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RESEARCH PAPER

# Propranolol enhances cell cycle-related gene expression in pressure overloaded hearts

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## BACKGROUND AND PURPOSE

Cell cycle regulators are regarded as essential for cardiomyocyte hypertrophic growth. Given that the β-adrenoceptor antagonist propranolol blunts cardiomyocyte hypertrophic growth, we determined whether propranolol alters the expression of cell cycle-related genes in mouse hearts subjected to pressure overload.

## EXPERIMENTAL APPROACH

Pressure overload was induced by transverse aortic constriction (TAC), whereas the expression levels of 84 cell cycle-related genes were assayed by real-time PCR. Propranolol (80 $mg \cdot kg^{-1} \cdot day^{-1}$) was administered in drinking water for 14 days.

## KEY RESULTS

Two weeks after surgery, TAC caused a 46% increase in the left ventricular weight-to-body weight (LVW/BW) ratio but no significant changes in cell cycle gene expression. Propranolol, at plasma concentrations ranging from 10 to 140 $ng \cdot mL^{-1}$, blunted the LVW/BW ratio increase in TAC mice, while significantly increasing expression of 10 cell cycle genes including mitotic cyclins and proliferative markers such as Ki67. This increase in cell cycle gene expression was paralleled by a significant increase in the number of Ki67-positive non-cardiomyocyte cells as revealed by immunohistochemistry and confocal microscopy. β-Adrenoceptor signalling was critical for cell cycle gene expression changes, as genetic deletion of β-adrenoceptors also caused a significant increase in cyclins and Ki67 in pressure overloaded hearts. Finally, we found that metoprolol, a $\beta_1$-adrenoceptor antagonist, failed to enhance cell cycle gene expression in TAC mice.

## CONCLUSIONS AND IMPLICATIONS

Propranolol treatment enhances cell cycle-related gene expression in pressure overloaded hearts by increasing the number of cycling non-cardiomyocyte cells. These changes seem to occur via $\beta_2$-adrenoceptor-mediated mechanisms.

### Abbreviations

Cdks, cyclin D-dependent kinases; Ki67, antigen identified by monoclonal antibody Ki-67; LVH, left ventricular hypertrophy; LVW/BW, left ventricular weight-to-body weight ratio; RT, reverse transcription; TAC, transverse aortic constriction; TSA, trichostatin A

## Introduction

When the heart experiences prolonged periods of pressure overload, it undergoes hypertrophic growth. Although the hypertrophic response to increased haemodynamic load is believed to be an adaptive process aimed at increasing cardiac pump function and decreasing ventricular wall stress, it greatly increases the risk of sudden death, ventricular

arrhythmias and heart failure. Thus, somewhat counterintuitively, limiting the hypertrophic response under work overload conditions could be beneficial.

Pressure overload generates biomechanical signals that converge on a number of intracellular signalling transduction pathways. These, in turn, regulate the hypertrophic response by altering the expression of hundreds of genes involved in the cell growth, in the cytoskeletal and matrix remodelling and in the regulation of energy metabolism. In particular, results from several studies indicate that cell cycle regulatory proteins associated with G1 phase such as cyclin D and cyclin D-dependent kinases (cdks) have an important role in the control of the cardiomyocyte hypertrophic growth. For example, in rat neonatal cardiomyocytes, cdk4/6-dependent phosphorylation of retinoblastoma protein is necessary for hypertrophic growth (Hinrichsen *et al.*, 2008). Additionally, specific inhibition of G1 phase cyclin or cyclin-dependent kinase activity (Nozato *et al.*, 2001; Busk *et al.*, 2002) as well as deletion of the cyclin D2 (Angelis *et al.*, 2008) have been shown to attenuate the development of cardiomyocyte hypertrophy in response to hypertrophic stimuli. Taken together, these data indicate that cyclins and cdks might be a potential drug target for the treatment of pathological cardiac growth.

We and others have demonstrated previously that propranolol, a β-adrenoceptor antagonist used for the management of systemic arterial hypertension, angina pectoris and certain types of cardiac arrhythmias, is able to significantly blunt the cardiac hypertrophic response to aortic banding-induced pressure overload in both rats and mice (Ostman-Smith, 1995; Marano *et al.*, 2002; Patrizio *et al.*, 2007). At this time, however, the molecular mechanisms responsible for this important effect of propranolol remain to be elucidated. Given the ability of propranolol to attenuate cardiac hypertrophy in response to pressure overload and the essential role that certain cyclins seem to have in cardiomyocyte hypertrophic growth, we postulated that propranolol treatment would have decreased the cardiac expression of genes that promote the progression of cell cycle such as the cyclins, subsequently leading to attenuation of cardiac hypertrophic response to pressure overload. Elucidation of gene expression changes driven by propranolol could not only provide novel insights in the pathophysiology of cardiac hypertrophy but also improve our understanding of the pharmacological properties of propranolol.

To address this issue, left ventricular hypertrophy was induced by transverse aortic constriction (TAC) in C57BL/6 mice, whereas the relative expression levels of 84 cell cycle-related genes were obtained by quantitative SYBR Green real-time PCR. Contrary to our hypothesis, we found that propranolol, at plasma concentrations ranging from 10 to 140 $ng \cdot mL^{-1}$, blunted cardiac hypertrophic growth in response to TAC but significantly increased expression of cell cycle-related genes including mitotic cyclins and proliferative markers such as Ki67. The results also revealed that these changes occurred in non-cardiomyocyte cells as evidenced by immunohistochemistry for Ki67. Since genetic deletion of β-adrenoceptors also caused a significant increase in the gene expression of cyclins and Ki67, β-adrenoceptor signalling appears to be critical for changes in cell cycle gene expression in pressure overloaded hearts.

# Methods

## Chemicals

(+/–)-Propranolol hydrochloride, (+/–)-metoprolol, (+)-tartrate and trichostatin A (TSA) were obtained from Sigma-Aldrich (St. Louis, MO), and isoflurane from Abbott (Pomezia, Italy).

## Animals and chronic administration of drugs

Male C57BL/6 (Harlan, San Pietro al Natisone, Italy) 12–14 week-old mice were used for most experiments. In a separate series of experiments, Scn5a heterozygous knockout (Scn5a KO) mice with 129/Sv genetic background, as well as male $\beta_1$- and $\beta_2$-adrenoceptor double knockout (KO) mice of 12–14 weeks old were also used (Rohrer *et al.*, 1999; Papadatos *et al.*, 2002). β-Adrenoceptor KO mice were on a FVB/C57/129/DBA genetic background, as attempts to breed these mice on a congenic background have not been successful (Bernstein *et al.*, 2005; and G. Marano, pers. data). For this reason, F2 generation β-adrenoceptor KO and wild-type (WT) mice with the same mixed genetic background were generated by inter-breeding β-adrenoceptor heterozygous KO mice (Bernstein *et al.*, 2005). Genotyping was performed on tail DNA using PCR standard protocols. All animal care and experimental procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and were approved by the local ethical committee of the Italian National Institute of Health.

Propranolol or metoprolol were administered in drinking water at the dose of about 80 and 100 $mg \cdot kg^{-1} \cdot day^{-1}$, respectively, for 14 consecutive days. The dosage was chosen on the basis of literature data (Asai *et al.*, 1999; Baumhäkel *et al.*, 2008). In preliminary experiments, we found that cardiac functional changes in response to the infusion of isoprenaline, a β-adrenoceptor agonist, at the dose of 30 $ng \cdot kg^{-1} \cdot min^{-1}$ did not occur in propranolol- or metoprolol-treated mice.

TSA was dissolved in ethanol and administered via daily i.p. injections at the dosage of 0.5 $mg \cdot kg^{-1} \cdot day^{-1}$ for 2 weeks as described previously (Kong *et al.*, 2006).

At the end of the protocol, the animals were killed, their hearts were quickly dissected and left ventricles (LV) as well as the other cardiac chambers were weighed. LVs only were frozen in liquid nitrogen and kept at –80°C, or fixed in 10% buffered formalin solution for further analysis.

## Mouse model of LV pressure overload

Pressure overload on the LV was induced by TAC as reported previously (Rockman *et al.*, 1991), with some modifications. Animals were anaesthetized with isoflurane (1.5–2.0% in 100% of oxygen), and the degree of aortic stenosis was about 60%. A control group of mice was subjected to a sham operation with an identical surgical procedure, but the ligature was not tightened.

To quantify the haemodynamic load imposed on the mouse LV after aortic banding, LV systolic pressure was measured with a 1.4-Fr micromanometer-tipped catheter (Millar Instruments, mod. SPR 839, Houston, TX, USA) by direct catheterization of the LV at the end of experiments. The procedure including pressure measurement took about 20 min.

Data were analysed with a software package for cardiovascular analysis (IOX 1.7; EMKA Technologies, Paris, France).

## Echocardiography

Mice were anaesthetized with isoflurane (1.0% in 100% of oxygen) and set in a supine position. Echocardiography was performed as previously described (Patrizio *et al.*, 2007). LV diastolic dimensions (LVDd) and LV end-systolic dimensions (LVDs) were measured. LV fractional shortening (%FS) was calculated as (LVDd – LVDs) / LVDd × 100.

## Radioreceptor binding assay of plasma propranolol concentration

Blood samples were collected by cardiac puncture within 15–20 s. The blood was placed in a tube containing 37.5 U of heparin, mixed and centrifuged. The plasma samples were kept at –20°C. The determination of plasma propranolol concentration was performed as described previously (Elkins *et al.*, 1986) with some modifications. Briefly, unextracted plasma, $^{125}$I-labelled pindolol ([$^{125}$I]-P) and $\beta_2$-adrenoceptors from stable transfected HEK-293 cells were used. Propranolol standards and nonspecific binding control were prepared by adding D,L-propranolol to drug-free mouse plasma. The displacement curves of [$^{125}$I]-P (Perkin-Elmer Life Sciences, Boston, MA, USA) by unlabelled (–)-propranolol to $\beta_2$-adrenoceptors were used as standard curves for determination of unknown amounts of this drug in treated and untreated samples. The radioreceptor binding assay was performed in 0.1 mL of 50 mM Tris–HCl and 0.1 mM EGTA–Tris (pH 7.4), for 90 min at room temperature using 1 μg of membrane protein prepared from stable transfected HEK-293 cells with $\beta_2$-adrenoceptors. The concentration of [$^{125}$I]-P was maintained constant at 50 pM in the presence of increasing concentrations of unlabelled ligands for the standard curves and without ligands for unknown samples. Reactions were terminated by filtration onto GF/B glass fibre filtering microplates (Filtermate 96, Packard Instrument Co., Meriden, CT, USA). Filters were washed three times in 1 mL of ice-cold 50 mM Tris–HCl (pH 7.4) and allowed to dry for a few hours. The plates were counted in a Packard Top Count after the addition of 50 μL of Microscint 20 (Perkin-Elmer Life Sciences) to each well. The data were computed using the four-parameter logistic equation (DeLean *et al.*, 1978). The lowest detectable concentration of propranolol that was significantly different from the zero standard was about 0.2 $\mu g \cdot L^{-1}$.

## Histological analysis

Hearts were fixed in 10% buffered formalin (BioOptica, Milan, Italy), embedded in paraffin and cut into 5 μm sections. Histological analysis was performed as described previously (Marano *et al.*, 2004). Specifically, LV sections were stained with haematoxylin and eosin for the measurement of myocyte cross-sectional area or by the sirius red / picric acid method to determine LV fibrosis by quantitative morphometry (Morphometric, Universal Imaging Corporation, Downingtown, PA, USA).

## Immunohistochemistry and confocal microscopy

Sections were deparaffinized in xylene, hydrated, rinsed in PBS and processed for immunostaining. Antigen retrieval was performed using a 10 mM Citrate–EDTA solution (pH 7.8) (UCS Diagnostics, Rome, Italy), 3 × 3 min, in the microwave at 650 W. Blocking of unspecific sites was performed using a PBS solution with 3% BSA 30 min at 37°C. Sections were subsequently incubated with primary antibodies for 75 min at 37°C. To detect cycling cells, we used an antibody directed against the nuclear protein Ki67 (Bethyl Labs, Montgomery, TX, USA), and Alexa Fluor®568-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). Slides were stained with Alexa Fluor®647-conjugated Wheat Germ Agglutinin (Invitrogen) to distinguish the sarcolemmal membrane. Nuclei were stained with DAPI (Invitrogen). Incubations were performed using a commercially available antibody diluent solution (Biocare Medical, Concord, CA, USA). Washing between immunostaining steps was performed with a PBS-Tween20 0.05% (v/v) solution.

For microscopic evaluation, slides were mounted using an anti-fade mounting medium (Biocare Medical). Analysis was conducted using an Olympus FV-1000 inverted spectral confocal microscope equipped with an Ultra-Plan Fluorite 40X Oil objective with 1.30 N.A and an Ultra-Plan Apochromat 60× Oil objective with 1.35 N.A. (Olympus Europa GmbH, Hamburg, Germany).

## RNA isolation and quantification

Total RNA was extracted from mouse LVs by using TRIzol (Invitrogen, Milan, Italy) and purified by using RNA purelink mini kit (Invitrogen, Milan, Italy). The concentration and purity of the RNA solution was determined by measuring its absorbance at 230, 260 and 280 nm using a NanoDrop spectrophotometer (Fisher Scientific, Milan, Italy), whereas its overall quality was analysed using the Agilent 2100 bioanalyser with an RNA LabChip (RNA 6000 Nano kit, Agilent, Milan, Italy). Total RNA was retrotranscribed by RT$^2$ First Strand Kit (SABiosciences Corporation, Frederick, MD). Mouse Cell Cycle RT$^2$ Profiler PCR Array (PAMM-020A) and RT$^2$ Real-Time SyBR Green/ROX PCR Mix were also used (SABiosciences Corporation). The full list of analysed genes is presented in Table 1. Real-time PCR was performed on ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA). The specificity of the SyBR Green assay was confirmed by melting point analysis.

Two selected genes (Brca1 and Cdkn2a) were also analysed by TaqMan real-time PCR (TaqMan, Applied Biosystems), a 5′nuclease chemistry-based real-time PCR assay that uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. In this case, cDNA was synthesized by using the High Capacity cDNA RT kit (Applied Biosystems). TaqMan reactions were carried out in 96-well plates using cDNA, TaqMan universal PCR mastermix, pre-optimized and pre-formulated TaqMan gene expressions assays (Assays on-Demand, Applied Biosystems) including specific primers and fluorescent probes for mouse and water to a final volume of 50 μL according to manufacturer's instructions. The codes for each gene expressions assay were derived from the online Applied Biosystems catalogue for TaqMan gene expression assays. No reverse transcriptase and no template controls were used to monitor for any contaminating amplification. Real-time PCR was performed on ABI Prism 7500 Sequence Detector (Applied Biosystems). Significant changes were determined using Student's unpaired

## Table 1

Mouse cell cycle real-time PCR array

| |
|---|
| G1 phase and G1/S transition: Camk2a, Camk2b, Gpr132, Itgb1, Mtbp, Myb (C-MYB), Nfatc1, Ppp2r3a, Ppp3ca, Skp2, Taf10, Slfn1. |
| S phase and DNA replication: Dnajc2 (Zrf2), Mcm2, Mcm3, Mcm4, Mki67, Mre11a, Msh2, Pcna, Rad17, Rad51, Sumo1. |
| G2 phase and G2/M transition: Dnajc2 (Zrf2), Chek1, Ppm1d. |
| M phase: Brca2, Ccna1 (Cyclin A1), Ccnb1, Cdc25a, Cdc25b, Cdk2, Nek2, Npm2, Pes1, Prm1, Rad21, Ran, Shc1, Smc1a (Smc1l1), Stag1, Terf1, Tnfsf5ip1, Wee1. |
| Cell cycle checkpoint and cell cycle arrest: Ak1, Apbb1, Brca2, Casp3, Cdk5rap1, Cdkn1a, Cdkn1b, Cdkn2a, Chek1, Cks1b, Ddit3 (CHOP), Dst, Gadd45a, Hus1, Inha, Macf1, Mad2l1, Mdm2, Msh2, Notch2, Pkd1, Pmp22, Ppm1d (WIP1), Rad9, Sesn2 (Sestrin 2), Sfn (Stratifin), Slfn1, Smc1a (Smc1l1), Tsg101. |
| Regulation of the cell cycle: Abl1, Brca2, Ccna1, Ccna2, Ccnb1, Ccnb2, Ccnc, Ccnd1, Ccne1, Ccnf, Cdk4, Cdkn1a, Cks1b, E2f1, E2f2, E2f3, E2f4, Gadd45a, Itgb1, Rad9, Ran, Sfn (Stratifin), Shc1, Skp2, Tfdp1, Tnfsf5ip1. |
| Negative regulation of the cell cycle: Apbb1, Atm, Brca1, Casp3, Cdkn2a, Inha, Rbl1, Rbl2, Trp53, Trp63. |

two-tailed *t*-test on $2^{-\Delta Ct}$ individual values according to Schmittgen and Livak (2008).

### *Statistical analysis*

Group means (±SEM) were calculated for all relevant variables. Statistical analysis was performed by ANOVA with Bonferroni's multiple comparison test for *post hoc* analyses when applicable or by Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

## Results

### *Propranolol alters cell cycle-related gene expression in pressure overloaded hearts*

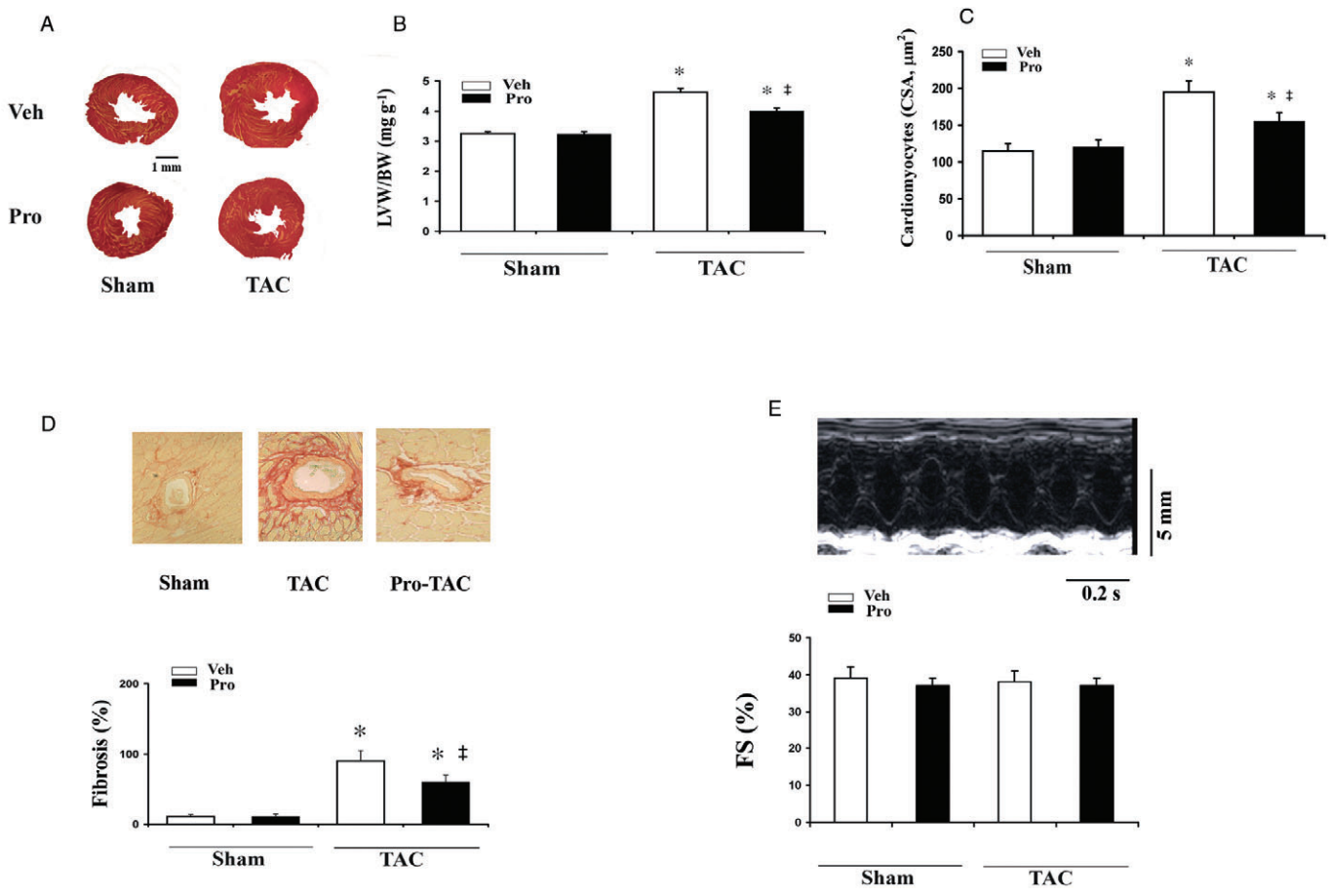
Two weeks after surgery, a significant increase in the LV systolic pressure was observed in TAC mice compared with sham-operated mice (101 ± 5 vs. 65 ± 3 mmHg, respectively; $n \geq 4$ for each group). TAC also caused cardiac hypertrophy as evidenced by a significant increase in the left ventricular weight to body weight (LVW/BW) ratio, the cardiomyocyte cross-sectional area and the amount of perivascular fibrosis (Figure 1A–D), associated with preserved cardiac systolic function (Figure 1E). Moreover, no gene was differentially expressed at significant levels in TAC LVs as compared with controls (Table 2). Propranolol reduced heart rate in both TAC and sham groups (–14% compared with sham mice), but no significant difference in the haemodynamic load was observed between untreated and propranolol-treated TAC mice (101 ± 5 vs. 95 ± 4 mmHg, respectively; $n \geq 4$ for each group). Propranolol administration also caused a significant

## Table 2

Propranolol increases the expression of 10 cell cycle-related genes in TAC mice

| | Brca1 | Cdkn2a | Chek1 | Rad51 | Slfn1 | Ccna2 | Ccnb1 | Ccnb2 | Mki67 | Nek2 |
|---|---|---|---|---|---|---|---|---|---|---|
| Fold of change | | | | | | | | | | |
| TAC ($n = 4$) | 1.2 ± 0.2 | 2.2 ± 0.3 | 1.8 ± 0.3 | 1.6 ± 0.3 | 1.2 ± 0.2 | 1.6 ± 0.2 | 2.1 ± 0.3 | 2.2 ± 0.4 | 1.7 ± 0.3 | 2.4 ± 0.2 |
| Pro-Sham ($n = 4$) | 1.0 ± 0.1 | 0.9 ± 0.1 | 1.2 ± 0.2 | 1.1 ± 0.1 | 0.9 ± 0.2 | 1.1 ± 0.2 | 0.9 ± 0.1 | 1.2 ± 0.1 | 1.1 ± 0.2 | 0.9 ± 0.2 |
| Pro-TAC ($n = 5$) | 3.9 ± 0.2* | 5.2 ± 0.2‡ | 4.5 ± 0.2‡ | 3.9 ± 0.2* | 2.5 ± 0.1* | 4.3 ± 0.2‡ | 6.2 ± 0.3* | 7.5 ± 0.3* | 4.9 ± 0.2‡ | 8.1 ± 0.4‡ |

*$P < 0.05$ versus Sham group, ‡$P < 0.01$ versus Sham group.
Real-time PCR data are shown as fold change (±SEM) relative to mean Sham values. In TAC mice, propranolol treatment caused a significant increase of cell cycle gene expression including both genes associated with cell proliferation and those known to negatively regulate cell cycle progression. For each group, individual data points expressed as $2^{-\Delta Ct}[2 - (Ctgene\ target - Ctgapdh)]$ were used for statistical analysis (Schmittgen and Livak, 2008).




## Figure 1

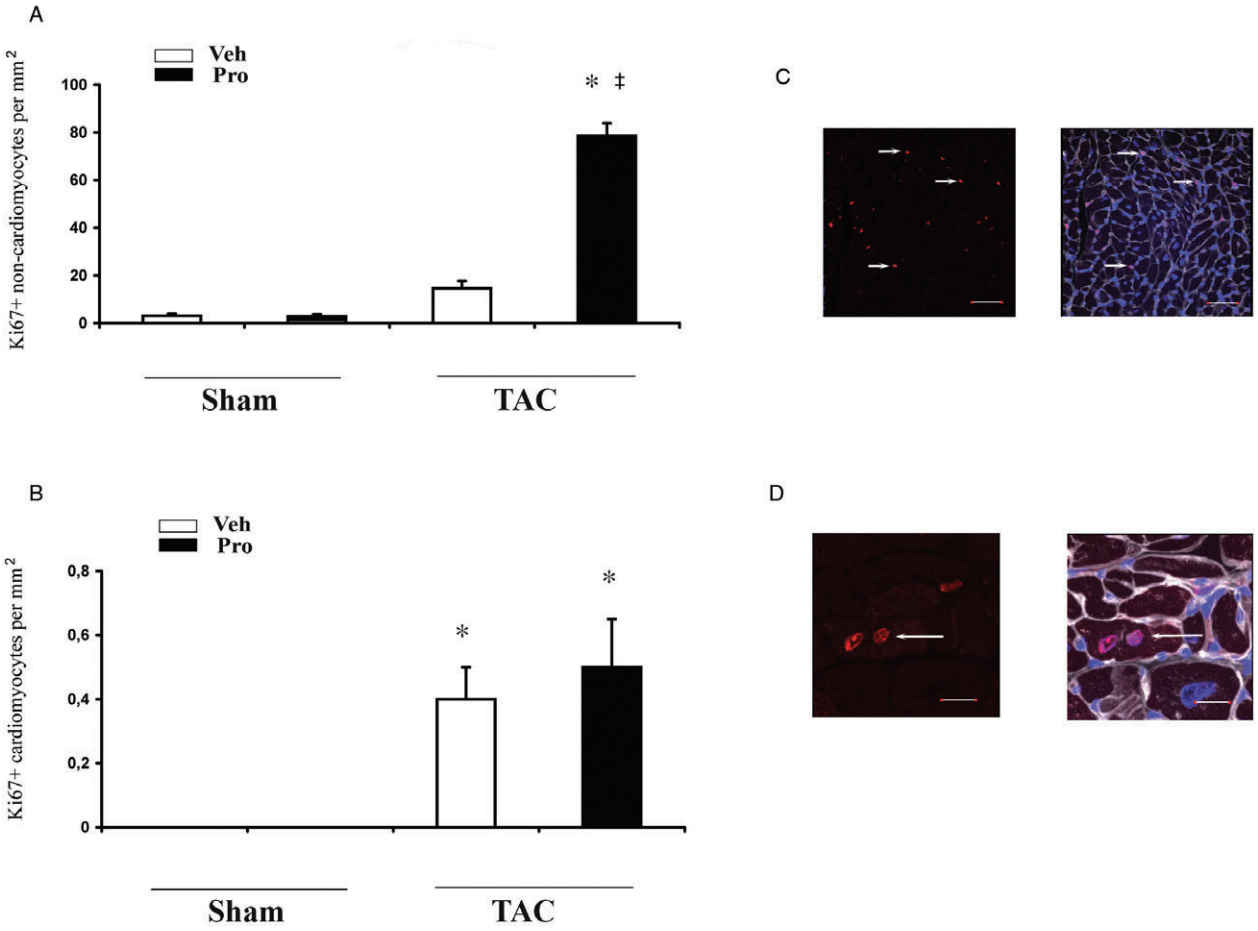
Propranolol blunts cardiac hypertrophic growth and increases cell cycle gene expression. (A) Representative haematoxylin and eosin staining of LV cross-sections from sham-operated and TAC mice, treated with or without propranolol, 2 weeks after surgery. (B) LV weight normalized to body weight (LVW/BW, mg·g$^{-1}$). A significant attenuation of hypertrophic response to TAC was observed in propranolol-treated mice ($n$ = 9 for each group). (C) Cross-sectional area (CSA) of cardiomyocytes measured as described. About 200 cardiomyocytes per group were analysed ($n$ = 4 for each group). (D) (upper) Representative fields of picrosirius red–stained left ventricular sections; (bottom) perivascular fibrosis, expressed as fibrosis area related to total vessel area, is attenuated by propranolol ($n$ = 4 for each group). (E) (upper) A representative transthoracic echocardiographic tracing; (bottom) echocardiographic analysis of hearts 2 weeks after surgery: FS, fractional shortening ($n$ = 9 for each group). Veh, vehicle; Pro, propranolol; Sham, sham operated mice. *$P$ < 0.05 versus the respective sham control; ‡$P$ < 0.05 versus TAC group.

attenuation of cardiac hypertrophic growth in response to TAC (Figure 1A–D) along with a significant increased expression (≥2.5-fold) of 10 cell cycle-related genes including the proliferative marker Ki67 and mitotic cyclins A2, B1 and B2 (Table 2). Despite significant blunting of hypertrophic growth, cardiac systolic function did not change (Figure 1E).

To corroborate the results obtained by SYBR Green PCR, we also used TaqMan PCR, which is one of the most popular alternatives to SYBR Green. TaqMan PCR revealed a very close correlation between the two methods for two selected genes (Brca1 and Cdkn2a) in terms of both the direction and magnitude of change as well as concordance in statistical significance (data not shown).

### *Propranolol increases Ki67-positive non-cardiomyocyte cells*

Cells other than cardiomyocytes account for approximately 70% of the total cell number in the heart, with the majority being fibroblasts. Given that propranolol increases Ki67 mRNA levels, we used an antibody directed against the nuclear protein Ki67, a proliferative marker that is expressed during all stages of the cell cycle excluding $G_0$, to quantify and individuate cycling cardiac cells. Ki67 expressing non-cardiomyocyte cells increased from 3.1 ± 0.9 cells mm$^{-2}$ in the sham group to 14.6 ± 3.1 cells mm$^{-2}$ 2 weeks after TAC, but not in a significant manner (Figure 2A). Conversely, Ki67 expressing non-cardiomyocyte cells increased significantly in TAC mice treated with propranolol (Figure 2A,C), which is in agreement with gene expression data (Table 2). Propranolol did not alter cycling non-cardiomyocyte cells in hearts from sham-operated mice (Figure 2A). Although there were no cycling cardiomyocytes in sham-operated mice, 0.4 ± 0.1 cardiomyocytes mm$^{-2}$ expressed Ki67 in mice after TAC (Figure 2B). A similar increase in Ki67 expressing cardiomyocytes was also observed in TAC mice treated with propranolol (Figure 2B,D).




## Figure 2

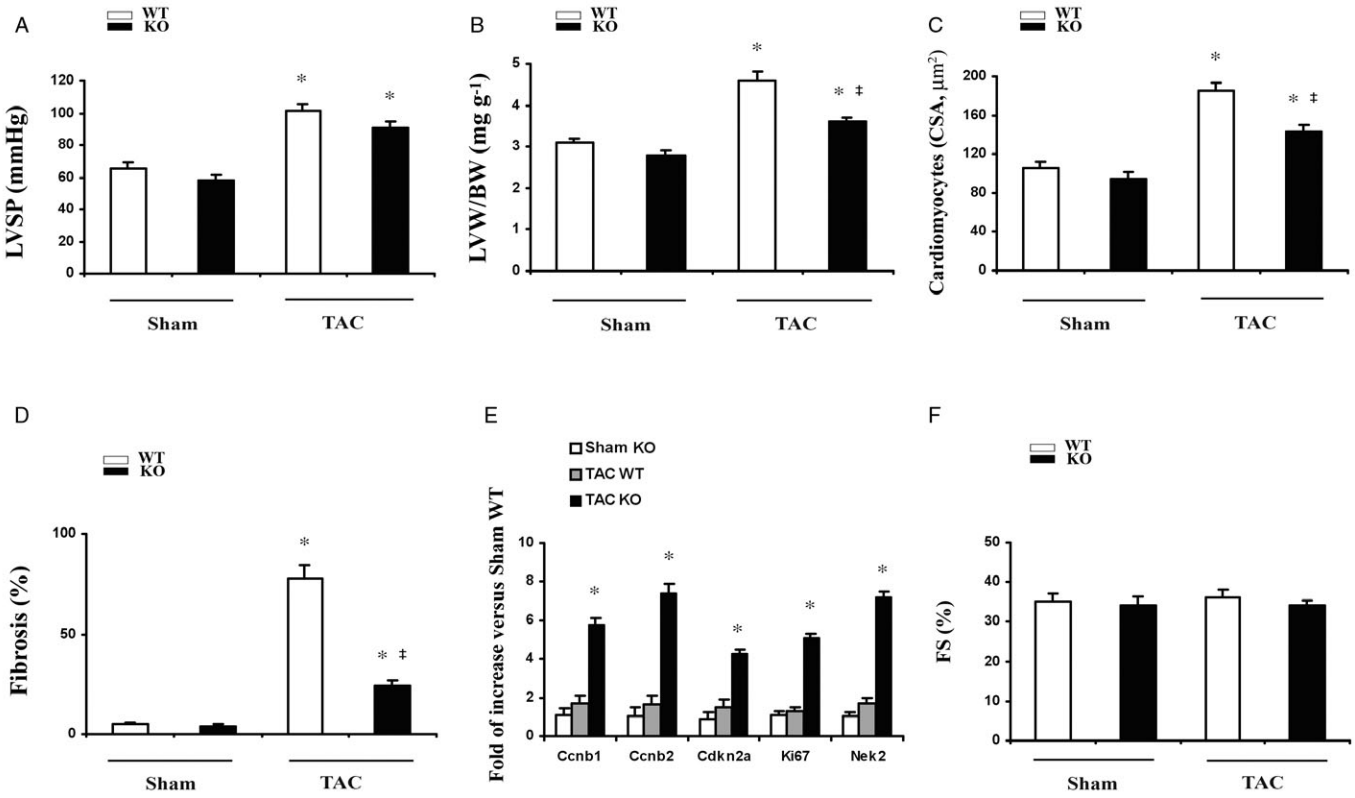
Effect of propranolol on Ki67-positive non-cardiomyocytes (A) and cardiomyocytes (B) in TAC mice. (C and D) Examples of left ventricular sections from TAC mice treated with propranolol in which co-immunostaining for the sarcolemmal membrane (white) and Ki67 (red) was performed. Nuclei were stained with DAPI (blue). Proliferating non-cardiomyocyte (C) and cardiomyocyte (D) nuclei are marked by arrows. Ki67-positive nuclei have a magenta colour in merged images (on the right). (D) Ki67-positive binucleated cardiomyocyte. Scale bars correspond to 50 and 20 μm for images acquired with the 40× (C) and the 60× (D) objectives, respectively. Experiments were performed on a minimum of three hearts per group. *$P < 0.05$ versus the respective sham control; ‡$P < 0.05$ versus TAC group.

### *Genetic deletion of β-adrenoceptors alters cell cycle gene expression*

The ability of β-adrenoceptor signalling to affect cell cycle gene expression was also evaluated in β-adrenoceptor KO mice, in which the predominant cardiac β-adrenoceptor subtypes are lacking (Rohrer *et al.*, 1999). Two weeks after surgery, a significant increase in the LV systolic pressure was observed in TAC mice, but there was no significant difference in haemodynamic load between WT and KO mice subjected to TAC (Figure 3A). KO mice showed significant attenuation of cardiac hypertrophic growth in response to TAC along with a significant increased expression of cell cycle-related genes including the proliferative marker Ki67 and mitotic B-type cyclins (Figure 3B–E) as compared to WT mice. No significant difference in cardiac systolic function was observed among the groups (Figure 3F).

### *$\beta_1$-adrenoceptor blockade is not required for propranolol effect on cell cycle gene expression*

Considering that propranolol binds to $\beta_1$- and $\beta_2$-adrenoceptors with equal affinity, we evaluated the contribution of $\beta_1$-adrenoceptor subtype to propranolol effects on the expression of cell cycle genes in chronically pressure overloaded hearts by administering metoprolol, a selective $\beta_1$-adrenoceptor antagonist. Metoprolol reduced heart rate in both TAC and sham groups (about −16% compared with sham mice), but no significant difference in the haemodynamic load was observed between untreated and metoprolol-treated TAC mice (101 ± 5 vs. 98 ± 3 mmHg, respectively; $n \geq 4$ for each group). At variance with propranolol, metoprolol failed to affect the cardiac growth response to TAC (Figure 4A,B) as well as the cardiac expression of cell cycle genes. Specifically, gene expression of B-type cyclins and Ki67 did not change in metoprolol-treated TAC mice (Figure 4C).




## Figure 3

β-Adrenoceptor genetic deletion increases expression of cell cycle-related genes in pressure overloaded hearts. (A) LV systolic pressure was measured as described ($n \geq 5$ for each group). (B) LVW/BW ($n = 6$ or 7 for each group). (C) CSA of cardiomyocytes. About 150–200 cardiomyocytes per group were analysed ($n = 3$ or 4 for each group). (D) Perivascular fibrosis ($n = 3$ or 4 for each group). (E) Transcript amount from left ventricles was quantified by SYBR Green real-time PCR. Data are shown as fold change relative to sham WT values. For each group, summary data of three independent experiments run in duplicate are shown. (F) Echocardiographic analysis ($n = 6$ or 7 for each group). WT, wild type mice; KO, β-adrenoceptor knockout mice. *$P < 0.05$ versus the respective sham control; ‡$P < 0.05$ versus TAC group.

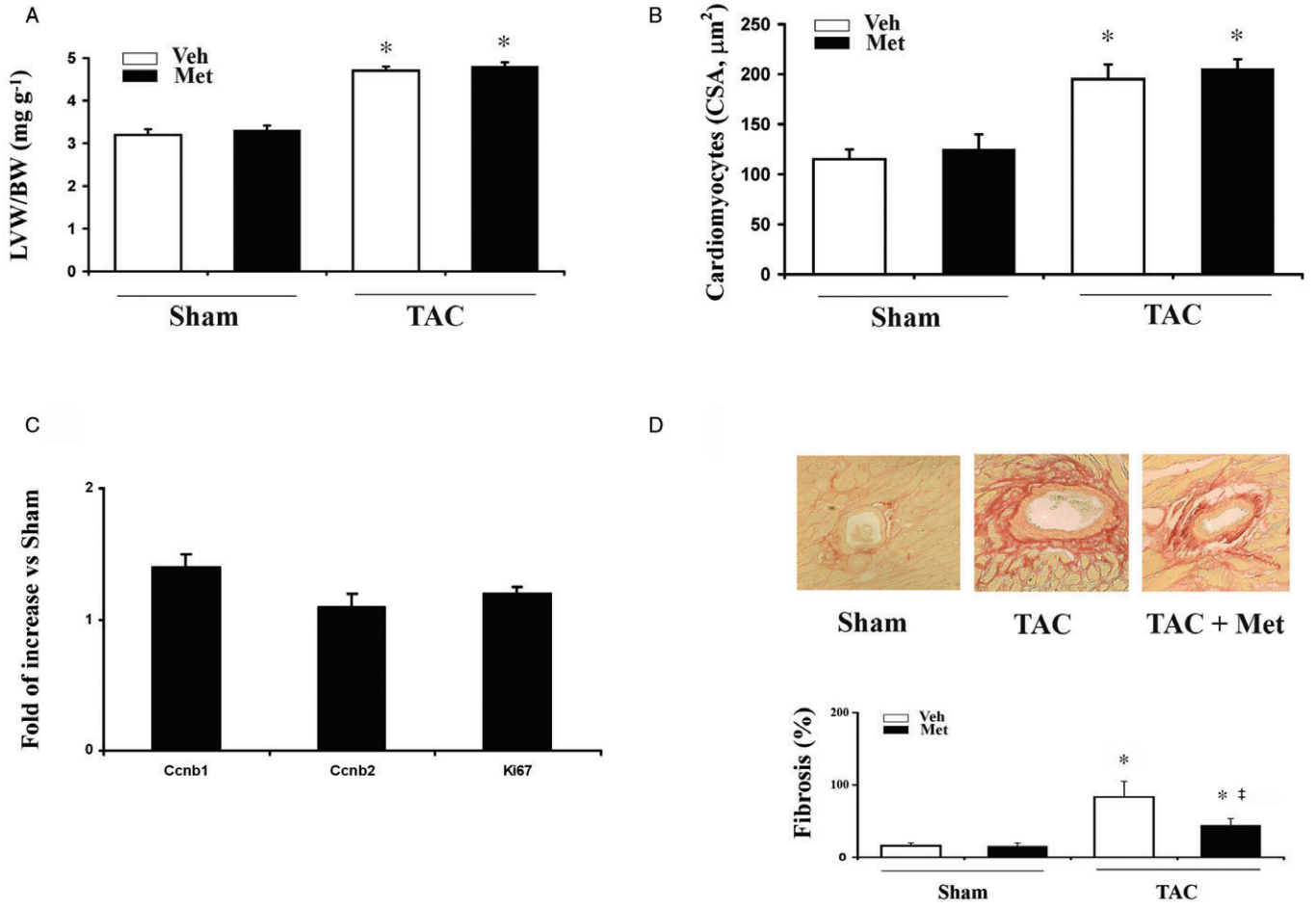
However, we found that metoprolol administration significantly reduced the amount of perivascular fibrosis in TAC mice (Figure 4D). Cardiac systolic function was unchanged (data not shown).

### *Propranolol effects on cell cycle gene expression are not a consequence of a blunted cardiac hypertrophic response to TAC*

In the present study, we also evaluated whether a blunted cardiac hypertrophic response to TAC was *per se* sufficient to alter the cell cycle gene expression. To address this issue, TSA, a histone deacetylase inhibitor endowed with anti-hypertrophic activity, was administered to TAC mice at the dose of 0.5 $mg \cdot kg^{-1} \cdot day^{-1}$ according to Kong *et al.* (2006). Although no significant difference in the haemodynamic load was observed between untreated and TSA-treated TAC mice (105 ± 4 vs. 107 ± 5 mmHg, respectively; $n \geq 4$ for each group), cardiac hypertrophic growth was blunted significantly in TSA-treated TAC mice as compared to controls (Figure 5A–C). However, no gene was differentially expressed at significant levels between sham and TSA-treated TAC mice. The effects on B-type cyclins and Ki67 gene expression are shown (Figure 5D). Moreover, despite significant blunting of hypertrophic growth, cardiac systolic function did not change (Figure 5E).

### *The membrane-stabilizing activity is not an obligatory prerequisite for changing cell cycle gene expression*

In addition to its ability to antagonize catecholamine-induced β-adrenoceptor stimulation in the heart, propranolol possesses other potentially beneficial properties unrelated to β-adrenoceptor blockade. For example, it displays membrane stabilizing activity; that is, it reduces cardiac sodium channel conductance. Therefore, to evaluate whether the membrane-stabilizing activity was *per se* responsible for the enhanced expression of cell cycle-related genes, TAC was induced in $Scn5a^{+/-}$ KO mice, in which heterozygous disruption of the cardiac sodium channel gene causes a significant reduction in sodium conductance (Papadatos *et al.*, 2002). No significant difference in the haemodynamic load was observed between KO and WT TAC mice (Figure 6A). Deletion of Scn5a gene failed to affect cardiac growth response to TAC (Figure 6B–D) as well as the cardiac expression of cell cycle genes. Specifically, gene expression of B-type cyclins and Ki67 did not




## Figure 4

Cell cycle gene expression is not altered by metoprolol. (A) LVW/BW ($n$ = 9 for each group). (B) CSA of cardiomyocytes as described ($n$ = 4 for each group). (C) Effects of metoprolol on gene expression of B-type cyclins (Ccnb1 and Ccnb2) and Ki67. Transcript amount from left ventricles was quantified by SYBR Green real-time PCR 14 days after TAC. For each group, summary data of three independent experiments run in duplicate are shown. (D) (top) Representative fields of picrosirius red–stained left ventricular sections. (bottom) Perivascular fibrosis ($n$ = 4 for each group). Veh-Sham and Veh-TAC groups are the same as in Figure 1. Met, metoprolol. *$P$ < 0.05 versus the respective sham control; ‡$P$ < 0.05 versus TAC.

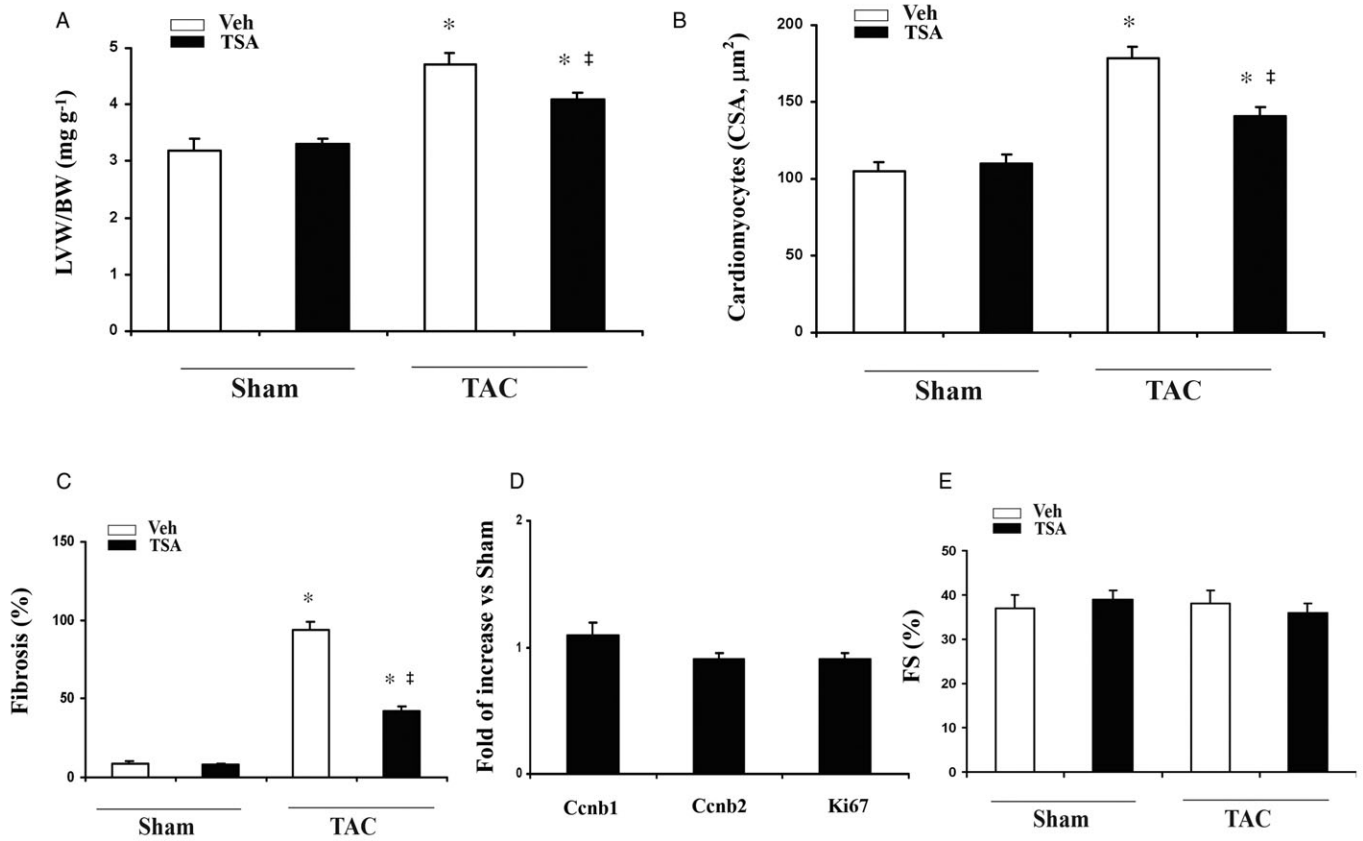
change (Figure 6E). Moreover, no significant difference in cardiac systolic function was observed between sham and TAC groups (data not shown).

### *Propranolol effects on cell cycle gene expression are observed at low plasma propranolol concentrations*

To evaluate whether propranolol effects on cell cycle gene expression were obtained at plasma concentrations that are usually achieved with the recommended therapeutic doses of this drug in humans, we determined plasma propranolol concentration by radioreceptor binding assay. We found that plasma drug concentrations in propranolol-treated TAC mice ranged from 10 to 140 ng·mL$^{-1}$ after 2 weeks of daily administration, which is within the range selective for β-adrenoceptor blockade.

## Discussion

In the present study, we demonstrated that propranolol enhances cell cycle-related gene expression in chronically pressure overloaded hearts by increasing the number of cycling non-cardiomyocyte cells. Our data also indicate that the effect of propanolol is mediated by occupation of $\beta_2$-adrenoceptors, rather than as a consequence of the $\beta_1$-blocking, anti-hypertrophic or stabilizing membrane effects of this drug. These findings were supported by several lines of evidence. Firstly, experiments with immunohistochemistry and confocal microscopy revealed that propranolol significantly increased Ki67-positive non-cardiomyocyte cells in pressure overloaded hearts, which concurs with gene expression data obtained by real-time PCR. Secondly, the effect of propranolol on cell cycle gene expression was directionally and quantitatively similar to that



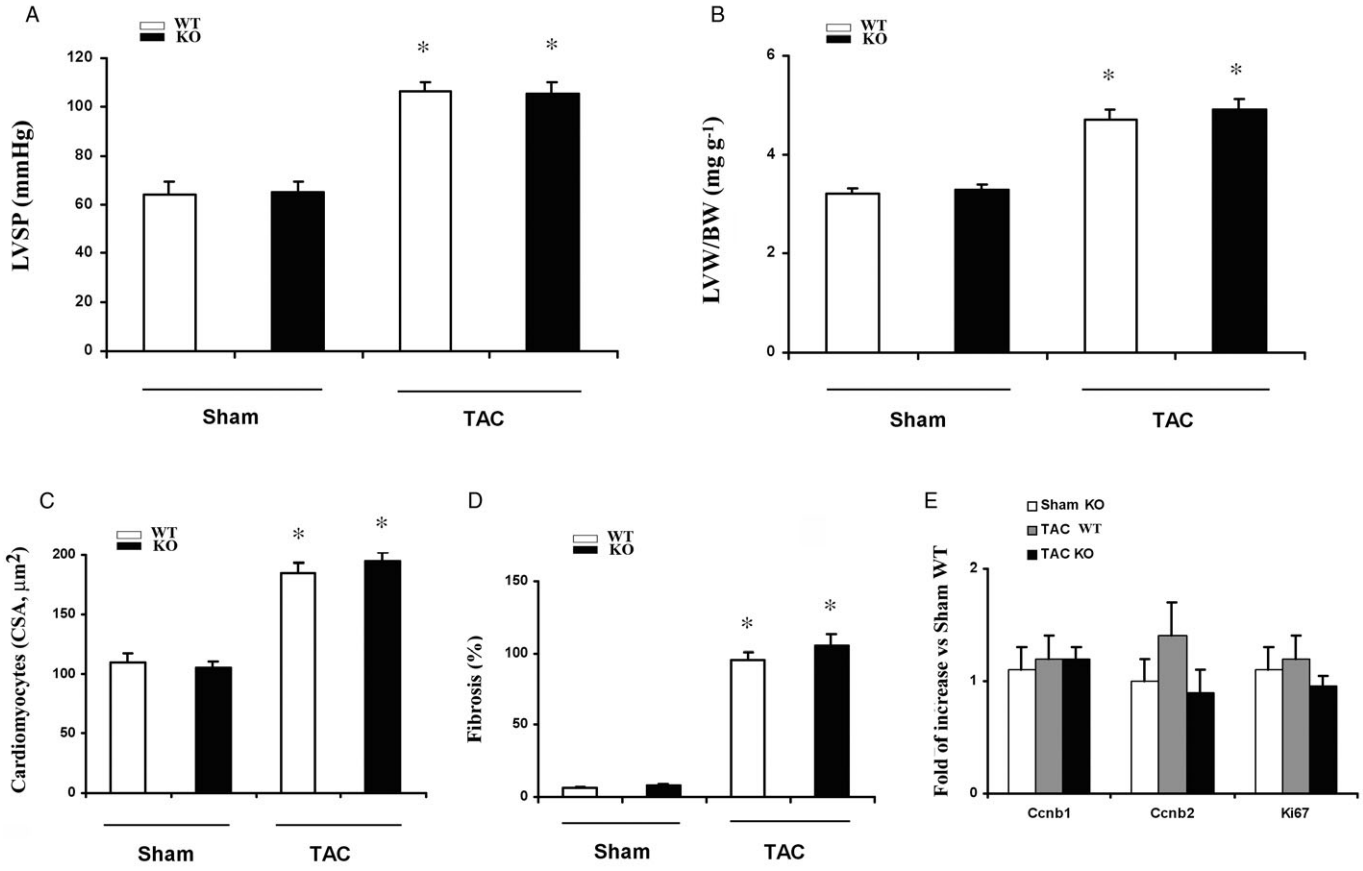

## Figure 5

TSA blunts cardiac hypertrophy without changing cell cycle gene expression. (A) LVW/BW ($n$ = 7 for each group). (B) CSA of cardiomyocytes ($n$ = 4 for each group). (C) Perivascular fibrosis; collagen accumulation is significantly blunted in TSA-treated TAC hearts ($n$ = 4 for each group). (D) Effects of TSA on gene expression of B-type cyclins and Ki67. Transcript amount from left ventricles was quantified by real-time PCR 2 weeks after TAC ($n$ = 3 for each group). (E) Echocardiographic analysis ($n$ = 7 for each group). *$P$ < 0.05 versus the sham operated group; ‡$P$ < 0.05 versus TAC.

observed in β-adrenoceptor KO mice subjected to TAC; this means that either the genetic deletion of β-adrenoceptors or their pharmacological blockade by the antagonist propranolol result in a similar biological response. Thirdly, metoprolol, a $\beta_1$-adrenoceptor antagonist, failed to change cell cycle gene expression in TAC mice. Fourthly, the attenuation of the cardiac hypertrophic response to TAC was not *per se* responsible for the enhanced expression of cell cycle-related genes. In fact, TSA, a histone deacetilase inhibitor endowed with anti-hypertrophic activity, failed to enhance cell cycle gene expression. Fifthly, $Scn5a^{+/-}$ KO mice, in which heterozygous disruption of the cardiac sodium channel gene causes a significant reduction in total cardiac sodium conductance thus mimicking the membrane stabilizing effect of propranolol, displayed no significant changes in cardiac cell cycle gene expression when subjected to TAC.

Previous studies have reported that hypertrophic stimuli increase the expression of cyclins in cardiomyocytes *in vitro* and *in vivo*. For example, certain D-type cyclins were up-regulated in neonatal cardiomyocytes in the presence of angiotensin II or phenylephrine (Busk *et al.*, 2002) as well as in LVs of mice subjected to aortic banding (Li *et al.*, 1998; Wagner *et al.*, 2004; Angelis *et al.*, 2008). In this study, we examined the gene expression of cyclins A1, A2, B1, B2, C, D1, E1 and F, using real-time PCR, but found no significant changes in LVs of C57BL/6 mice 2 weeks after TAC. Except for cyclin D1, which was found either to increase (Wagner *et al.*, 2004) or to decrease (Busk *et al.*, 2002) in animals subjected to aortic banding, our data are in accordance with previous reports. Li *et al.* (1998) reported that cyclins A, D1 and E protein levels were undetectable 6 weeks after aortic banding in Wistar rats. Also, Wagner *et al.* (2004) showed that there was no increase in gene expression of cyclins B and E 3 weeks after TAC in FVB mice. Contrasting outcomes among our study and others about the expression pattern of the cyclin D1 could be the result of different sites of banding as well as timing-, or species-related differences.

Several investigations have demonstrated that aortic constriction-induced pressure overload promotes cardiac fibroblast activation (i.e. proliferation of cardiac fibroblasts and their transdifferentiation to myofibroblasts). Cycling fibroblasts are found to peak at 5–7 days after aortic constriction remaining modestly increased at 2–4 weeks after aortic constriction (Kuwahara *et al.*, 2002; Takeda *et al.*, 2010). Since we found that propranolol administration was associated with a marked increase in cycling non-cardiomyocyte cells in




## Figure 6

Cell cycle gene expression is not altered by Scn5a gene deletion. (A) LV systolic pressure ($n \geq 5$ for each group). (B) LVW/BW ($n = 6$ or 7 for each group). (C) CSA of cardiomyocytes ($n = 3$ or 4 for each group). (D) Perivascular fibrosis ($n = 3$ or 4 for each group). (E) Transcript amount from left ventricles was quantified by SYBR Green real-time PCR. Data are shown as fold change relative to sham WT values. For each group, summary data of three independent experiments run in duplicate are shown. *$P < 0.05$ versus the sham operated group.

pressure overloaded hearts 2 weeks after TAC, an important question is whether propranolol promotes proliferation of cardiac fibroblasts, which constitute greater than 90% of non-cardiomyocyte cells in the heart, and cardiac fibrosis. However, this scenario seems to be unlikely for several reasons. Firstly, propranolol did not increase either cell cycle gene expression or cycling non-cardiomyocyte cells in hearts from sham-operated mice (Table 2 and Figure 2). Secondly, propranolol attenuated cardiac remodelling by blunting cardiomyocyte hypertrophy as well as cardiac fibrosis (Figure 1). Thirdly, results from another laboratory indicate that long-term treatment with propranolol (6–7 months) has beneficial effects on cardiac function and remodelling in a transgenic model of cardiac hypertrophy and fibrosis (Asai *et al.*, 1999). Fourthly, a recent study showed that propranolol reduces liver fibrosis in a murine model of sclerosing cholangitis, thus suggesting that its antifibrotic effects are not limited to cardiac tissue (Strack *et al.*, 2011). Given that propranolol treatment decreased cardiac fibrosis, but it is also associated with an increased number of cycling non-cardiomyocyte cells in pressure overloaded hearts 2 weeks after TAC, one possibility that remains to be explored in future studies is that propranolol could inhibit fibroblast transdifferentiation to myofibroblasts, but not fibroblast proliferation under pressure overload conditions. Then, considering that fibroblast transformation into myofibroblasts is associated with enhanced matrix production and decreased matrix turnover, the net result would be an increase in the number of cycling undifferentiated fibroblasts and a decrease in interstitial cardiac fibrosis.

Some important clinical effects of propranolol depend on its ability to competitively antagonize catecholamine-induced β-adrenoceptor stimulation in the heart. However, several studies have demonstrated that propranolol possesses other potentially beneficial properties. Indeed, it possesses membrane-stabilizing activity, in that it reduces sodium channel conductance and antioxidant properties that can protect cells and tissues against oxidative injury. These properties, which are unrelated to β-adrenoceptor blockade because both L- and D-propranolol isomers appear to be equipotent, are manifest at high concentrations. For example, plasma concentrations up to 1000 ng·mL$^{-1}$ may be required for the control of ventricular arrhythmias (Murray *et al.*, 1990). In the current study, we found that propranolol reduces cardiac growth response to pressure overload at low plasma concentrations. Additionally, we observed no signifi-

cant differences in LVW/BW ratio between wild-type and Scn5a$^{+/-}$ KO mice under pressure overload conditions. Collectively, these results suggest that the direct membrane effects are not critical for the anti-hypertrophic effect of propranolol.

Two additional aspects of our study are worth commenting on. Firstly, the effects of propranolol on cardiac gene expression could be secondary to effects arising from β-adrenoceptor blockade in other tissues, for example changes in renin–angiotensin status. Our results suggest that the effects of propranolol on the expression of the cell cycle-related genes in the heart are not secondary to changes of renin secretion or, more generally, to the blockade of the response elicited by $\beta_1$-adrenoceptor activation given that metoprolol, a $\beta_1$-adrenoceptor antagonist, failed to change cell cycle gene expression in TAC mice. Thus, propranolol could influence cell cycle gene expression in the heart via $\beta_2$-adrenoceptor antagonism in other tissues. However, it remains difficult to establish under *in vivo* conditions whether the cardiac effects of propranolol on cell cycle gene expression are due mainly to the blockade of cardiac β-adrenoceptors or are secondary to β-adrenoceptor blockade in other tissues. Secondly, the profile of gene expression observed in propranolol-treated TAC mice was broader than that detected in TAC β-adrenoceptor KO mice. In fact only a subset of the genes up-regulated by propranolol was also found overexpressed in β-adrenoceptor KO mice (Ccnb1, Ccnb2, Cdkn2a, Ki67 and Nek2). It is possible that this incomplete match may result from differences in genetic background between the two mouse strains and/or from secondary effects arising from indirect actions of the genetic deletion in other tissues that might in turn contribute to the overall profile of gene expression of the heart.

In conclusion, propranolol treatment increases the number of cycling non-cardiomyocyte cells via β-adrenoceptor-mediated mechanisms. Since these effects were obtained at plasma concentrations, which are usually achieved with the recommended therapeutic doses of this drug in humans, they could have clinical relevance under pressure overload conditions.

## Acknowledgements

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## Conflicts of interest

None.

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