

# Cardiac myocyte $\beta$ 3-adrenergic receptors prevent myocardial fibrosis by modulating oxidant stress-dependent paracrine signaling

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## 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 remodelling, particularly fibrosis in response to haemodynamic 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 haemodynamic 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 connective tissue growth factor [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 haemodynamic 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

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## Translational perspective

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

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.

Cardiac beta3 AR exerts effects that are antipathetic to those of 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.

## Introduction

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

Recent lineage tracing experiments have identified specific populations of fibroblasts that contribute myocardial fibrosis.<sup>5,6</sup> They concluded to a major participation of resident cardiac fibroblasts developmentally deriving from epicardium or endocardial endothelium.<sup>6</sup> Upon pressure overload, local proliferation and subsequent myodifferentiation of these resident fibroblasts produce myocardial fibrosis, emphasizing the importance of signalling within 'fibrogenic niches'.<sup>7</sup> 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,<sup>8</sup> in which their contractile effects are antipathetic to those of beta1-2AR.<sup>9</sup> Although prolonged activation or high heterologous expression of beta1AR exacerbates adverse myocardial remodeling,<sup>10</sup> this opposing effect of beta3AR suggests unique protective properties for this isotype.<sup>11</sup> 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 connective tissue growth factor (CTGF)/CCN2 expression was downregulated by beta3AR through its coupling to neuronal nitric oxide synthase (nNOS) and anti-oxidant effects in cardiac myocytes.

## Methods

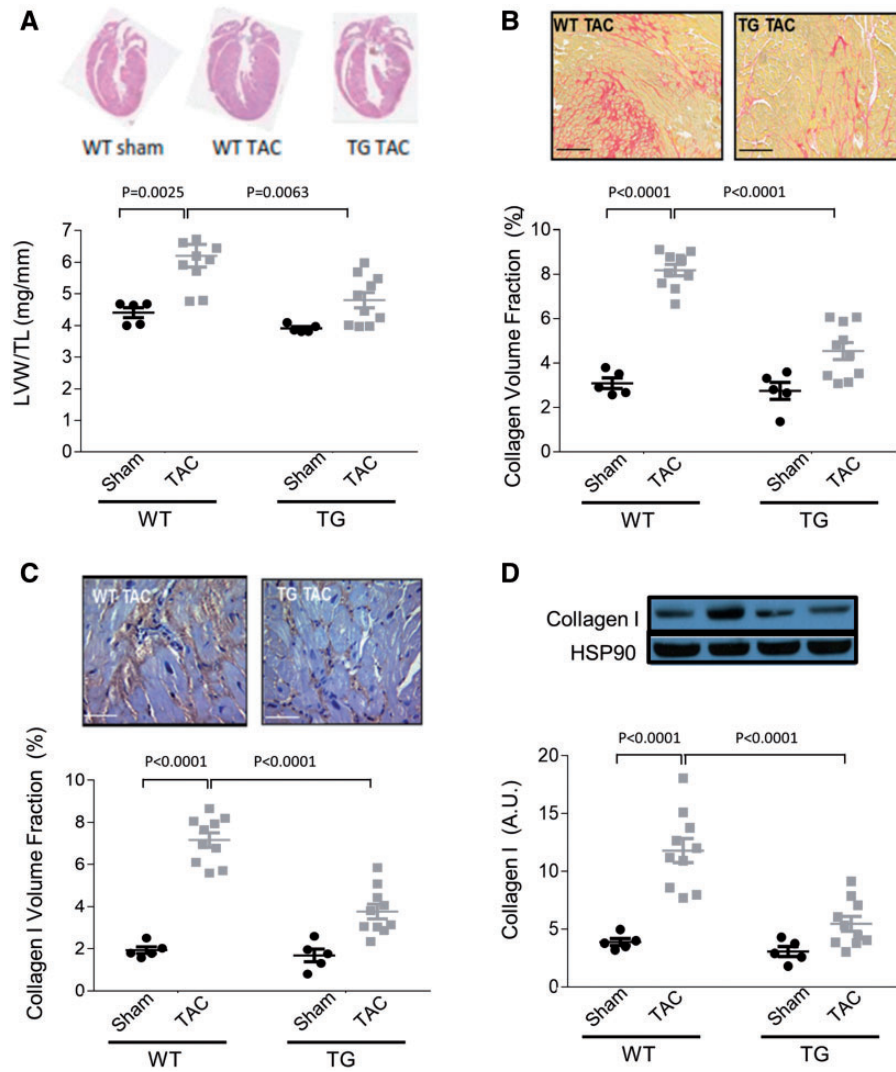
Expanded methods are available in the *Supplementary material online*.

## Results

### Expression of the beta3-adrenergic receptors in cardiac myocytes protects from myocardial fibrosis under haemodynamic 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 *Figure 1A*. Note that, in our model, the abundance of transgenic human beta3AR proteins was comparable to that observed in human ventricular extracts (see Supplementary material online, *Figure S1G*) and that transcripts levels of *Adrb1* and *Adrb2* were unchanged between *ADRB3*-tg and WT, both at baseline and after TAC (see Supplementary material online, *Figure S1A* and *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 (see Supplementary material online, *Figure S2*). *ADRB3*-tg mice developed a moderate hypertrophic response which was significantly milder than WT (*Figure 1A*). Histological analysis showed that myocardial fibrosis was strikingly attenuated in *ADRB3*-tg (*Figure 1B*). This was confirmed by quantification of collagen volume fraction, as well as of collagen type 1 both by immunohistochemical analysis and western blotting (*Figure 1C* and *D*). Capillary density and inflammatory cells (CD45<sup>+</sup>) infiltration were comparable between genotypes. No differences in apoptosis were observed between genotypes 9 weeks post-TAC (see Supplementary material online, *Figure S3*).

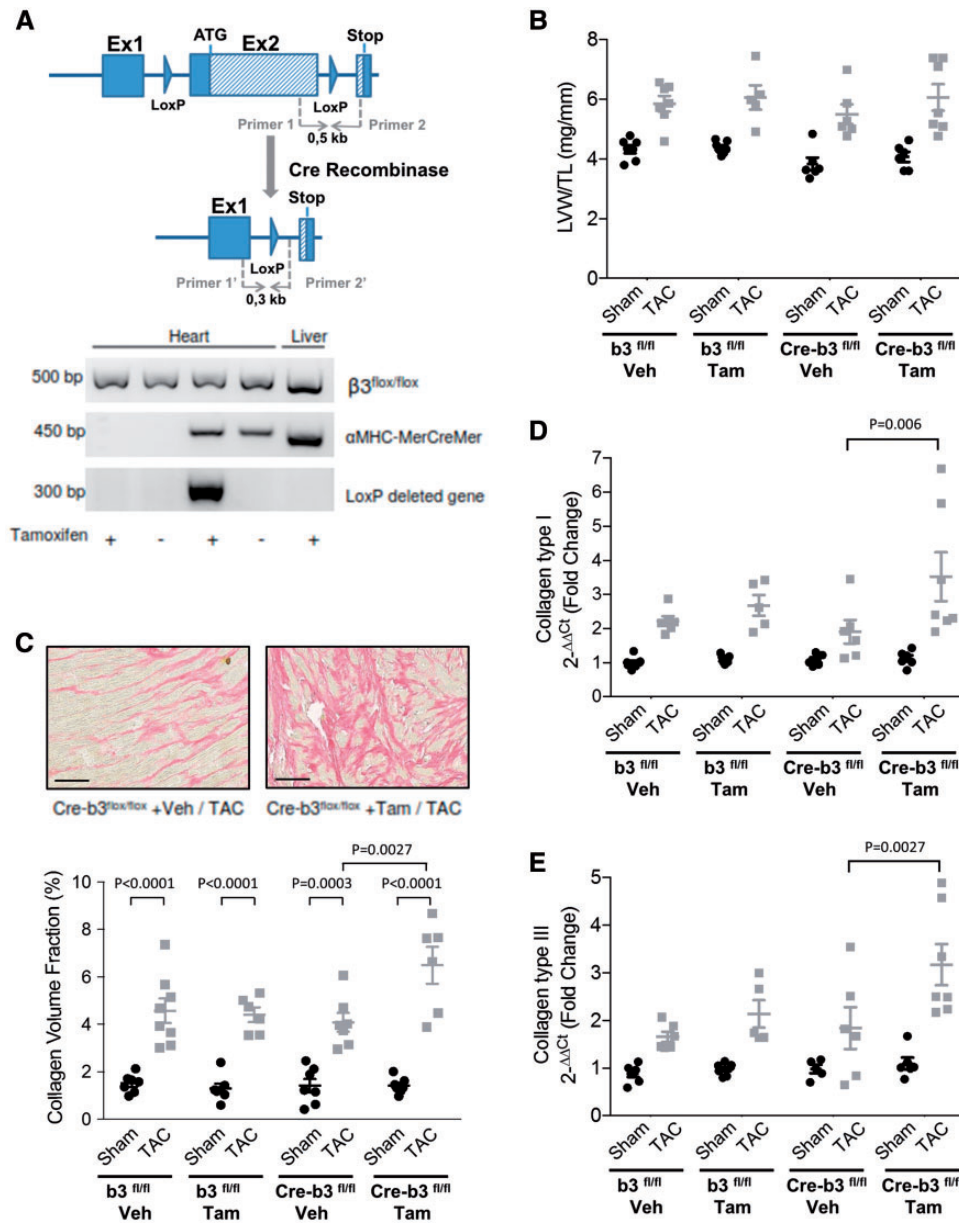
To further confirm the causal role of the cardiac *Adrb3*, we analyzed the phenotype of mice with cardiac myocyte-specific deletion of the mouse *Adrb3* using an inducible Cre-lox system. Mice with exon 2 of *Adrb3* flanked by 2 lox-P sites were generated and back-crossed in the C57Bl/6j background for 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) (*Figure 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



**Figure 1**  $\beta$ 3-adrenergic receptors 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  $\times 20$ ; scale: 100  $\mu$ m). (Lower) Collagen volume fraction from three 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 three sections per heart. (D) (Upper) Immunoblotting for collagen type I 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  $\pm$  SEM ( $n = 5$ –10 mice per group). Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparison test.

in double-transgenic mice but did not *per se* induce fibrosis in all controls tested (see Supplementary material online, Figure S4). Heterozygous *alpha-MHC-MerCreMer*<sup>+/+</sup>, homozygous *Adrb3*<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> and double-transgenic mice without tamoxifen were submitted to TAC (or sham) in parallel as controls. Note that transcripts levels of *Adrb1* and *Adrb2* were unchanged between single or double transgenics with tamoxifen, both at baseline and after TAC (see Supplementary material online, Figure S1C and D). As expected,

single transgenic mice and double transgenics without tamoxifen developed TAC-induced hypertrophy (Figure 2B) and fibrosis (Figure 2C–E) to a comparable extent. However, after tamoxifen treatment, double transgenics developed similar hypertrophy (Figure 2B) but a higher degree of fibrosis compared with all other controls (including double transgenics without tamoxifen) (see Supplementary material online, Figure C–E). This included higher collagen volume fraction (Figure 2C), increased collagen type I (Figure 2D) and type III (Figure 2E).



**Figure 2**  $\beta 3$ -adrenergic receptors deletion in mouse cardiac myocytes exacerbates the development of myocardial fibrosis under pressure overload. (A) Characterization of *Adrb3*<sup>flx/flx</sup> ( $b3^{fl/fl}$ ) mice. (Upper) PCR strategy to detect allele recombination after tamoxifen treatment of  $b3^{flx/flx}$ /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  $b3^{flx/flx}$ /aMHC-MerCreMer mice, and not in tamoxifen-treated  $b3^{flx/flx}$  only mice. (B) Left ventricular mass (LVM) normalized to tibial length (TL) from  $b3^{flx/flx}$ /aMHC-MerCreMer mice (Cre- $b3^{fl/fl}$ ) 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 (picosirius red) in Cre- $b3^{fl/fl}$  treated with tamoxifen (Tam, right) or not (Veh, left panel) to induce *Adrb3* deletion and submitted to TAC (Magnification  $\times 20$ ; scale bar: 100  $\mu m$ ). (Lower) Collagen volume fraction from three sections per heart. (D, E) Collagen type I (D) and collagen type III (E) mRNA expression in cardiac extracts from Cre- $b3^{fl/fl}$  and  $b3^{fl/fl}$  treated with tamoxifen (Tam) or not (Veh) post TAC or Sham surgery. Dot-plots represent data from individual animals, as well as mean  $\pm$  SEM ( $n = 6-8$  mice per group). Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparison test.

## Expression of beta3-adrenergic receptors 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 (Figure 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 (see Supplementary material online, Figure S1E and F). When control CM (non-infected or GFP-expressing) were pre-stimulated with the alpha-adrenergic agonist, phenylephrine (PE), their conditioned media induced proliferation (Figure 3B) and myofibroblasts differentiation (detected as alpha-smooth muscle actin expression, Figure 3D and E), but no significant effect on migration (Figure 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 (see Supplementary material online, Figure S5).

In 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 (see Supplementary material online, Figure S5D and 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 vs. *ADRB3*, a number of up- or down-regulated proteins were listed (Table 1), 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 (see Supplementary material online, Figure S6A) 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 (see Supplementary material online, Figure S6B and 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 (see Supplementary material online, Figure S6D).

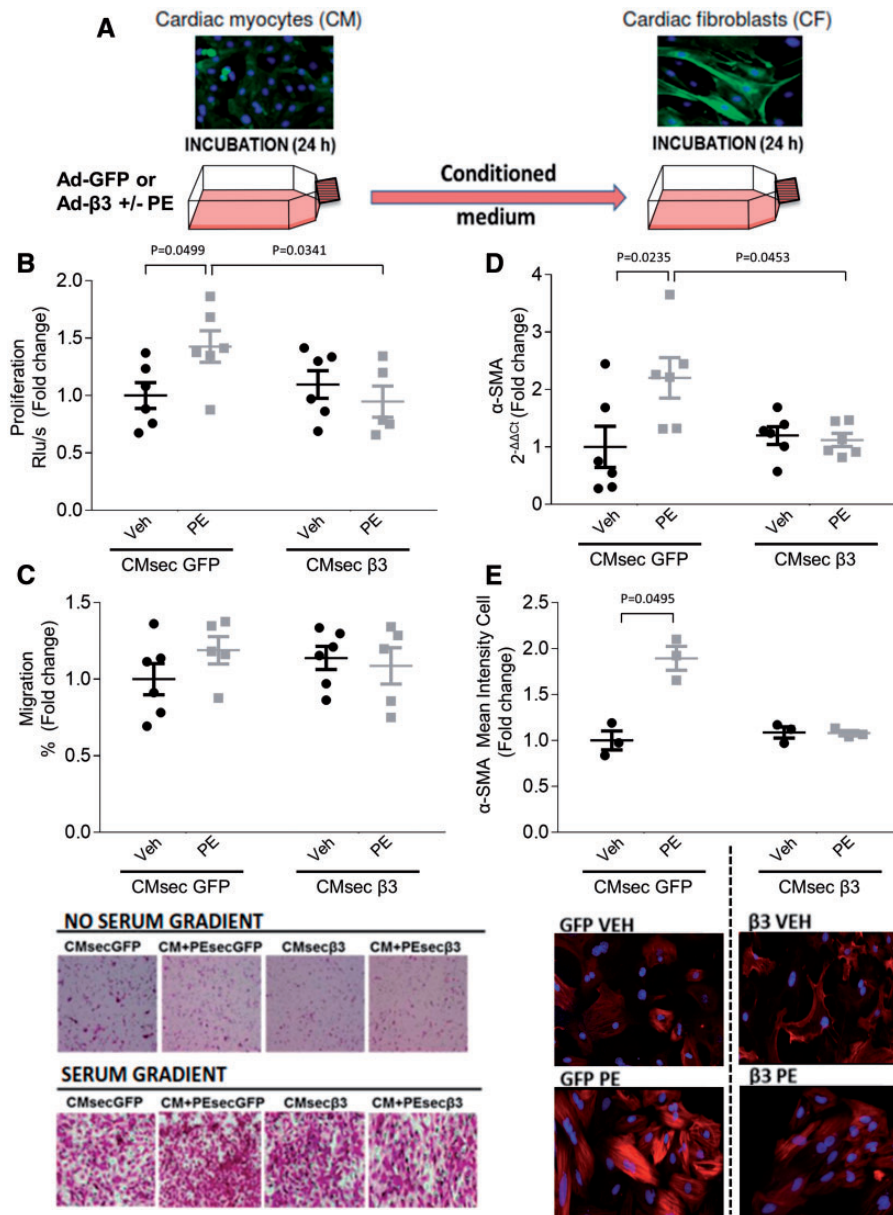
To validate the functional importance of CTGF as paracrine mediator for the effect of PE, CTGF expression was downregulated by siRNA in cardiac myocytes and their conditioned media tested on fibroblasts (see Supplementary material online, Figure S6E and G). Conditioned media from PE- and siRNA-treated myocytes reduced procollagen 1 expression (see Supplementary material online, Figure S6F) (without effect on proliferation, Figure 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 Figure 4A and B, upon TAC, the expression of CTGF was decreased in cardiac extracts from *ADRB3*-tg (Figure 4A), but significantly increased in hearts from TAM-treated *alpha-MHC-MerCreMer*<sup>0/+</sup>; *Adrb3*<sup>flx/flx</sup> (Figure 4B), compared with controls.

## Reactive oxygen species-dependent production of connective tissue growth factor in cardiac myocytes: role of beta3AR

The expression of CTGF in fibroblasts is regulated by reactive oxygen species (ROS)-dependent signalling.<sup>12–14</sup> 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 (see Supplementary material online, Figure S7E). Further, we observed that treatment of cardiac myocytes with the anti-oxidant N-acetyl-cysteine (NAC) abrogated their expression of CTGF upon PE stimulation (see Supplementary material online, Figure S7A); and that superfusion of fibroblasts with culture media from such NAC-treated myocytes inhibited their expression of procollagen type 1 (see Supplementary material online, Figure S7B). 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 (see Supplementary material online, Figure S7C). Consistently, myocardial ROS production was significantly reduced in *ADRB3*-TG after TAC (see Supplementary material online, Figure S7D) compared with WT.

## Neuronal nitric oxide synthase mediates anti-oxidant effects downstream beta3-adrenergic receptor and beta3-adrenergic receptor's protective paracrine effects

We next tested whether NOS downstream beta3AR is involved in this anti-oxidant protection. As shown in Figure 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.

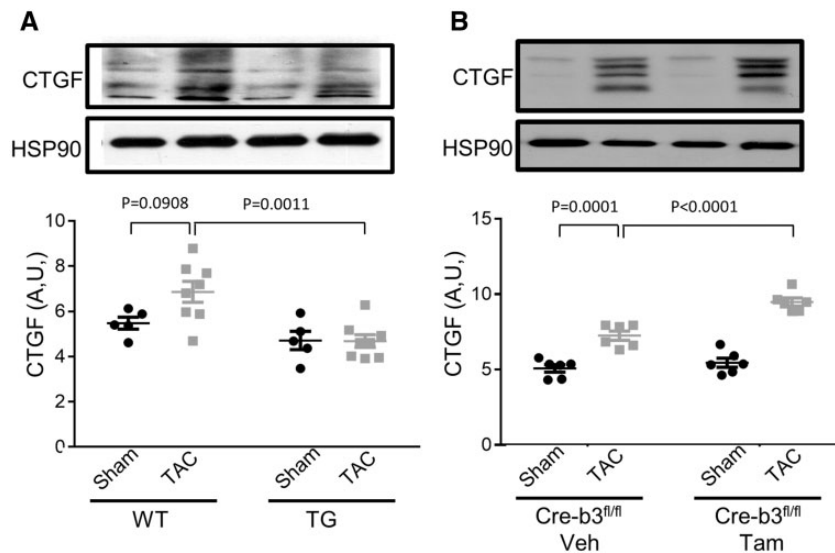


**Figure 3** Expression of ADRB3 in cardiac myocytes attenuates their paracrine pro-fibrotic effect on fibroblasts. (A) Schematic representation of the *in vitro* superfusion assay for paracrine signaling between neonatal cardiac myocytes (CM) and neonatal cardiac fibroblasts (CF). CF in serum-free conditions were incubated 24 h with media conditioned by cultured CM after adenoviral transduction of the human ADRB3 or GFP (Ad-GFP, Ad-β3) 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 (β3) 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  $\pm$  SEM (B–D)  $n = 5–6$  different preparations; two-way ANOVA followed by Tukey’s multiple comparison test.

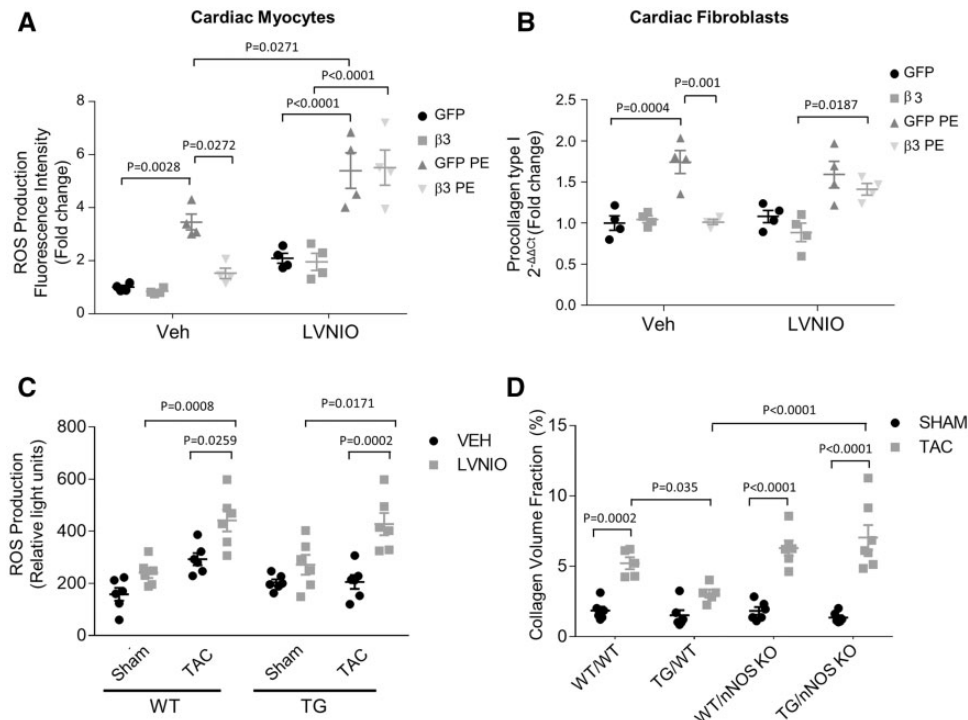
**Table 1** Differential regulation of proteins identified in the secretome of ADRB3-expressing cardiac myocytes compared with GFP controls

Protein name	UniProt ID	Peptide count	Confidence score	Ratio B3-PE vs. GFP-PE	ANOVA (p)	GFP ( $\times 10^6$ )	GFP-PE ( $\times 10^6$ )	B3 ( $\times 10^6$ )	B3-PE ( $\times 10^6$ )
Fibronectin	FINC_RAT	17	1190.61	0.48	2.77E-09	6.250	7.860	6.520	3.800
Thrombospondin-1	TSP1_MOUSE	10	669.94	0.51	1.09E-10	1.660	5.040	1.830	2.580
Connective tissue growth factor	CTGF_RAT	9	495.18	0.37	2.57E-09	0.993	9.410	2.000	3.470
Galectin-3-binding protein	LG3BP_RAT	7	474.64	0.50	5.58E-06	2.270	1.670	1.600	0.833
Collagen alpha-1(III) chain	CO3A1_RAT	5	298.65	0.54	3.60E-05	0.774	0.821	1.020	0.444
Laminin subunit gamma-1	LAMC1_MOUSE	4	289.89	0.49	4.67E-04	0.832	0.879	0.596	0.428
Lactadherin	MFGM_RAT	5	267.14	0.39	4.70E-07	0.430	0.615	0.475	0.241
Clusterin	CLUS_RAT	5	263.59	0.40	0.01	1.600	2.200	1.860	0.869
Rab GDP dissociation inhibitor beta	GDIB_MOUSE	6	260.84	2.94	1.25E-04	0.400	0.244	0.372	0.717
Superoxide dismutase [Cu-Zn]	SODC_RAT	2	223.52	3.49	4.26E-07	2.380	1.550	2.360	5.410
Chondroitin sulfate proteoglycan 4	CSPG4_RAT	6	213.96	1.65	4.05E-09	0.149	0.384	0.177	0.632
Collagen alpha-1(I) chain	CO1A1_MOUSE	3	212.38	0.51	4.07E-04	0.901	1.100	1.250	0.560
Plasminogen activator inhibitor 1	PAI1_RAT	3	210.49	0.60	3.26E-05	0.684	2.820	1.090	1.690
Peroxiredoxin-1	PRDX1_MOUSE	3	179.82	3.33	4.12E-04	0.787	0.628	0.834	2.090
Amyloid beta A4 protein	A4_MOUSE	4	143.25	0.39	1.30E-09	0.202	0.430	0.155	0.169
Latent-transforming growth factor beta-binding protein 2	LTBP2_RAT	2	124.83	0.48	7.82E-04	0.044	0.085	0.042	0.040
Fibrillin-1	FBN1_MOUSE	3	116.42	0.42	2.72E-05	0.294	0.318	0.227	0.132
Laminin subunit beta-2	LAMB2_RAT	3	99.85	0.52	1.57E-03	0.134	0.098	0.104	0.051

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



**Figure 4** *Adrb3* 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 *Adrb3* (TAM-treated  $b3^{flx/flx}$ ; aMHC-MerCreMer<sup>0/+</sup> [Cre- $b3^{fl/fl}$  Tam] vs. vehicle-treated  $b3^{flx/flx}$ ; aMHC-MerCreMer<sup>0/+</sup> [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  $\pm$  SEM from 5 to 8 mice. Statistical significance was determined by two-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 *ADRB3* ( $\beta 3$ ) 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 *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 *ADRB3*-TG mice (right) after TAC or SHAM operation ( $n = 6$  hearts per group). (D) Myocardial fibrosis (collagen volume fraction) in hearts from *ADRB3*-tg or *ADRB3*<sup>O/+</sup>;*Nos1*<sup>-/-</sup> mice (vs. respective littermate controls) after TAC or SHAM operation. ( $n = 5$ –7 mice per group). A–D, by two-way ANOVA followed by Tukey's multiple comparison test.

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 *Figure 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 (*Figure 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 *Figure 5D*, although *ADRB3*<sup>O/+</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>O/+</sup>;*Nos1*<sup>-/-</sup> (TG/nNOS KO) compared with WT/*Nos1*<sup>-/-</sup> (WT/nNOS KO).

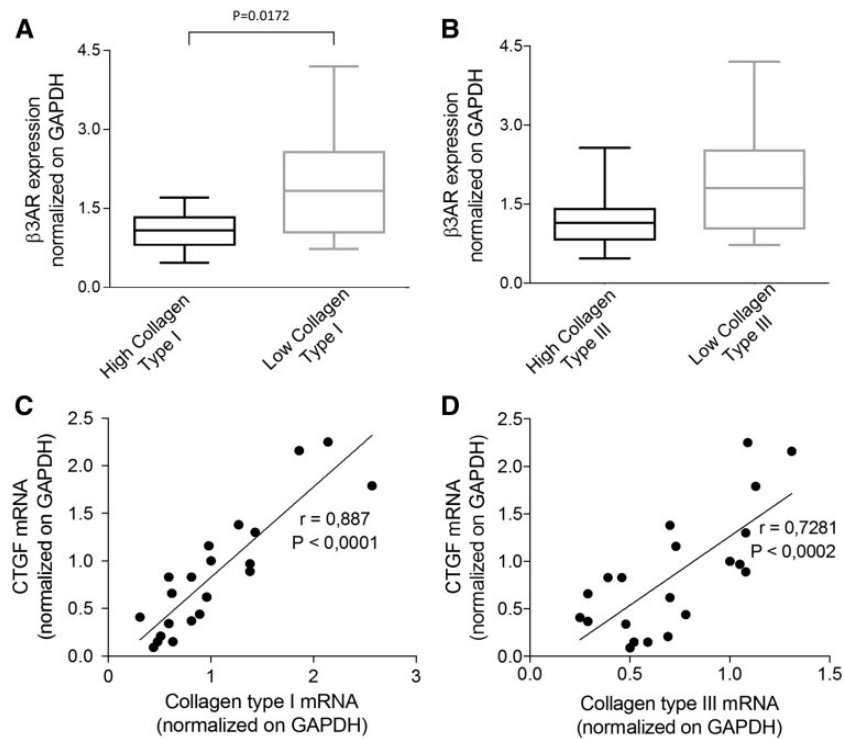
### Beta3-adrenergic receptor expression is inversely related with myocardial fibrosis in human ventricular biopsies

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 Supplementary material online, *Table S1*. As shown in *Figure 6A*, the expression of *ADRB3* was almost double in biopsies with the lowest *COL1A1* levels, and a similar trend was seen in biopsies with lowest *COL3A1* (*Figure 6B*). Of note, *CTGF* expression was also closely related to *COL1A1* (*Figure 6C*) and *COL3A1* (*Figure 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



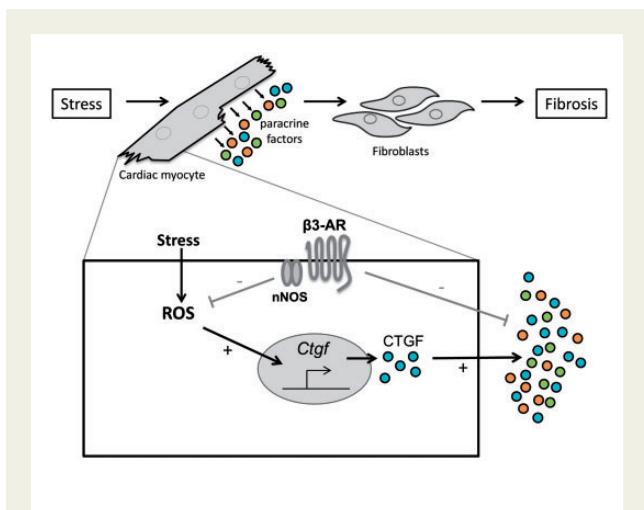


**Figure 6** The abundance of beta3AR is inversely related to myocardial fibrosis in human ventricular biopsies from patients with valvular disease. (A, B) Comparison of ADRB3 mRNA abundance in ventricular biopsies from patients with high or low Collagen type I (A) and Collagen type III (B) mRNA expression ( $n = 10$  and  $11$ , respectively). Statistical significance was determined by unpaired t-test. Univariate correlation between CTGF mRNA and Collagen type I (C) and Collagen type III (D) in the 21 biopsies.

fibrosis in response to haemodynamic (TAC) stress; (ii) conversely, *Adrb3* genetic deletion specifically in cardiac myocytes (Tamoxifen-treated alpha-MHC<sup>MerCreMer</sup>/*Adrb3*<sup>fl/fl</sup> 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 signalling 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 (Figure 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 signalling 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 1). CTGF (CCN2), also downregulated by the beta3AR in our study, has been widely implicated in fibrogenesis in many organs, including the heart.<sup>15</sup> 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.<sup>16</sup> Accordingly, genetic models with cardiac-specific CTGF overexpression<sup>17–19</sup> or *Ctgf* deletion<sup>19</sup> have yielded divergent remodeling phenotypes depending on the type of stress imposed (e.g. ischaemia/reperfusion vs. Angiotensin II infusion vs. TAC). Nevertheless, cardiac-specific *Ctgf* deletion did attenuate fibrosis in the context of TAC and TGFbeta overexpression.<sup>19</sup> Likewise, siRNA downregulation of *Ctgf* in our study significantly attenuated the pro-fibrotic effect of PE (see Supplementary material online, Figure S6), 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 four 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.<sup>20</sup> One of these is thrombospondin-1 (TSP1)<sup>21</sup> that was



**Figure 7** Cardiac beta3AR modulates stress-induced paracrine signaling to fibroblasts. Haemodynamic or neurohormonal stress on cardiac myocytes induces their production of cytokines and matrix proteins that activate fibroblasts, resulting in interstitial fibrosis. Cardiac beta3AR modulates paracrine signals to reduce fibrosis. In particular, beta3AR coupling to neuronal nitric oxide synthase reduces stress-induced ROS production and downstream *Ctgf/Ccn2* transcripts and protein expression (among others), thereby attenuating the synergistic effects of CTGF on paracrine pro-fibrotic factors.

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 1). 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<sup>22</sup> and downregulated by administration of blocking antibodies targeting CCN2/CTGF in the TAC model,<sup>23</sup> suggesting that cardiac NOS may prevent fibrosis by inhibiting CCN2/CTGF and subsequent PAI-1 and collagen production. As shown in the Table 1, *ADRB3* expression reciprocally increased the expression of chondroitin sulfate proteoglycan 4 (also known as NG2). NG2 binds PDGF-AA<sup>24</sup> and promotes angiogenesis through autocrine regulation of VEGF expression.<sup>25</sup> Upregulation of NG2 by *ADRB3* could explain our previous observations of pro-angiogenic effects of beta3AR activation.<sup>26</sup>

Cardiac fibrosis and CTGF expression were correlated with ROS in our models, as observed by others e.g. in human myxomatous mitral valve remodelling.<sup>13</sup> 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,<sup>27,28</sup> including eNOS and nNOS.<sup>29,30</sup> The latter protects eNOS from oxidative uncoupling by S-glutathionylation, thereby maintaining NO bioavailability and downstream signalling.<sup>31</sup> nNOS, in turn, was shown to inhibit ROS production in cardiac myocytes through cGMP-dependent inactivation of xanthine oxidoreductase.<sup>32</sup>

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<sup>33</sup> and humans,<sup>34</sup> possibly as a protective mechanism. Notably, the coupling of beta3AR may be preserved under adrenergic stress because of relative resistance to desensitization.<sup>35</sup> This makes beta3AR an attractive target for the therapeutic use of new, more specific beta3AR agonists currently in clinical use for non-cardiovascular indications.<sup>36</sup> 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,<sup>37</sup> where ROS and fibrosis are clearly implicated in the generation and maintenance of atrial fibrillation.<sup>38,39</sup> Therefore, our findings may guide future therapeutic uses of current and new specific beta3AR agonists for myocardial protection.

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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