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A Novel Immunomodulator, FTY-720 Reverses Existing Cardiac Hypertrophy and Fibrosis from Pressure Overload by Targeting NFAT Signaling and Periostin

Wei Liu, MD, PhD[1], **Min Zi, MD**[2], **Hoyee Tsui, Bsc**[1,2], **Sanjoy K Chowdhury, MBBS**[1], **Leo Zeef, PhD**[1], **Qing-Jun Meng, MD, PhD**[1], **Mark Travis, PhD**[1], **Sukhpal Prehar, MSc**[2], **Andrew Berry, PhD**[2], **Neil Hanley, MD, PhD**[2], **Ludwig Neyses, MD**[2], **Rui-Ping Xiao, MD, PhD**[3], **Delvac Oceandy, MD, PhD**[2], **Yunbo Ke, PhD**[4], **R. John Solaro, PhD**[4], **Elizabeth J. Cartwright, PhD**[2,*], **Ming Lei, MD, PhD**[2,*], and **Xin Wang, MD, PhD**[1,*]

[1]Faculty of Life Sciences, The University of Manchester, UK

[2]Faculty of Medical and Human Sciences, The University of Manchester, UK

[3]Institute of Molecular Medicine, Peking University, Beijing 100871, China

[4]Department of Physiology and Biophysics and Center for Cardiovascular Research, University of Illinois at Chicago, USA

Abstract

Background—Hypertension or aortic stenosis causes pressure overload, which evokes hypertrophic myocardial growth. Sustained cardiac hypertrophy eventually progresses to heart failure. Growing evidence indicates that restraining hypertrophy could be beneficial; here we discovered that FTY-720, an immuno-modulator for treating multiple sclerosis, can reverse existing cardiac hypertrophy/fibrosis.

Methods and Results—Male C57/Bl6 mice underwent transverse aortic constriction (TAC) for 1 week followed by FTY-720 treatment for 2 weeks under continuing TAC. Compared to vehicle-treated TAC hearts, FTY-720 significantly reduced ventricular mass, ameliorated fibrosis and improved cardiac performance. Mechanistic studies led us to discover that FTY-720 appreciably inhibited NFAT activity. Moreover, we found that in primary cardiomyocytes (rat and human) pertussis toxin (PTX, G_i-coupled receptor inhibitor) substantially blocked the anti-hypertrophic effect of FTY-720. This observation was confirmed in a mouse model of pressure overload. Interestingly, gene array analysis of TAC-hearts revealed that FTY-720 profoundly decreased gene expression of a group of matricellular proteins, of which periostin was prominent. Analysis of periostin protein expression in TAC-myocardium, as well as in rat and human cardiac fibroblasts confirmed the array data. Moreover, we found that FTY-720 treatment or knockdown of periostin protein was able to inhibit TGF-β responsiveness and decrease collagen expression.

Conclusions—FTY-720 alleviates existing cardiac hypertrophy/fibrosis through mechanisms involving negative regulation of NFAT activity in cardiomyocytes and reduction of periostin expression allowing for a more homeostatic extracellular compartment milieu. Together, FTY-720 or its analogues could be a promising new approach for treating hypertrophic/fibrotic heart disease.

Correspondence to: Dr. Xin Wang, Faculty of Life Sciences, University of Manchester, Michael Smith Building, Oxford Street, Manchester, M13 9PT, xin.wang@manchester.ac.uk, Tel: 0044-161- 2755616, Fax: 0044-161-2755082.
*Equal senior authors

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Keywords

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Increased blood pressure, which occurs in approximately one-third of the global adult population, has become a major public health problem[1]. Aortic stenosis is commonly caused by atherosclerotic disease which damages and stiffens the aortic valve, thereby reducing orifice area[2]. Both hypertension and aortic stenosis can result in significant left ventricular (LV) pressure overload, which evokes myocardial hypertrophy[1, 2]. There is widespread agreement that sustained hypertrophy with increased extracellular matrix (ECM) deposition is an important intermediate step leading to heart failure (HF)[3, 4]. Growing clinical and experimenta/l evidence suggests that suppression/reversal of the hypertrophic process may be beneficial even if pressure overload remains present[5, 6]. In humans from the onset of cardiac hypertrophy to the end stage of HF it usually takes relatively long time. This leaves a large time window, in which a patient displaying early cardiac hypertrophy could be treated to slow or even reverse the progression of hypertrophic remodeling. However, to date there are limited treatments available to reverse cardiac hypertrophy.

FTY-720 is a synthetic sphingosine analogue, recently approved by the FDA as a drug (named Gilenya) for treating relapsing multiple sclerosis. FTY-720 undergoes phosphorylation by sphingosine kinases (SPHKs), and phosphorylated FTY-720 competes with sphingosine 1-phosphate (S1P) to bind to G protein-coupled sphingosine 1-phosphate receptors (S1PRs) to regulate diverse biological activities[7–9]. We have previously demonstrated that FTY-720 is able to prevent the initiation of cardiac hypertrophy[10]. However, clinically it is difficult to anticipate the onset of cardiac hypertrophy; therefore more useful treatments would aim to reverse/alleviate existing hypertrophy. To this end, in experiments reported here we investigated whether FTY-720 can reverse existing hypertrophy/fibrosis and its mechanism of action.

We discovered that FTY-720 profoundly reverses existing hypertrophy/fibrosis through negative regulation of NFAT activity in cardiomyocytes through G_i signaling and reduction of periostin expression in the ECM, which renders a favourable milieu for myocytes, leading to improved cardiac performance. Our study provides new insights into the potential medical use of FTY-720 or its analogues for treating hypertrophic/fibrotic heart disease associated with hypertension, atherosclerotic/ rheumatic valvular lesion and cardiomyopathy.

Methods

Detailed methods echocardiography, hemodynamic analysis, histology, immunoblot analyses, quantitative PCR, immunocytochemistry, luciferase assay and Affymetrix gene expression array are available in supplemental material.

Animal Models of Pressure Overload and FTY-720 Treatment

Pressure overload by TAC was used to induce cardiac hypertrophy in male C57/Bl6 mice (8–10 weeks old). One week after surgery, TAC- or sham-operated mice were randomized into different cohorts for intra-peritoneal injection of FTY-720 (10μg/g/day) or vehicle for further two weeks. To determine whether FTY-720 requires Gi-signaling for limiting hypertrophy, after one week of TAC, C57/Bl6 mice were treated with FTY-720 (10μg/g/day) alone, or along with PTX, or PTX alone (30ng/g/every 3 days for two weeks). Hypertrophic responses at the end of the treatment were analyzed by echocardiography, hemodynamic analysis and biochemical analysis.

Echocardiography and Hemodynamic Analysis

Mice were anesthetized with Avertin (200 mg/kg) via intra-peritoneal injection. Transthoracic M-mode echocardiographic recordings were performed using an Acuson Sequoia C256 system (Siemens). Three measurements taken at end-systole and end-diastole were averaged to calculate intraventricular septal thickness (IVS), left ventricular posterior wall thickness (PW), left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD) and fractional shortening (FS %). *In vivo* hemodynamic analysis was performed using a pressure volume system (Millar Instruments). We used a 1.4F pressure-volume catheter (SPR-839) following a protocol described previously[10]. Pressure-volume (P-V) loops and a variety of hemodynamic parameters were measured to evaluate cardiac contractile and diastolic functions.

Neonatal Rat Cardiomyocytes and Human Embryonic Cardiomyocytes

Neonatal rat cardiomyocytes (NRCMs) were isolated from 1–2 day old Sprague-Dawley rats and plated in culture medium at a field density of 2×10^6 cells/well for treatments and subsequent analyses. The University of Manchester research ethics committee approved the collection of human tissue for cell culture. Human embryonic cardiomyocytes (HECMs) were prepared from spontaneous aborted human foetuses (9–12 weeks) and isolated HECMs were cultured at a density of 2×10^6 cells/well for various treatments and analyses.

Neonatal Rat Cardiac Fibroblasts and Human Adult Cardiac Fibroblasts

Neonatal rat cardiac fibroblasts (NRCFs) were prepared from the hearts of Sprague-Dawley rats at 1–2 days old. Human adult cardiac fibroblasts (HACFs) were purchased from Promo Cell Co. and cultured in conditioned medium supplemented with insulin (5μg/ml) and basic fibroblast growth factor (1ng/ml).

Affymetrix Gene Expression Array

Gene expression arrays for sham hearts and TAC-hearts treated with or without FTY-720 were performed with Mouse Genome 430 2.0 array chip (Affymetrix). The details of procedure and data analysis are provided in the methods section of the online data supplement.

Data Analysis

Data distribution normality was examined by Kolgomorov-Smirnov test. One-way or Two-way ANOVA followed by Bonferonni post-hoc tests were used for statistical comparisons among multiple groups, as appropriate. Comparisons between two groups were performed using Student's t-test. P-values <0.05 were considered statistically significant. Data are expressed as mean ± SEM.

Results

FTY-720 Reverses Existing Cardiac Hypertrophy and Fibrosis

1 week of TAC was sufficient to induce steady cardiac hypertrophy (Supplement figure I). At this time point FTY-720 or vehicle was administered for 2 weeks whilst continuation of TAC. Under the similar pressure gradients (30 mmHg), TAC-mice receiving FTY-720 manifested less hypertrophy than mice receiving vehicle, evidenced by a significant decrease in heart weight/tibia length (HW/TL) ratio and a substantial reduction in cross-sectional area of TAC/FTY-720 cardiomyocytes with respect to the TAC/vehicle cardiomyocytes (Figure 1A–B). This diminution in hypertrophy was confirmed by quantitative PCR analysis of hypertrophic biomarker genes. Transcript expression of atrial natriuretic peptide (*ANP*) and regulator of calcineurin 1 variants 4 (*RCAN1.4*) was

significantly down-regulated in the TAC/FTY-720 hearts (Figure 1C). Cardiac remodeling was also greatly improved in the FTY-720 treated hearts. Masson's trichrome staining barely detected interstitial fibrosis in the TAC/FTY-720 hearts (Figure 1D). Leukocyte infiltration was analysed by immunohistochemistry that revealed very few infiltrating macrophages and neutrophils in TAC/FTY-720 myocardium (Figure 1E). Moreover, we found that even compared to 1 week-TAC hearts, FTY-720-treated hearts exhibited less hypertrophic remodeling (Supplement Table I).

Next we tested whether Pak1 is involved in the ability of FTY-720 to reverse hypertrophy. The same FTY-720 treatment protocol was applied to $Pak1^{cko}$ and control mice ($Pak1^{f/f}$). FTY-720 decreased TAC-induced hypertrophic remodeling in $Pak1^{f/f}$ mice, whereas despite FTY720 treatment, $Pak1^{cko}$ displayed similar extent of hypertrophic remodeling, as well as comparable cardiac structure and function with respect to vehicle-treated $Pak1^{cko}$ mice (Supplement Figure II-III). These data indicate that FTY-720 reverses hypertrophy via a Pak1-dependent mechanism.

FTY-720 Improves Cardiac Performance

Reduced cardiac hypertrophy by FTY-720 treatment was substantiated by echocardiographic analysis. Compared to the TAC only group, dPW and dIVS at end-diastole were consistently decreased in the TAC/FTY-720 ventricles (Figure 2A–B). Enlargement of ventricular chambers at both end-systole and -diastole (LVESD and LVEDD), as well as decreased FS % were observed after 3 weeks of TAC (Figure 2C–E), indicating impaired cardiac function. Such compromised cardiac function was significantly improved upon FTY-720 treatment (Figure 2C–E).

To further assess the effect of FTY-720 on cardiac function, invasive hemodynamic analysis was carried out. P-V loops were measured before and during transient inferior vena-cava occlusion. The sham condition had the most leftward P-V loops, whereas 3 weeks of TAC induced a prominent rightward shift of the loops, while FTY-720 treatment brought this shift back (Figure 2F), indicating 3 weeks of TAC caused a chamber dilation, and FTY-720 treatment ameliorated this deterioration. Notably, ejection fraction (EF%) and end-systolic elastance (Ees) were significantly higher whereas left ventricular end-diastolic volume (Ved) and end-diastolic pressure (Ped) were decreased by FTY-720 treatment (Table). FTY-720 was reported to cause a transient slowing in heart rate (HR) in human subjects[11], however we did not detect slowed HR at the time of recording. Also, FTY-720 did not alter blood pressure (BP) and left ventricular peak pressure (Pes) (Table).

FTY-720 Attenuates NFAT Activity

We next investigated the mechanistic action of FTY-720 at the cellular and molecular level. FTY-720-treated NRCMs showed obviously smaller cell surface area than NRCMS treated with PE only (Supplement Figure IV). In addition, FTY-720 resulted in less ANP-positive NRCMs compared to the respective control groups (Supplement Figure IV). These results demonstrate that FTY-720 exerts a direct effect on cardiomyocyte hypertrophy.

Led by the observed down-regulation of RCAN 1.4 mRNA level in the TAC/FTY-720 heart, we next examined whether FTY-720 has an ability to regulate NFAT activity. Immunofluorescence staining demonstrated that PE stimulation caused NFAT nuclear translocation, whereas FTY-720 treatment mobilized its movement from the nucleus to the cytoplasm (Figure 3A–B). Knockdown of Pak1 by shPak1 potentiated PE-induced NFAT nuclear accumulation, but under these conditions lacking Pak1, FTY-720 was unable to reverse this effect (Figure 3A–B). Also, FTY-720 failed to block NFAT nuclear translocation in NRCMs expressing constitutively active calcineurin (Figure 3C). Together,

these results suggest that FTY-720 does not have a direct effect on calcineurin and that the reversal of hypertrophy occurs by Pak1 activation, which is likely through negative regulation of NFAT activity.

FTY-720 Targets G_i-Signaling

FTY-720 is able to interact both G_q- and G_i-coupled receptors depending on biological context[12, 13]. G_q stimulation of cardiac hypertrophy is well documented[14, 15], and thus by inference we asked that FTY-720 may work through G_i-dependent signaling to exert its anti-hypertrophic function. Firstly, we tested whether FTY-720 phosphorylation by sphingosine kinases (SPHKs) is a precondition for its anti-hypertrophic function. Western blotting demonstrated that FTY-720 induced Pak1 phosphorylation whereas SPHK inhibitor 2 blocked it (Figure 4A), suggesting conversion to FTY-720-P is required for its function. To determine whether FTY-720-induced Pak1 phosphorylation depends on its interaction with S1PR, we pre-treated NRCMs with of S1P with increasing doses followed by 1h treatment of FTY-720. As shown, FTY-720 alone induced marked Pak1 phosphorylation, whereas pre-treatment with S1P blocked this phosphorylation with a significant effect at 200 nM (Figure 4A). Next we found FTY-720 induced whereas PTX blocked Pak1 phosphorylation (Figure 4B). To translate this finding to a scenario relevant in humans, the above experiment was carried out in HFCMs. We found that PTX counteracted FTY-720-induced Pak1 phosphorylation in HFCMs (Figure 4B). Furthermore, we observed that HFCMs receiving PTX treatment had an increased ANP expression comparable to that in PE-treated cells, and significantly more than that in FTY-720-treated HFCMs (Figure 4C).

Next we tested our hypothesis in a pressure overloaded mouse model. Mice undergoing TAC for 1 week were then administered with FTY-720 for 2 weeks with or without PTX. In agreement with *in vitro* data, we found that PTX treatment offset the protective effect of FTY-720, causing hypertrophy comparable to that induced by 3 weeks of TAC in control animals (Figure 4D–E). Echocardiographic analysis corroborated changes in these treatment groups (Figure 4F–H and Supplement Table II). The beneficial effect of FTY-720 on cardiac performance was also diminished in the presence of PTX (Figure 4H). Notably, treatment with PTX alone did not cause any change in cardiac structure and function in sham mice, also TAC-stressed mice receiving PTX only did not display further hypertrophy (Figure 4D-H and Supplement Table II). Taken together, our data demonstrate that FTY-720 works through PTX-sensitive G_i-coupled receptors to activate Pak1, thereby antagonizing NFAT activity for reversing hypertrophy.

Periostin is Involved in Reduced Fibrotic Response Following FTY-720 Treatment

In view of our observations that FTY-720 efficiently ameliorated TAC-induced interstitial fibrosis, we then performed Affymetrix gene array analysis of TAC-hearts to probe the mechanism underpinning this beneficial effect of FTY-720. Of 45,000 analysed transcripts, 56 genes were significantly altered ($p<0.05$) as a result of both TAC and FTY-720 treatment. Using the DAVID functional annotation tool, we found that a large set of these differentially expressed genes fell into a biological category involving extracellular matrix organization and/or inflammation response, such as periostin (Postn), collagens (Col), thrombospondin-4 (Thbs4), IL-6, pleiotrophin (Ptn) and tumor necrosis factor receptor superfamily member 11b (Tnfrsf11b) (Figure 5 and Supplement Table III). Among these altered genes periostin was prominent; its mRNA expression was significantly increased with hypertrophic remodeling (5.4-fold increase, $P<0.01$) but decreased by FTY-720 treatment (nearly 3-fold decrease, $P<0.01$).

To corroborate the array results, we examined protein expression of periostin. The periostin level in sham-operated hearts was undetectable, but in TAC-hearts it was markedly induced,

however this induction was reduced upon FTY-720 treatment (Figure 6A). Furthermore, we detected that expression of *collagen 1α2*, *3α1* and *5α2* was significantly induced by TAC stress, but decreased by FTY-720 treatment (Figure 6B). Next we examined whether FTY-720 could directly affect periostin expression in cardiac fibroblasts. NRCFs and HACFs were incubated with angiotensin II (Ang II) with or without FTY-720. After 24h incubation, we detected considerably higher periostin expression in response to Ang II in both cell types, while FTY-720 decreased its expression (Figure 6C). TGF-β signaling can profoundly influence ECM deposition through inducing expression of pro-fibrotic proteins[16–18]. Although transcript expression of major TGF-β family members appeared not to be altered in the gene array analysis, we extended our investigation to the examination of TGF-β activation. We determined that expression of activated TGF-β1 (25 kDa) and Smad 2 phosphorylation were substantially increased upon TAC stress, but decreased in response to FTY-720 treatment (Figure 6D). Moreover, we used a TGF-β responsive luciferase-reporter system to determine whether FTY-720 is able to block TGF-β activation. NRCFs were co-cultured with TGF-β responsive reporter cells, which contained a plasminogen activator inhibitor-1 (PAI-1) promoter fused to the luciferase-reporter gene[19]. After 24h treatment of Ang II with or without FTY-720, we detected a significantly higher luciferase activity in response to Ang II alone; whereas in the presence of FTY-720 the luciferase activity was much lower, indicating the presence of an FTY-720 inhibition of TGF-β responsiveness (Figure 6E).

Periostin is known to be a TGF-β-inducible matrix protein; conversely periostin itself has important roles in TGF-β activation and collagen production[20–23]. We then investigated whether periostin affects TGF-β responsiveness in cardiac fibroblasts. Periostin was knocked down by si-Postn in NRCFs, which were then exposed to Ang II for 48h. We detected that expression of activated TGF-β1 and Smad 2 phosphorylation were appreciably increased in response to Ang II stimulation, but Ang II failed to induce TGF-β activation when periostin was absent (Figure 6F). Furthermore, we found that periostin knockdown diminished Ang II-induced gene expression of collagen 1a2 and 5a2 (Figure 6F).

Finally we tested whether FTY-720 induced periostin reduction involves Pak1 signaling. Interestingly, we observed that FTY-720 was unable to reduce Ang II induced-periostin expression in NRCFs with Pak1 knockdown (Supplement Figure VA–B). In contrast, overexpression of Pak1 in NRCFs was sufficient to decrease periostin expression by Ang II stimulation (Supplement Figure VB). NFAT activity was also examined in NRCFs, but we found that FTY-720 had no effect on Ang II-induced NFAT activity (Supplement Figure VC). Moreover, excessive amount of S1P was shown to prevent Pak1 phosphorylation by FTY-720 in NRCFs; however, PTX had no effect on this phosphorylation (Supplement Figure VD-E). These data suggest that FTY-720 likely requires S1PRs and Pak1 for its anti-fibrotic effect; however signaling system used by FTY-720 for reducing fibrotic response in cardiac fibroblasts seems different as it operates in cardiomyocytes for antagonising hypertrophy.

Discussion

In this study we identified a new dual-mechanism system used by FTY-720 for treating hypertrophic/fibrotic heart disease. The major findings of this study are: (1) Phosphorylated FTY-720 through G_i-coupled S1PRs conveys anti-hypertrophic signals involving Pak1/NFAT signalling in cardiomyocytes; (2) Periostin seems to be a primary effector of FTY-720 in the ECM. By this dual-mechanism system FTY-720 reverses hypertrophy, reduces the fibrotic response, restores ECM integrity and improves cardiac performance (Figure 7).

FTY-720, G_i- Coupled Receptors and NFAT Signaling in Cardiomyocyte Hypertrophy

Being part of the G-protein coupled receptor family, S1P receptors (S1PRs) can activate different signaling cascades by coupling with heterotrimeric G-proteins[12, 13]. $S1P_1$ receptor selectively couples to G_i-proteins, whereas $S1P_3$ is a promiscuous receptor available for all major G-protein subtypes[12, 13]. Cardiomyocytes express both $S1P_1$ and $S1P_3$[24, 25]. Here we found that exposure of NRCMs or C57/Bl6 mice to PTX rendered FTY-720 ineffective at reducing hypertrophic response to either PE or TAC. This effect was similar in the context of human cardiomyocytes. Moreover, excessive S1P was found to block FTY-720 effect on Pak1 phosphorylation. Thus, we believe that FTY-720 inhibits hypertrophy likely through G_i-coupled S1PRs and this mechanism can be used by cardiomyocytes across different species.

The NFAT family of transcription factors is a nodal control point of the cardiac hypertrophic response. Our data suggest that FTY-720 appears to be related to the negative regulation of NFAT transcriptional responsiveness through signaling involving Pak1. To determine whether the suppression of NFAT activity is directly responsible for anti-hypertrophic effect of FTY-720, future experiments are needed to test FTY-720 effect in mouse models with targeted inhibition or activation of NFAT signaling.

FTY-720 and Periostin in Fibrotic Response

ECM components constitute a fibrous skeleton for maintaining overall ventricular geometries and proper myocyte organization. Long-standing pressure overload associated with hypertension, valvular disease and cardiomyopathy all induce remodeling of the ECM[26]. In other words, these major forms of heart diseases often develop greater hypertrophy with overabundant fibrosis and ventricular disobedience. As described above, we successfully identified periostin, which was significantly altered in correlation to TAC stress and FTY-720 treatment.

Periostin is a secreted ECM protein with minimum expression in adult ventricles at baseline, but it is dramatically re-expressed largely by cardiac fibroblasts after pressure overload or myocardial infarction[21, 27]. As a crucial adhesion molecule periostin is important for regulating ECM integrity and modulating hypertrophy through its ability to bind with multiple ECM components and integrins[27]. In genetic modification studies, periostin knockout ($Pn^{-/-}$) resulted in reduced fibrosis and hypertrophy following pressure overload[28, 29], whereas periostin overexpressing transgenic mice developed spontaneous hypertrophy with aging[28, 29]. Akin to these findings, we observed that periostin expression was massively increased by TAC stress, but its expression was decreased with a concomitant reduction in fibrosis and hypertrophy following FTY-720 treatment. Moreover, FTY-720 treatment led to a reduction in Ang II-induced periostin expression, whilst periostin knockdown resulted in blunted TGF-β activation and decreased collagen expression in cardiac fibroblasts. This finding is consistent with previous observations that not only is periostin induced by TGF-β; periostin itself can activate TGF-β signaling[20, 22].

It has been demonstrated in lung and skin that fibrotic remodeling can occur from activation of latent TGF-β in the ECM rather than from accrued transcription[30, 31]. Consistently we demonstrated that the mechanical force of pressure overload activated latent TGF-β whereas FTY-720 inhibited its activation. Given that periostin is an important activator of the TGF-β signaling pathway, we hypothesize that reduced periostin by FTY-720 treatment is possibly attributable to blunted TGF-β activation, which in turn further down-regulates periostin expression. By modulating this signaling feedback loop FTY-720 reduces fibrotic response and restores ECM integrity.

Apart from matricellular proteins, we also found a set of cytokines responding to FTY-720 treatment, such as IL-6, Ptn and Tnfrsf11b. Notably, IL-6 is a potent hypertrophic factor signaling through gp130 in response to pressure overload[32]. The ability of FTY-720 to suppress IL-6 expression was also reported in an atherosclerosis mouse model[33]. It is plausible that reduction of IL-6 or other cytokines and matrix proteins may participate in some extent to the high capacity of FTY-720 to reserve cardiac hypertrophy and associated fibrosis.

FTY-720 and Cardiac Performance

One important criterion for anti-hypertrophic drug is its capability of improving cardiac performance. Our data indicates that FTY-720 likely ameliorates functional deterioration evident by increased FS% and EF%, whereas on another point, Ea was decreased by FTY-720. Given similarity in pressure gradient and no changes in HR, it could suggest a possibility that FTY-720 may affect systemic resistance, which gives rise to a lower net "afterload". Although we did not observe any alterations in BP in various treated groups, whether FTY-720 has effects on vascular tone was not investigated directly in this study. It is plausible that FTY-720 may work through interactions with both myocardium and vasculatures, resulting in improved cardiac function. Another interesting point is the effect of FTY-720 on diastolic function. Although FTY-720 significantly reduced fibrotic response with an early sign of improvement of compliance, however, some major diastolic parameters, like Tau and dP/dt_{min} remained unchanged. This incongruity may be related to the fact that in mice the amount of fibrosis does not always generally reflect the extent of diastolic stiffness in left ventricles. Future study using a chronic and severe TAC model (9–16 weeks) may provide a better evaluation for FTY-720 efficacy on both systolic and diastolic functions.

Clinical Potential and Future Study

A growing number of clinical and experimental studies have discovered elevated periostin expression in the hearts of atherosclerotic and rheumatic valvular heart disease, hypertrophic cardiomyopathy and Marfan syndrome[34, 35], suggesting an important role for periostin in the pathogenesis of these heart diseases. Suggested by our current findings, further investigations are needed to test FTY-720 efficacy in animal models mimicking these hypertrophic/fibrotic heart diseases. Another interesting point is that some efforts have been made to alleviate ECM remodeling by interfering with the TGF-β pathway over the past few years[6]; however pleiotropic effects of TGF-β render its clinical use somewhat difficult. It appears therefore that inhibition of periostin by FTY-720 may be more optimal than global TGF-β blockage. Although we here demonstrated that Pak1 is involved in FTY-720 induced periostin diminution in cardiac fibroblasts, however action mechanisms underlying FTY-720 reduced fibrotic response, with regards to G-protein subtype and downstream molecules are largely unknown and required further investigations.

Conclusion

Reversal of hypertrophy and fibrosis by FTY-720 might occur through a dual-mechanism system involving negative regulation of NFAT activity in cardiomyocytes and diminution of periostin expression in the ECM. Two mechanisms can work in a reinforcing manner leading to better cardiac performance. Overall, this study shows promise for the clinical use of FTY-720 or its analogues for hypertrophic/fibrotic heart disease.

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References

1. Drazner MH. The progression of hypertensive heart disease. Circulation. 2011; 123:327–334. [PubMed: 21263005]
2. Houser SR, Margulies KB, Murphy AM, Spinale FG, Francis GS, Prabhu SD, Rockman HA, Kass DA, Molkentin JD, Sussman MA, Koch WJ. Animal models of heart failure: a scientific statement from the American Heart Association. Circ Res. 2012; 111:131–150. [PubMed: 22595296]
3. Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, the ugly. Annu Rev Physiol. 2003; 65:45–79. [PubMed: 12524460]
4. Frey N, Katus HA, Olson EN, Hill JA. Hypertrophy of the heart: a new therapeutic target? Circulation. 2004; 109:1580–1589. [PubMed: 15066961]
5. McKinsey TA, Kass DA. Small-molecule therapies for cardiac hypertrophy: moving beneath the cell surface. Nat Rev Drug Discov. 2007; 6:617–635. [PubMed: 17643091]
6. Koitabashi N, Kass DA. Reverse remodeling in heart failure-mechanisms and therapeutic opportunities. Nat Rev Cardiol. 2011; 9:147–157. [PubMed: 22143079]
7. Brinkmann V. FTY-720: Mechanism of action and potential benefit in organ transplantation. Yonsei Med J. 2004; 45:991–997. [PubMed: 15627289]
8. Kahan BD. FTY720: From bench to bedside. Transplant Proc. 2004; 36:531S–543S. [PubMed: 15041402]
9. Chiba K. FTY720, a new class of immunomodulator, inhibits lymphocyte egress from secondary lymphoid tissues and thymus by agonistic activity at sphingosine 1-phosphate receptors. Pharmacol Ther. 2005; 108:308–319. [PubMed: 15951022]
10. Liu W, Zi M, Naumann R, Ke Y, Ulm S, Jin J, Taglieri DM, Prehar S, Gui J, Xiao RP, Neyses L, Soralo J, Cartwright EJ, Lei M, Wang X. Pak1 is a novel signaling regulator attenuating cardiac hypertrophy in mice. Circulation. 2011; 124:2702–2715. [PubMed: 22082674]
11. Schmouder R, Serra D, Wang YB, Kovarik JM, DiMarco J, Hunt TL, Bastien MC. FTY720: Placebo-controlled study of the effect on cardiac rate and rhythm in healthy subjects. J Clin Pharmacol. 2006; 46:895–904. [PubMed: 16855074]
12. Sensken SC, Staubert C, Keul P, Levkau B, Schoneberg T, Graler MH. Selective activation of G alpha i mediated signaling of S1P3 by FTY720-phosphate. Cell Signal. 2008; 20:1125–1133. [PubMed: 18313900]
13. Brinkmann V, Lynch KR. FTY720: targeting G-protein-coupled receptors for sphingosine 1-phosphate in transplantation and autoimmunity. Curr Opin Immunol. 2002; 14:569–575. [PubMed: 12183155]
14. Adams JW, Brown JH. G-protein in growth and apoptosis: lessons from the heart. Oncogene. 2001; 20:1626–1634. [PubMed: 11313910]
15. Salazer NC, Chen J, Rockman HA. Cardiac GPCRs: GPCR signaling in heathy and failing hearts. Biochim Biophys Acta. 2007; 1768:1006–1018. [PubMed: 17376402]
16. Berk BC, Fujiwara K, Lehoux S. ECM remodelling in hypertrophive heart disease. J Clin Invest. 2007; 117:568–575. [PubMed: 17332884]
17. Leask A. TGFbeta, cardiac fibroblasts, and the fibrotic response. Cardiovasc Res. 2007; 74:207–212. [PubMed: 16919613]
18. Massague J. TGF-beta signal transduction. Annu Rev Biochem. 1998; 67:753–791. [PubMed: 9759503]
19. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB. An assay for transforming growth factor-β using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. Anal Biochem. 1994; 216:276–284. [PubMed: 8179182]
20. Snider P, Hinton RB, Mereno-Rodriguez RA, Wang J, Rogers R, Lindsley A, Li F, Ingram DA, Menick D, Field L, Firulli AB, Molkentin JD, Markwald R, Conway SJ. Periostin is required for

maturation and extracellular matrix stabilization of noncardiomyocyte lineages of the heart. Circ Res. 2008; 102:752–760. [PubMed: 18296617]

21. Snider P, Standley KN, Wang J, Azhar M, Doetschman T, Conway SJ. Origin of cardiac fibroblasts and the role of periostin. Circ Res. 2009; 105:934–947. [PubMed: 19893021]
22. Sidhu SS, Yuan S, Innes AL, Kerr S, Woodruff PG, Hou L, Muller SJ, Fahy JV. Roles of epithelial cell-derived periostin in TGF-β activation, collagen production, and collagen gel elasticity in asthma. Proc Natl Acad Sci USA. 2010; 107:14170–14175. [PubMed: 20660732]
23. Lorts A, Schwanekamp JA, Baudino TA, McNally EM, Molkentin JD. Deletion of periostin reduces muscular dystrophy and fibrosis in mice by modulating the transforming growth factor-β pathway. Proc Natl Acad Sci USA. 2012; 109:10978–10983. [PubMed: 22711826]
24. Takuwa Y, Okamoto Y, Yoshioka K, Takuwa N. Sphingosin-1-phosphate signaling and biological activities in the cardiovascular system. Biochimica et Biophysica Acta. 2008; 1781:483–488. [PubMed: 18472021]
25. Kennedy S, Kane KA, Pyne NJ, Pyne S. Targeting sphingosine-1-phosphate signalling for cardioprotection. Curr Opin Pharmacol. 2009; 9:194–201. [PubMed: 19070545]
26. Burlew BS, Weber KT. Cardiac fibrosis as a cause of diastolic dysfunction. Herz. 2002; 27:92–98. [PubMed: 12025467]
27. Cownway SJ, Molkentin JD. Periostin as a heterofunctional regulator of cardiac development and disease. Curr Genomics. 2008; 9:548–555. [PubMed: 19516962]
28. Oka T, Xu J, Kaiser RA, Melendez J, Hambleton M, Sargent MA, Lorts A, Brunskill EW, Dorn GW II, Conway SJ, Aronow BJ, Robbins J, Molkentin JD. Genetic manipulation of periostin expression reveals a role in cardiac hypertrophy and ventricular remodelling. Circ Res. 2007; 101:313–321. [PubMed: 17569887]
29. Lorts A, Schwanekamp JA, Elrod JW, Sargent MA, Molkentin JD. Genetic manipulation of periostin expression in the heart does not affect myocyte content, cell cycle activity, or cardiac repair. Circ Res. 2009; 104:e1–e7. [PubMed: 19038863]
30. Hinz B. Tissue stiffness, latent TGF-beta activation, and mechanical signal transduction: implications for the pathogenesis and treatment of fibrosis. Curr Rheumatol Rep. 2009; 11:120–126. [PubMed: 19296884]
31. Goodwin A, Jenkins G. Role of integrin-mediated TGFbeta activation in the pathogenesis of pulmonary fibrosis. Biochem Soc Trans. 2009; 37:849–854. [PubMed: 19614606]
32. Wollert KC, Drexler H. The role of interleukin-6 in the failing heart. Heart Fail Rev. 2001; 6:95–103. [PubMed: 11309528]
33. Nofer JR, Bot M, Brodde M, Taylor PJ, Salm P, Brinkmann V, van Berkel T, Assmann G, Biessen EAL. FTY720, a synthetic sphingosine 1 phosphate analogue, inhibits development of atherosclerosis in low-density lipoprotein receptor-deficient mice. Circulation. 2007; 115:501–508. [PubMed: 17242282]
34. Hakuno D, Kimura N, Yoshioka M, Mukai M, Kimura T, Okada Y, Yozu R, Shukunami C, Hiraki Y, Kudo A, Ogawa S, Fukuda K. Periostin advances atherosclerotic and rheumatic cardiac valve degeneration by inducing angiogenesis and MMP production in humans and rodents. J Clin Invest. 2010; 120:2292–2306. [PubMed: 20551517]
35. Teekakirkui P, Eminaga S, Toka O, Alcalai R, Wang L, Wakimoto H, Nayor M, Konno T, Gorham JM, Wolf CM, Kim JB, Schmitt JP, Molkentin JD, Norris RA, Tager AM, Hoffman SR, Markwald RR, Seidman CE, Seidman JG. Cardiac fibrosis in mice with hypertrophic cardiomyopathy is mediated by non-myocyte proliferation and requires Tgf-β. J Clin Invest. 2010; 120:3520–3529. [PubMed: 20811150]

CLINICAL SUMMARY

Regardless of pathological stimuli, cardiac hypertrophy per se is a critical intermediate step for the development of heart failure, which affects approximately 2.5% of the population worldwide. In humans, the time from the onset of cardiac hypertrophy to the end stage of HF is usually prolonged. This leaves a large time window, in which a patient displaying early cardiac hypertrophy could be treated to slow or even reverse the progression of hypertrophic remodeling. However, to date there are limited treatments available to reverse cardiac hypertrophy. In this study we discovered FTY-720, a synthetic sphingosine analogue, recently approved by the FDA as a drug (named Gilenya) for treating relapsing multiple sclerosis, can profoundly reverses existing hypertrophy and associated fibrosis through a novel dual-mechanism system involving negative regulation of NFAT activity in cardiomyocytes and diminution of periostin expression in the extracellular matrix, allowing for a more homeostatic extracellular compartment milieu. Two mechanisms can work in a reinforcing manner leading to better cardiac performance. Overall, this study shows promise for the clinical use of FTY-720 or its analogues for hypertrophic/fibrotic heart disease.

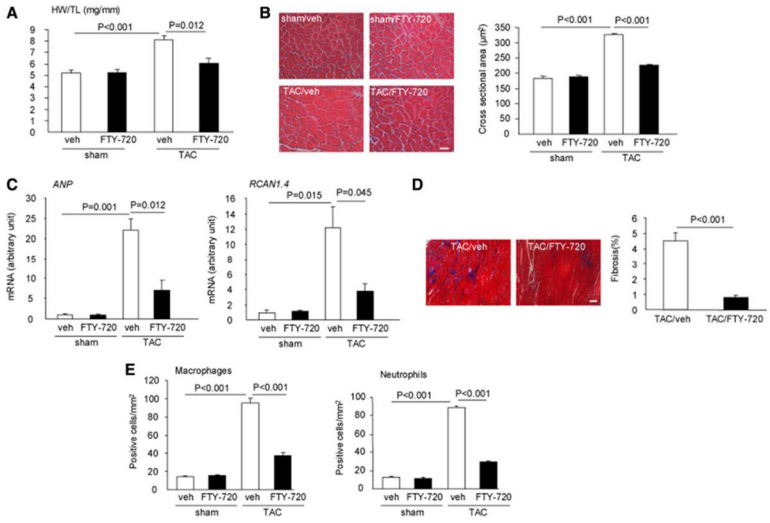

Figure 1.

FTY-720 reverses cardiac hypertrophy and interstitial fibrosis caused by pressure overload. (A) HW/TL ratios and (B) Measurements of mean cross-sectional area of cardiomyocytes (scale bar: 20μm) demonstrate that TAC increased whereas FTY-720 reduced cardiac hypertrophy. (C) qPCR analysis of *ANP* and *RCAN1.4*. The data is normalized to the GAPDH. (D) Masson's trichrome staining (scale bar: 50μm) shows that FTY-720 remarkably reduced TAC-induced fibrosis. Quantification of the relative area of fibrosis is expressed as percentage fibrosis. (E) Quantification of immunostaining of heart sections with anti-Mac-3 and anti-neutrophil antibodies indicates increased populations of macrophages and neutrophils in TAC-stressed ventricular myocardium, whereas FTY-720 treatment ameliorated this TAC-provoked leucocyte infiltration. N=8–12 per group, data is presented as mean ± SEM.

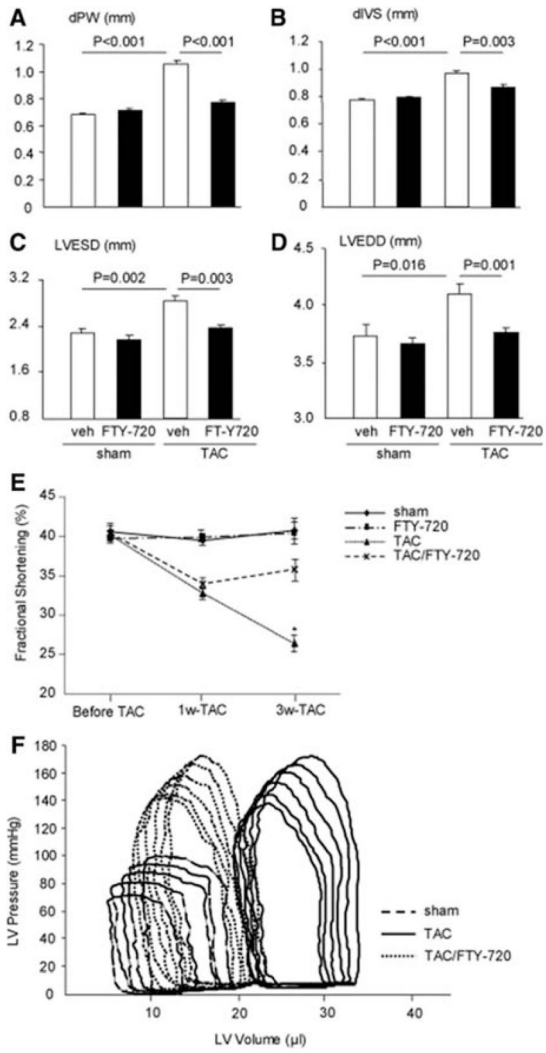

Figure 2.

Echocardiography and hemodynamic analysis demonstrate the beneficial effect of FTY-720 on cardiac function. TAC by 3 weeks caused thickening of LV posterior wall (A) and intraventricular septum (B), whereas FTY-720 diminished ventricular wall thickness (A–B). (C–D) Chamber dimensions at end-systole and end-diastole were enlarged in TAC stressed-hearts, while ventricular chamber of FTY-720-treated hearts at both stages became smaller. (E) Cardiac function indexed by FS % was measured at 1 and 3 weeks of TAC, indicating that FS% was decreased by TAC stress, but improved by 2 weeks of FTY-720 treatment. (F) TAC for 3 weeks induced a prominent rightward shift of the P-V loops, whereas FTY-720 treatment brought the loops leftward. N=8–12 per group, data is presented as mean ± SEM.

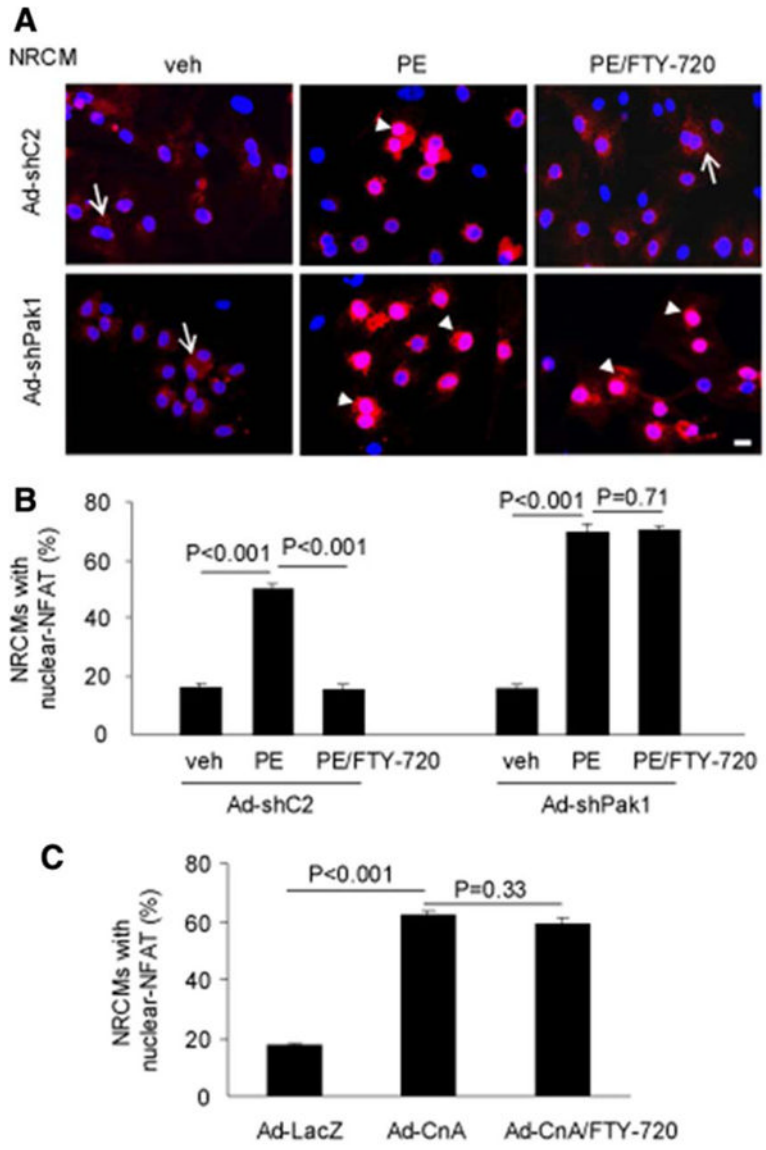

Figure 3.

FTY-720 negatively regulates NFAT activity through Pak1 activation in cardiomyocytes. (A) Representative fluorescent images show the cytoplasmic-nuclear localization of endogenous NFATc4 in NRCMs infected with Ad-shPak1 or Ad-shC2 followed by treatment with PE ± FTY-720, which demonstrate FTY-720 inhibited PE-induced NFAT nuclear retention, whereas such inhibitory effects were eliminated in NRCMs with Pak1 knockdown. Red staining is for NFAT, blue for visualizing the nuclei, arrows point to cytoplasmic NFAT; arrowheads show nuclear localization of NFAT (scale bar: 20μm). (B) These results were quantified from approximately 500 infected cardiomyocytes per group and presented as the percentage of cells with nuclear-NFAT. (C) Immunostaining of NFAT4c was also applied to NRCMs infected by Ad-CnA with or without FTY-720. Ad-LacZ was used as a control virus. Quantitative data is presented as the percentage of cells expressing nuclear-NFAT. N=5 per group, data is presented as mean ± SEM.

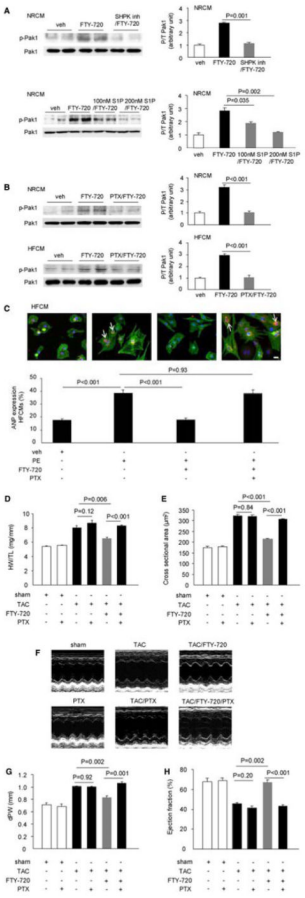

Figure 4.

FTY-720 antagonizes hypertrophy through a G_i-dependent mechanism. (A) Protein extracts from NRCMs treated with FTY-720 ± SPHK inhibitor 2 (SPHK inh) or ± various doses of S1P were examined by immunoblotting for total and phosphorylated (Thr 423) Pak1. (B) Immunoblotting demonstrates effect of FTY-720 alone, or in combination with PTX on Pak1 phosphorylation at Thr 423 in NRCMs and HFCMs. The ratios of P/T Pak1 are presented by the bar graphs. (C) Representative fluorescent images show triple staining of ANP in HFCMs receiving PE ± FTY-720, or combination with PTX (red staining for ANP, pointed by arrows; green for α-actinin; blue for DAPI to highlight the nuclei, scale bar: 20μm). Quantification of ANP-expressing cells is presented by the bar graph. (D–E) PTX blocks the anti-hypertrophic effect of FTY-720, demonstrating by increased HW/TL ratio and larger cardiomyocyte surface area in comparison to the TAC/FTY-720 heart. (F) M-mode echocardiographic tracings from the mice receiving various treatments. (G–H) By echocardiography left ventricular PW and EF% confirm that PTX blocks the beneficial effect of FTY-720 in antagonizing cardiac hypertrophy. N=6–8 per group, data is presented as mean ± SEM.

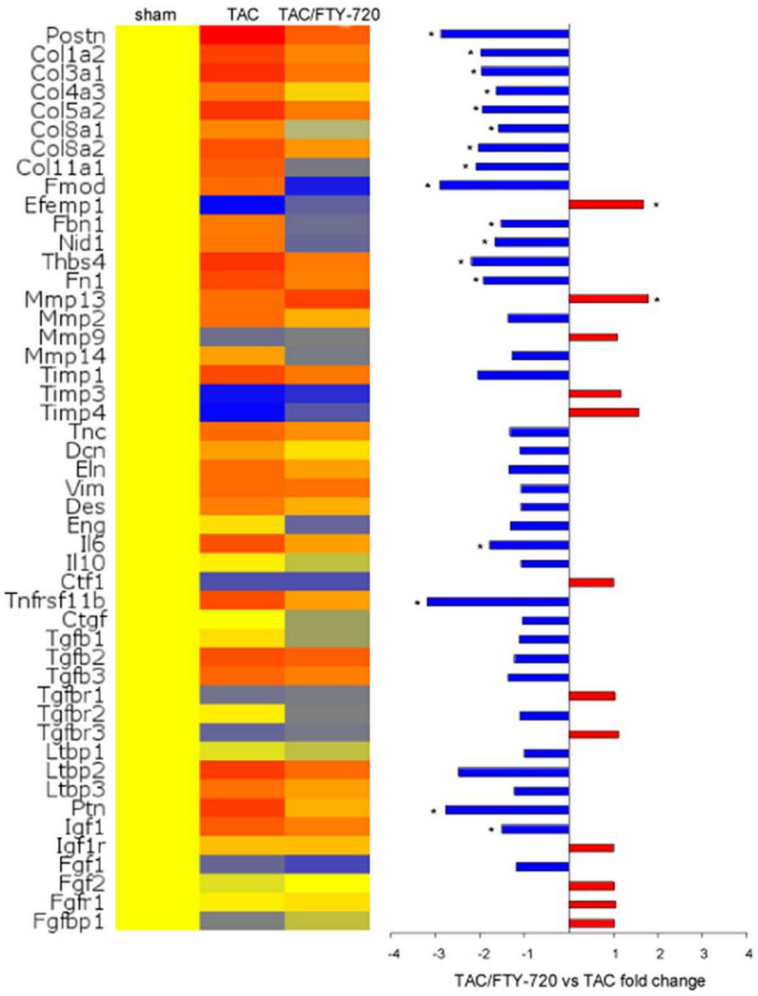

Figure 5.

A diagram shows differentially expressed genes (in a category of ECM organization/ inflammation response) by Affymetrix gene array. Gene expression profiling in sham hearts was normalized to yellow, which is defined as unchanged. Orange and red represent increased expression, and grey and blue represent decreased expression. Fold change of expression is shown for TAC/FTY-720 versus TAC only (n=2 for each group). All genes identified as significantly altered ($P<0.05$) by 1.5 fold or more were marked as *.

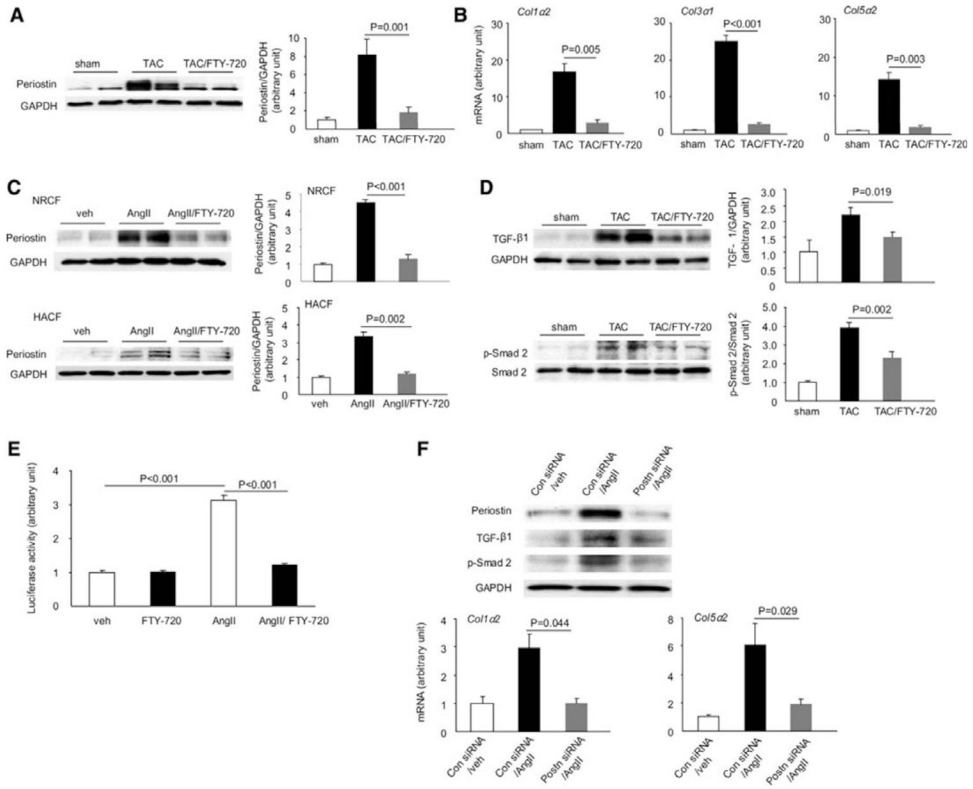

Figure 6.

FTY-720 reduces fibrotic response and improves extracellular compartment milieu. (A) Immunoblotting shows FTY-720 profoundly diminished TAC-induced periostin expression. GAPDH expression is the protein loading control. The ratio of periostin/GAPDH is represented by the bar graph. (B) qPCR analysis validates decreased gene expression of *Col1α2*, *Col3α1* and *Col5α2* in the heart receiving FTY-720 treatment. The data are normalized to the GAPDH content. (C) Immunoblot analyses show that the protein level of periostin in NRCFs and HACFs was increased by Ang II and decreased by additional FTY-720 treatment. The ratios of periostin/GAPDH are represented by the bar graphs. (D) Expression of activated TGF-β1 and Smad 2 phosphorylation were substantial increases upon TAC stress, whilst both decreased in response to FTY-720 treatment. The ratios of TGF-β1/GAPDH and phosphorylated/total Smad 2 are represented by the bar graphs. (E) Increased luciferase activity reflecting TGF-β activation was detected upon Ang II stimulation, whereas FTY-720 caused a reduction in luciferase activity. (F) Protein lysates were prepared for analysis of siRNA-mediated knockdown of periostin (upper panel). Periostin knockdown resulted in blunted TGF-β activation (upper panel) and down-regulated gene expression of *Col1α2* and *Col5α2* (lower panel). N=5–8 per group, data is presented as mean ± SEM.

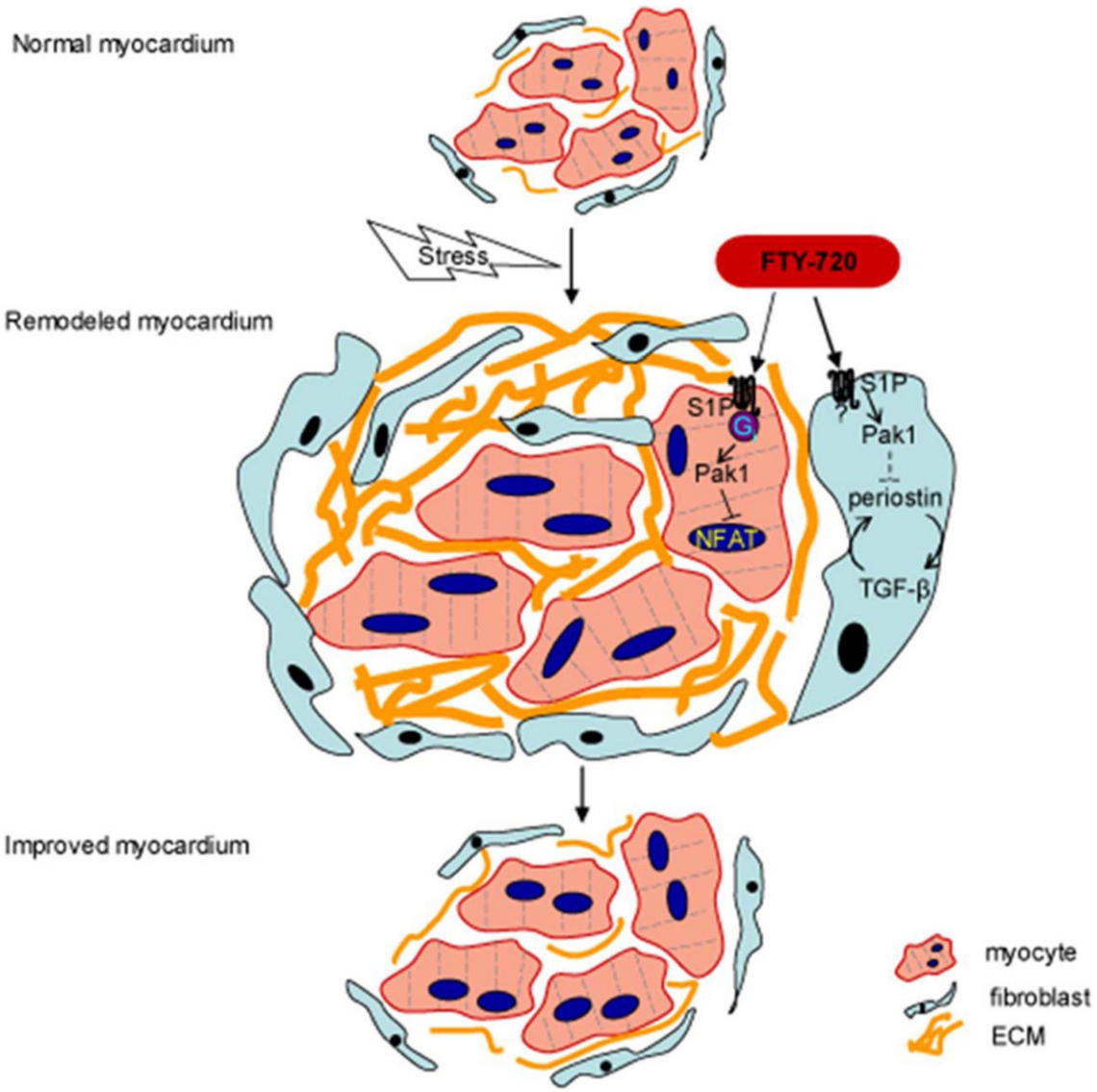

Figure 7.
Proposed model shows the reversal of hypertrophy and fibrosis by FTY-720 through a dual-mechanism system involving negative regulation of NFAT activity in cardiomyocytes and diminution of periostin expression in the ECM.

Table

Hemodynamic analysis demonstrates improved cardiac performance following FTY-720 treatment

	sham/veh	sham/FTY720	TAC/veh	TAC/FTY720	P value, 4 groups	P value, TAC/veh vs sham/veh	P value, TAC/FTY720 vs TAC/veh
HR (/min)	421±15	415±7	446±19	428 ±12	0.254	…	…
BPmax (mmHg)	103.78±5.73	102.65±7.11	144.76±8.74	123.57±6.52	0.01	0.011	0.104
BPmin (mmHg)	65.02±3.34	60.78±6.35	59.87±3.08	58.06±2.69	0.374	…	…
Ves (μl)	11.02±2.39	9.10±3.36	19.74±1.53	7.80±2.33	0.004	0.014	0.006
Ved (μl)	28.07±2.78	24.32±3.62	35.12±1.34	26.87±1.98	0.011	0.034	0.019
Pes (mmHg)	87.36±6.57	90.56±8.75	113.56±4.05	108.63±2.86	0.005	0.006	0.329
Ped (mmHg)	5.44±0.38	6.12±1.01	11.68±1.29	8.05±0.68	<0.001	0.002	0.016
SV (μl)	17.05±1.12	15.22±1.93	15.38±2.06	19.08±1.62	0.046	0.495	0.205
Ea (mmHg/μl)	5.20±0.52	6.12±0.31	8.64±0.82	6.01±0.41	0.016	0.024	0.035
EF (%)	62.94±6.83	67.51±9.22	40.63±4.77	74.26±6.82	0.005	0.045	0.012
CO (ml/min)	7.23±0.68	6.29±0.75	6.15±1.14	7.84±0.59	0.322	…	…
dP/dt max (mmHg/sec)	7946±481	8293±902	5959±635	5495±420	0.007	0.039	0.541
Normalized Ees (mmHg/μl/g)	27.09±2.32	25.55±1.79	35.78±1.94	55.23±4.55	0.002	0.017	0.007
MSW (mmHg)	70.50±4.59	78.61±7.78	138.73±8.97	134.74±17.39	<0.001	<0.001	0.878
dP/dt min (mmHg/sec)	−6494±356	−6059±581	−5506±270	−5312±315	0.003	0.045	0.085
Tau (ms)	6.61±0.48	6.86±0.50	8.33±0.49	9.68±0.72	0.026	0.034	0.229

HR, heart rate; LV mass, left ventricular mass; BP, blood pressure; Ves, end-systolic volume; Pes, end-systolic pressure; Ved, end-diastolic volume; Ped, end-diastolic pressure; SV, stroke volume; Ea, arterial elastance; EF, ejection fraction; CO, cardiac output; dP/dtmax indicates peak rate of pressure rise; Ees, LV end-systolic elastance (stiffness); MSW, preload recruitable stroke work; dP/dtmin, peak rate of pressure decline; Tau, time constant of relaxation normalized to heart rate. N=8–12 per group, data is presented as mean ± SEM.