CTGF in fibroblasts is regulated by reactive oxygen species (ROS)-dependent signalling (12-14). Accordingly, when ROS measurements from all mice in our models were correlated with collagen volume fraction (CVF) in the same hearts, linear regression analysis showed a proportional increase in CVF with higher ROS production (Suppl Fig 7E). Further, we observed that treatment of cardiac myocytes with the anti-oxidant N-acetyl-cysteine (NAC) abrogated their expression of CTGF upon PE stimulation (Suppl Fig 7A); and that superfusion of fibroblasts with culture media from such NAC-treated myocytes inhibited their expression of procollagen type 1 (Suppl Fig 7B). Therefore, we reasoned that beta3AR expression may exert anti-oxidant effects in cardiac myocytes resulting in less expression of CTGF under stress. We verified this in homotypic cultures of GFP- or ADRB3-expressing cardiac myocytes, in which ADRB3 expression significantly reduced ROS production after PE stimulation (Suppl Fig 7C).
Consistently, myocardial ROS production was significantly reduced in ADRB3-TG after TAC (Suppl Fig 7D) compared with WT.

4. Neuronal Nitric Oxide Synthase (nNOS) mediates anti-oxidant effects downstream beta3AR and beta3AR’s protective paracrine effects

We next tested whether NOS downstream beta3AR is involved in this anti-oxidant protection. As shown in Fig 5A, specific nNOS inhibition with N5-(1-imino-3-butenyl)-L-ornithine (L-VNIO) increased the ROS signals and, importantly, abrogated the protective effect of beta3AR expression. This suggested that the anti-oxidant effect of beta3AR is mediated by activation of nNOS in cardiac myocytes.

To verify the impact of nNOS-mediated anti-oxidant effect on paracrine signalling, culture media conditioned by myocytes treated (or not) with L-VNIO and stimulated with PE were incubated on fibroblasts, from which the expression of procollagen type 1 was measured. As shown in Fig 5B, co-treatment of myocytes with L-VNIO and PE fully abrogated the inhibitory effect of beta3AR on procollagen 1 expression in fibroblasts (whereas L-VNIO alone had no independent effect). We confirmed the involvement of nNOS by analysing ROS production from myocardial extracts from ADRB3-tg (vs WT) mice submitted (or not) to TAC (Fig 5C); treatment of extracts with L-VNIO strongly increased ROS signals from TAC hearts and, notably, abrogated the protection previously observed in ADRB3-tg. To add genetic proof of the causal involvement of Nos1, we cross-bred our ADRB3-tg with Nos1<sup>-/-</sup> mice and examined their phenotype at 3 weeks post-TAC (compared to their respective controls). As shown in Fig 5D, although ADRB3<sup>+/0</sup>; Nos1<sup>+/+</sup> (TG/WT) developed less fibrosis than WT/Nos1<sup>+/+</sup> (WT/WT) controls, illustrating again the protection by cardiac ADRB3 expression, this protection was completely lost in ADRB3<sup>+/0</sup>; Nos1<sup>-/-</sup> (TG/nNOS KO) compared with WT/Nos1<sup>-/-</sup> (WT/nNOS KO).

5. Beta3AR expression is inversely related with myocardial fibrosis in human ventricular biopsies
Finally, we compared the abundance of ADRB3 mRNA and myocardial fibrosis, measured as COL1A1 and COL3A1 mRNA expression ex vivo in ventricular biopsies obtained at the time of operation in patients that underwent valve surgery. Patients characteristics are reported in Suppl Table 1. As shown in Fig 6 A, the expression of ADRB3 was almost double in biopsies with the lowest COL1A1 levels, and a similar trend was seen in biopsies with lowest COLIA3 (Fig 6B). Of note, CTGF expression was also closely related to COL1A1 (Fig 6C) and COLIA3 (Fig 6D) expression in the same biopsies.

**DISCUSSION**

The main findings of this study are as following; i. expression of the human ADRB3 in cardiac myocytes (ADRB3-tg) attenuates cardiac fibrosis in response to hemodynamic (TAC) stress; ii. conversely, Adrb3 genetic deletion specifically in cardiac myocytes (Tamoxifen-treated alpha-MHCMerCreMer/Adrb3fl/fl mice) exacerbates TAC-induced cardiac fibrosis; iii. the protection by ADRB3 expression is replicated by superfusion of cardiac fibroblasts with the secretome of ADRB3-expressing myocytes, implying that beta3AR modulates paracrine signallning to attenuate the pro-fibrotic phenotype; iv. proteomic analysis of the secretome from ADRB3-expressing cardiac myocytes identifies downregulation of several secreted growth factors or matricellular proteins involved in fibrosis; among these, CTGF production is reduced by ADRB3 expression in myocytes in vitro and in vivo after TAC; v. this reduction is mediated by the anti-oxidant effect of beta3AR through its coupling to neuronal NOS (nNOS); vi. accordingly, the beta3AR protection from TAC-induced fibrosis is lost in Nos1-deficient mice (Fig 7); vii. in human ventricular biopsies, ADRB3 expression is inversely related to the degree of myocardial fibrosis.

The pattern of diffuse fibrosis post-TAC is accompanied with early proliferation (days 4-7) of specific resident fibroblasts populations in situ. This underscores the importance of local paracrine signallning for the control of the fibrogenic response, as suggested from our data. Several signalling peptides were identified as differentially regulated by the beta3AR in the myocyte secretome, many of which regulate TGFbeta signalling, such as the latent TGFbeta-binding
protein 2 (Table). CTGF (CCN2), also downregulated by the beta3AR in our study, has been widely implicated in fibrogenesis in many organs, including the heart (15). Although both cytokines can be produced by cardiac myocytes, CTGF does not simply replicate or function as a downstream effector of TGFbeta signalling but its effects vary according to the type of stress or cytokines context (16). Accordingly, genetic models with cardiac-specific CTGF overexpression (17-19) or Ctgf deletion (19) have yielded divergent remodeling phenotypes depending on the type of stress imposed (e.g. ischemia/reperfusion vs. Angiotensin II infusion vs. TAC). Nevertheless, cardiac-specific Ctgf deletion did attenuate fibrosis in the context of TAC and TGFbeta overexpression (19). Likewise, siRNA downregulation of Ctgf in our study significantly attenuated the pro-fibrotic effect of PE (Suppl Fig 6), albeit not completely. Although compensatory increases in other factors probably account for the incomplete phenotypes in these genetic experiments, this also underscores the role of CCN2 as a modulator, rather than mediator of cardiac fibrosis. As other members of the CCN group, CCN2 contains 4 distinct modules, including a thrombospondin homology domain (module III) and heparin binding domain (module IV) that enable its modulation of the signalling of other co-secreted molecules (20). One of these is thrombospondin-1 (TSP1)(21) that was similarly down-regulated as CCN2 by ADRB3 expression in our model. TSP1 and CCN2 also control extracellular matrix deposition, consistent with our observation of parallel downregulation of fibronectin, collagen type 1 and 3, laminin and fibrillin-1 (Table). Another regulator of extracellular matrix remodelling, Plasminogen Activator Inhibitor-1 (PAI1) was also downregulated by ADRB3 expression. Notably, PAI1 expression is activated by systemic NOS inhibition (22) and downregulated by administration of blocking antibodies targeting CCN2/CTGF in the TAC model (23), suggesting that cardiac NOS may prevent fibrosis by inhibiting CCN2/CTGF and subsequent PAI-1 and collagen production. As shown in the Table, ADRB3 expression reciprocally increased the expression of chondroitin sulfate proteoglycan 4 (also known as NG2).NG2 binds PDGF-AA (24) and promotes angiogenesis through autocrine regulation of VEGF expression (25). Upregulation of NG2 by ADRB3 could explain our previous observations of pro-angiogenic effects of beta3AR activation (26).
Cardiac fibrosis and CTGF expression were correlated with ROS in our models, as observed by others e.g. in human myxomatous mitral valve remodelling (13). Notably, both were exacerbated upon nNOS inhibition, which also abrogated the protection by ADRB3 expression in vitro and in vivo. We and others previously demonstrated beta3AR coupling to NOS in cardiac myocytes (27, 28), including eNOS and nNOS (29, 30). The latter protects eNOS from oxidative uncoupling by S-glutathionylation, thereby maintaining NO bioavailability and downstream signalling (31). nNOS, in turn, was shown to inhibit ROS production in cardiac myocytes through cGMP-dependent inactivation of xanthine oxidoreductase (32).

The relevance of these findings to human cardiac remodelling is reinforced by our additional observation that myocardial fibrosis is lower in patients with higher ADRB3 expression in ventricular biopsies. We had previously shown that contrary to beta1-2ARs, the expression of beta3AR is upregulated in stressed cardiac myocytes from rodents (33) and humans (34), possibly as a protective mechanism. Notably, the coupling of beta3AR may be preserved under adrenergic stress because of relative resistance to desensitization (35). This makes beta3AR an attractive target for the therapeutic use of new, more specific beta3AR agonists currently in clinical use for non-cardiovascular indications (36). One of these, mirabegron, is currently tested for “re-purposing” in patients with structural cardiac disease at risk of developing heart failure with preserved ejection fraction (ClinicalTrials.gov NCT02599480), for which myocardial fibrosis is a key pathogenic factor. Of note, beta3AR is robustly expressed in human atrial muscle (37), where ROS and fibrosis are clearly implicated in the generation and maintenance of atrial fibrillation (38, 39) Therefore, our findings may guide future therapeutic uses of current and new specific beta3AR agonists for myocardial protection.

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**Conflict of interest:** none declared

**REFERENCES**


Figure Legends

Figure 1. β3-AR expression in cardiac myocytes prevents the development of myocardial fibrosis under pressure overload.

A) (Upper) Representative pictures of pressure overload-induced cardiac hypertrophy in mouse hearts from wild-type controls (WT) and heterozygous ADRB3-tg (TG) after transverse aortic constriction (TAC) at 9 weeks; (Lower) Left ventricular mass (LVM) normalized to tibial length (TL) from WT and ADRB3-tg (TG) post-TAC or Sham surgery.

B) (Upper) Myocardial fibrosis (picrosirius red) in a WT mouse (left) and an heterozygous ADRB3-tg (TG) transgenic mouse (right) after TAC (Magnification x20; scale: 100 µm). (Lower) Collagen volume fraction from 3 sections per heart.

C) (Upper) Immunostaining for collagen type I (immunoperoxidase method) in cardiac sections from a WT mouse (left) and an ADRB3-tg transgenic mouse (right) after TAC. (Lower) Collagen volume fraction from 3 sections per heart.

D) (Upper) Immunoblotting for collagen type 1 in cardiac extracts from ADRB3-tg compared with WT mice after TAC. (Lower) Densitometric quantification of immunoblots.

(A-D) dot-plots represent data from individual animals, as well as mean ± SEM (n=5-10 mice per group). Statistical significance was determined by 2-way ANOVA followed by Tukey’s multiple comparison test.
Figure 2. β3-AR deletion in mouse cardiac myocytes exacerbates the development of myocardial fibrosis under pressure overload.

A) Characterization of Adrb3\textsuperscript{flox/flox} (b\textsubscript{3}\textsuperscript{fl/fl}) mice. (Upper) PCR strategy to detect allele recombination after tamoxifen treatment of b\textsubscript{3}\textsuperscript{flox/flox} /aMHC-MerCreMer mice. Recombination results in the amplification of a “null” gene band of 300 bp. (Lower) Recombination PCR from heart (left) and liver (right): the “null” allele appears only in heart, but not liver from tamoxifen-treated b\textsubscript{3}\textsuperscript{flox/flox} /aMHC-MerCreMer mice, and not in tamoxifen-treated b\textsubscript{3}\textsuperscript{flox/flox} only mice.

B) Left ventricular mass (LVM) normalized to tibial length (TL) from b\textsubscript{3}\textsuperscript{flox/flox} /aMHC-MerCreMer mice (Cre-b\textsubscript{3}\textsuperscript{fl/fl}) treated with tamoxifen (Tam) or not (Veh) post TAC or Sham surgery.

C-D-E: Cardiac myocyte-specific Adrb3 deletion exacerbates myocardial fibrosis after TAC. C) (Upper) Myocardial fibrosis (picrosirius red) in Cre-b\textsubscript{3}\textsuperscript{fl/fl} treated with tamoxifen (Tam, right) or not (Veh, left panel) to induce Adrb3 deletion and submitted to TAC (Magnification x20; scale bar: 100 μm). (Lower) Collagen volume fraction from 3 sections per heart. D-E: Collagen type I (D) and collagen type III (E) mRNA expression in cardiac extracts from Cre-b\textsubscript{3}\textsuperscript{fl/fl} and b\textsubscript{3}\textsuperscript{fl/fl} treated with tamoxifen (Tam) or not (Veh) post TAC or Sham surgery. Dot-plots represent data from individual animals, as well as mean ± SEM (n=6-8 mice per group). Statistical significance was determined by 2-way ANOVA followed by Tukey’s multiple comparison test.

Figure 3. Expression of ADRB3 in cardiac myocytes attenuates their paracrine pro-fibrotic effect on fibroblasts.

A) Schematic representation of the in vitro superfusion assay for paracrine signaling between neonatal cardiac myocytes (CM) and neonatal cardiac fibroblasts (CF). CF in serum-free conditions were incubated 24 hours with media conditioned by cultured CM after adenoviral transduction of the
human *ADRB3* or *GFP* (Ad-GFP, Ad-beta3) and treated or not with phenylephrine (PE+/−).

Conditioned media from non-infected (NI) and GFP-expressing CM treated or not with PE were used as controls. As no difference was observed between NI and GFP-CM, only the results with the latter are presented in subsequent Figures.

(B-E) Effects of conditioned media (secretome, CMsec) from cardiac myocytes (CM) expressing *ADRB3* (b3) or GFP, treated with phenylephrine (PE) (or vehicle, Veh) on (B) cardiac fibroblasts proliferation, (C) serum-induced migration (from trans-well assay, below), and (D-E) expression of α-smooth muscle actin transcripts (D) quantified by RT-qPCR and proteins (E) by immunostaining (below), calculated as the mean intensity of red labeling (α-SMA) normalized to the number of cells (DAPI); n=3 different preparations; Mann-Whitney. Data are expressed as fold-change over values in control CF incubated in CMsec from vehicle-treated GFP-expressing CM (CMsec GFP). Dot-plots represent data from biological replicates as well as mean ± SEM (B-D) n=5-6 different preparations; 2-way ANOVA followed by Tukey’s multiple comparison test.

**Figure 4. Adb3 regulates myocardial expression of CTGF after TAC in vivo.**

(A) Myocardial expression of CTGF protein after TAC is reduced in *ADRB3*-TG vs. WT controls. (B) Conversely, myocardial CTGF after TAC is increased after cardiac myocyte-specific ablation of *Adb3* (TAM-treated b3^fl/fl^, aMHC-MerCreMer^0/+^ [Cre-b3^fl/fl^ Tam] vs vehicle-treated b3^fl/fl^,aMHC-MerCreMer^0/+^ [Cre-b3^fl/fl^ Veh]. (A, B, Upper) Western blots of CTGF, normalized to hsp90. (A, B, lower) Quantification reported as densitometric arbitrary units. A-B: Dot-plots represent biological replicates, as well as mean ± SEM from 5-8 mice. Statistical significance was determined by 2-way ANOVA followed by Tukey’s multiple comparison test.

**Figure 5. The anti-oxidant and anti-fibrotic effect of cardiac ADRB3 is mediated by nNOS**
A) ROS measurements (DCF fluorescence, as fold change over GFP-Veh) in cardiac myocytes (CM) expressing \textit{ADRB3} (b3) or GFP, stimulated (or not) with phenylephrine (PE), after incubation with the nNOS inhibitor, L-VNIO (or vehicle, Veh). (n=4 preparations).

B) Pro-collagen type 1 mRNA expression in cardiac fibroblasts upon incubation with secretomes from cardiac myocytes expressing \textit{ADRB3} (or GFP), treated with LVNIO (or vehicle, Veh) and stimulated (or not) with PE. Data are reported as fold change over untreated control (Veh) (n=4 preparations).

C) ROS production (lucigenin assay) in left ventricular tissue homogenates from WT (left) and \textit{ADRB3}-TG mice (right) after TAC or SHAM operation (n=6 hearts per group).

D) Myocardial fibrosis (collagen volume fraction) in hearts from \textit{ADRB3}^{-}tg or \textit{ADRB3}^{-/}^{-};\textit{Nos1}^{-/-} mice (vs respective littermate controls) after TAC or SHAM operation. (n=5-7 mice per group). A through D, by 2-way ANOVA followed by Tukey’s multiple comparison test.

\textbf{Figure 6. The abundance of beta3AR is inversely related to myocardial fibrosis in human ventricular biopsies from patients with valvular disease}

(A-B) Comparison of \textit{ADRB3} mRNA abundance in ventricular biopsies from patients with high or low Collagen type I (A) and Collagen type III (B) mRNA expression (n=10 and 11, respectively). Statistical significance was determined by unpaired t-test. Univariate correlation between \textit{CTGF} mRNA and Collagen type I (C) and Collagen type III (D) in the 21 biopsies.

\textbf{Figure 7. Cardiac beta3AR modulates stress-induced paracrine signaling to fibroblasts}

Hemodynamic or neurohormonal stress on cardiac myocytes induces their production of cytokines and matricellular proteins that activate fibroblasts, resulting in interstitial fibrosis. Cardiac beta3AR modulates paracrine signals to reduce fibrosis. In particular, beta3AR coupling to neuronal NOS
reduces stress-induced ROS production and downstream Ctgf/Ccn2 transcripts and protein expression (among others), thereby attenuating the synergistic effects of CTGF on paracrine profibrotic factors.
**Table**: Differential regulation of proteins identified in the secretome of ADRB3-expressing cardiac myocytes compared with GFP controls

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<tr>
<td>Thrombospondin-1</td>
<td>TSP1_MOUSE</td>
<td>10</td>
<td>669.94</td>
<td>0.51</td>
<td>1.09E-10</td>
<td>1.660</td>
<td>5.040</td>
<td>1.830</td>
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<td>Connective tissue growth factor</td>
<td>CTGF_RAT</td>
<td>9</td>
<td>495.18</td>
<td>0.37</td>
<td>2.57E-09</td>
<td>0.993</td>
<td>9.410</td>
<td>2.000</td>
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<td>Galectin-3-binding protein</td>
<td>LG3BP_RAT</td>
<td>7</td>
<td>474.64</td>
<td>0.50</td>
<td>5.58E-06</td>
<td>2.270</td>
<td>1.670</td>
<td>1.600</td>
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<tr>
<td>Collagen alpha-1(III) chain</td>
<td>CO3A1_RAT</td>
<td>5</td>
<td>298.65</td>
<td>0.54</td>
<td>3.60E-05</td>
<td>0.774</td>
<td>0.821</td>
<td>1.020</td>
<td>0.444</td>
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<td>Laminin subunit gamma-1</td>
<td>LAMC1_MOUSE</td>
<td>4</td>
<td>289.89</td>
<td>0.49</td>
<td>4.67E-04</td>
<td>0.832</td>
<td>0.879</td>
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<td>Lactadherin</td>
<td>MFGM_RAT</td>
<td>5</td>
<td>267.14</td>
<td>0.39</td>
<td>4.70E-07</td>
<td>0.430</td>
<td>0.615</td>
<td>0.475</td>
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<tr>
<td>Clusterin</td>
<td>CLUS_RAT</td>
<td>5</td>
<td>263.59</td>
<td>0.40</td>
<td>0.01</td>
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<td>Rab GDP dissociation inhibitor beta</td>
<td>GDIB_MOUSE</td>
<td>6</td>
<td>260.84</td>
<td>2.94</td>
<td>1.25E-04</td>
<td>0.400</td>
<td>0.244</td>
<td>0.372</td>
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<td>Superoxide dismutase [Cu-Zn]</td>
<td>SODC_RAT</td>
<td>2</td>
<td>223.52</td>
<td>3.49</td>
<td>4.26E-07</td>
<td>2.380</td>
<td>1.550</td>
<td>2.360</td>
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<tr>
<td>Chondroitin sulfate proteoglycan 4</td>
<td>CSPG4_RAT</td>
<td>6</td>
<td>213.96</td>
<td>1.65</td>
<td>4.05E-09</td>
<td>0.149</td>
<td>0.384</td>
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<td>Collagen alpha-1(I) chain</td>
<td>CO1A1_MOUSE</td>
<td>3</td>
<td>212.38</td>
<td>0.51</td>
<td>4.07E-04</td>
<td>0.901</td>
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<td>Plasminogen activator inhibitor 1</td>
<td>PAI1_RAT</td>
<td>3</td>
<td>210.49</td>
<td>0.60</td>
<td>3.26E-05</td>
<td>0.684</td>
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<td>Peroxiredoxin-1</td>
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<td>3</td>
<td>179.82</td>
<td>3.33</td>
<td>4.12E-04</td>
<td>0.787</td>
<td>0.628</td>
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<td>Amyloid beta A4 protein</td>
<td>A4_MOUSE</td>
<td>4</td>
<td>143.25</td>
<td>0.39</td>
<td>1.30E-09</td>
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<td>Latent-transforming growth factor beta-binding protein 2</td>
<td>LTPB2_RAT</td>
<td>2</td>
<td>124.83</td>
<td>0.48</td>
<td>7.82E-04</td>
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<td>0.085</td>
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<td>Fibrillin-1</td>
<td>FBN1_MOUSE</td>
<td>3</td>
<td>116.42</td>
<td>0.42</td>
<td>2.72E-05</td>
<td>0.294</td>
<td>0.318</td>
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<td>Laminin subunit beta-2</td>
<td>LAMB2_RAT</td>
<td>3</td>
<td>99.85</td>
<td>0.52</td>
<td>1.57E-03</td>
<td>0.134</td>
<td>0.098</td>
<td>0.104</td>
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</tbody>
</table>

Cardiac myocytes were transfected with an adenovirus coding ADRB3 (B3) or GFP, then treated with phenylephrine (PE) or vehicle for 24h. Proteome profiling was performed on the myocytes-conditioned media. Listed are proteins unequivocally quantified based on at least 2 peptides. The mean of normalized abundance (based on 3 independent experiments) was used to calculate the ratio B3-PE/GFP-PE.
Figure 3

A. Cardiac myocytes (CM) and Cardiac fibroblasts (CF) incubation for 24 hours.

B. Proliferation: CMsec GFP and CMsec β3 with Veh or PE.

C. Migration: CMsec GFP and CMsec β3 with Veh or PE.

D. \( \alpha \)-SMA: CMsec GFP and CMsec β3 with Veh or PE.

E. \( \alpha \)-SMA mean intensity: CMsec GFP and CMsec β3 with Veh or PE.

NO SERUM GRADIENT

- CMsec GFP
- CM+PEsec GFP
- CMsec β3
- CM+PEsec β3

SERUM GRADIENT

- CMsec GFP
- CM+PEsec GFP
- CMsec β3
- CM+PEsec β3

P-values:
- B: P=0.0499, P=0.0341
- D: P=0.0235, P=0.0453
- E: P=0.0495
Figure 4

A

CTGF
HSP90

P = 0.0908
P = 0.0011

CTGF (A.U.)

Sham TAC Sham TAC
WT TG

B

CTGF
HSP90

P = 0.0001
P < 0.0001

CTGF (A.U.)

Sham TAC Sham TAC
Cre-b3fl/fl Veh Cre-b3fl/fl Tam
Fig 5

A. Cardiac Myocytes

B. Cardiac Fibroblasts

- ROS Production (Fluorescence intensity, Fold change)
- Procollagen type I (Fold change)

C. ROS Production (Relative light units)

D. Collagen Volume Fraction (%)
Fig 6

A

β3AR expression normalized on GAPDH

High Collagen Type I
Low Collagen Type I

P=0.0172

B

β3AR expression normalized on GAPDH

High Collagen Type III
Low Collagen Type III

C

CTGF mRNA (normalized on GAPDH)

Collagen type I mRNA (normalized on GAPDH)

r = 0.887
P < 0.0001

D

CTGF mRNA (normalized on GAPDH)

Collagen type III mRNA (normalized on GAPDH)

r = 0.7281
P < 0.0002
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