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Pak1 as a Novel Therapeutic Target for Anti-Hypertrophic Treatment in the Heart

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Abstract

Background—Stress-induced hypertrophic remodeling is a critical pathogenetic process leading to heart failure. While many signal transduction cascades are demonstrated as important regulators to facilitate the induction of cardiac hypertrophy, the signaling pathways for suppressing hypertrophic remodeling remain largely unexplored. In this study, we identified p21-activated kinase 1 (Pak1) as a novel signaling regulator which antagonizes cardiac hypertrophy.

Methods and Results—Hypertrophic stress applied to primary neonatal rat cardiomyocytes (NRCMs), or murine hearts caused the activation of Pak1. Analysis of NRCMs expressing constitutively active Pak1 or in which Pak1 was silenced disclosed that Pak1 played an anti-hypertrophic role. To investigate the *in vivo* role of Pak1 in the heart, we generated mice with a cardiomyocyte-specific deletion of Pak1 ($Pak1^{cko}$). When subject to 2 weeks of pressure overload, $Pak1^{cko}$ mice compared to controls, developed greater cardiac hypertrophy with attendant blunting of JNK activation, and these knockout mice underwent the transition into heart failure when prolonged stress was applied. In addition, chronic angiotensin II infusion also caused increased cardiac hypertrophy in $Pak1^{cko}$ mice. Moreover, we discovered that the Pak1 activator FTY720, a sphingosine-like analogue, was able to prevent pressure overload-induced hypertrophy in wild-type mice, without compromising their cardiac functions. Meanwhile FTY720 failed to exert such

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an effect on Pak1^{cko} mice, suggesting that the anti-hypertrophic effect of FTY720 likely acts through Pak1 activation.

Conclusions—These results, for the first time, establish Pak1 as a novel anti-hypertrophic regulator and suggest that it may be a potential therapeutic target for the treatment of cardiac hypertrophy and heart failure.

Keywords

Cardiac hypertrophy; heart failure; signal transduction; stress

Introduction

Cardiac hypertrophy is a critical pathogenetic process leading to heart failure, with an incidence and prevalence that is rapidly increasing worldwide. The life time risk of heart failure is one in five amongst both men and women. Cardiac hypertrophy is characterized as proliferation-independent cardiomyocyte growth, which bears some similarity to tumour growth. To date, many oncogenes have been demonstrated to positively regulate cardiac hypertrophy. For example, aberrant activation of Ras (small guanine nucleotide-binding protein) is a step in the development of many types of cancers [1]. Cardiac overexpression of constitutively active Ras manifested ventricular hypertrophy[2]. This evidence indicates that the signaling programs regulating cell proliferation may be closely related to the programs that control growth of postmitotic adult cardiomyocytes.

One of these signaling programs, which may be critical in both aberrant growth in cancer and in cardiac hypertrophy, is a cascade involving p21-activated kinase 1 (Pak1), for which there is provocative evidence for a role in tumour formation[3, 4], yet the role of Pak1 in cardiac hypertrophy signaling remains largely unexplored. The Pak family is a group of evolutionarily conserved serine/threonine protein kinases consisting of six isoforms, subdivided into two groups. Pak1 belongs to the group I subfamily, and was first discovered as a major binding partner for small GTPases Rac1 and Cdc42[5]. When cells face inciting stimuli, Cdc42/Rac1 becomes activated via exchange of GDP for GTP[6], and activated Cdc42/Rac1 binds to Pak1, which in turn induces the activation of Pak1[7]. At the last count, approximately 40 proteins in various cell types have been identified as downstream effectors of Pak1 reflecting the range of its biological activities, including regulation of cell proliferation, cell survival and cell motility[8]. Pak1 is abundant in the heart. Important findings by us and others lay the groundwork for suggesting the significant physiological roles of this kinase in the heart[9-12]. In the present study, we aimed to examine our hypothesis that Pak1 may play an important role in cardiac hypertrophy, and in the transition to heart failure, and to investigate whether Pak1 is a potential therapeutic target for anti-hypertrophic treatment.

Using both primary cardiomyocytes and Pak1^{cko} mice, we discovered that Pak1 acts as a novel signaling hub relaying anti-hypertrophic and survival signals from small GTPases to the JNK cascade in the heart. Furthermore, we observed that application of FTY720, a sphingosine-like synthetic analogue with an ability to activate Pak1[13, 14], prevented the development of cardiac hypertrophy in load stressed wild-type mice, and its anti-hypertrophic effect was likely due to its function to activate Pak1. Overall, these data demonstrate for the first time that Pak1 activation exerts a beneficial effect by resisting stress-induced hypertrophic remodeling, and Pak1 may thus be a potential therapeutic target for anti-hypertrophic treatment.

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Methods

More detailed methods are available in the online Data Supplement.

Adenoviral Infection of NRCMs

Adenovirus-mediated gene transfection of NRCMs was performed by methods described previously[15]. Adenovirus expressing shPak1 (CTGTTCTGGATGTGTTGGAAT) was generated using the BLOCK-iT adenoviral RNAi expression system (Invitrogen). Adenoviruses expressing caPak1 (constitutively active Pak1), or NFAT-luciferase reporter gene (Ad-NFAT-Luc) were described elsewhere[10, 16]. Ad-caMKK7 (constitutively active MKK7) was a kind gift from Dr. Hiroki Aoki (Kurume University, Japan). After infection, NRCMs were subjected to immunocytochemistry, quantitative RT-PCR, immunoblot analyses and luciferase reporter assay to investigate the role of Pak1 in cardiac hypertrophy.

Generation of $Pak1^{f/f}$ and $Pak1^{cko}$ Mice

Pak1 genomic DNA was used for constructing a gene targeting vector with two LoxP sites flanking exon 3 of *pak1.* 129Sv-derived R1 embryonic stem (ES) cell transfection, selection, screening and blastocyst injection were performed as described previously[17]. Germline transmitting chimeras were generated and mated with C57BL/6 females to produce heterozygous Pak1 flox mice, which were back-crossed into a C57BL/6 background for 5 generations to obtain homozygous Pak1 flox ($Pak1^{f/f}$) mice. To generate $Pak1^{cko}$ mice, $Pak1^{f/f}$ mice were mated with mice expressing Cre under α myosin heavy chain (αMHC) promoter (αMHC-Cre)[18]. Quantitative RT-PCR, immunoblotting and immunostaining were used to verify Pak1 deletion. All mice used in this study were maintained in a pathogen-free facility at the University of Manchester. The animal studies were performed in accordance with the UK Home Office and institutional guidelines.

Hypertrophy Models and FTY720 Administration

Cardiac hypertrophy was induced by administration of angiotensin II (Ang II, Sigma-Aldrich) at 1μg/g/day for 14 days via osmotic mini-pumps (Alzet) implanted subcutaneously in 8-10 week old male $Pak1^{cko}$ mice and their littermates ($Pak1^{f/f}$ mice), or by transverse aortic constriction (TAC) as previously described[15, 16, 19]. For FTY720 (2-amino-2-[2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride) administration, on the second day after the operation, TAC- or sham-operated wild-type mice or $Pak1^{cko}$ mice (C57BL/6 background, 8-10 week old male) were randomized into different groups for intra-peritoneal injection of FTY720 (10μg/g/day, Cayman Chemical) or vehicle (saline) for 5 days. FTY720-LD_{50} [50% lethal dose] is 300 μg/g. 7 days after the operation (5 days post FTY720 injection), hearts were taken from different experimental groups and the hypertrophic responses were analyzed by histology, quantitative RT-PCR, echocardiography and hemodynamic analysis.

Data Analysis

Two-way ANOVA followed by Bonferonni corrected post-hoc t-test was used for comparisons among multiple groups. Comparisons between two groups were performed using Student's *t*-test. P-values <0.05 are considered statistically significant. Data are expressed as mean ± SEM. Where sample sizes were < 5, tests were also conducted using ranked data. In all cases statistical conclusion were the same. For simplicity we present only the parametric results.

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Results

Pak1 Regulates the JNK Pathway and Antagonizes NFAT-Mediated Hypertrophy in NRCMs

In order to investigate the biological role of Pak1 in cardiac hypertrophy, we first determined activation of endogenous Pak1 by various hypertrophic agonists. Stimulation of NRCMs for 30 min with angiotensin II (Ang II, 10 μM), phenylephrine (PE, 30 μM), or isoproterenol (ISO, 10 μM) each significantly increased Pak1 phosphorylation of Thr-423 in the T-loop of Pak1, which is indicative of Pak1 activation (Figure 1A). Pak1 phosphorylation was also appreciably increased in ventricular tissues of wild type mice subjected to transverse aortic constriction (TAC) for 2 weeks (Figure 1A). We next assessed whether Pak1 exerts a pro-hypertrophic or an anti-hypertrophic effect in response to hypertrophic stimuli in NRCMs. NRCMs were infected with the Ad-caPak1 (constitutively active Pak1) or control adenovirus Ad-GFP prior to 48 h PE treatment. Unexpectedly, Ad-caPak1 abrogated the pro-hypertrophic effect of PE, showing a significantly smaller cell surface area concomitant with a nearly 2-fold down-regulation of *ANP* mRNA expression (Figure 1B-C). Furthermore, we examined whether activated Pak1 affects NFAT transcriptional activity, which plays a central role in regulating cardiac hypertrophy. In line with results reported above, adenoviral infection of the NFAT-luciferase reporter (Ad-NFAT-Luc) in control NRCMs (infected with Ad-LacZ) led to enhanced NFAT reporter activity following PE stimulation. However, infection of Ad-caPak1 did not lead to any increase in NFAT activity despite PE stimulation (Figure 1D). To corroborate these data, we adopted a gene knockdown system in NRCMs, where Pak1 expression was deleted by 85% following infection with Ad-shPak1; expression of Pak2 and Pak3 (close Pak family isoforms) remained unchanged (Figure 2A). Compared to NRCMs infected with scrambled shRNA (Ad-shC2), PE induced significantly greater increases in cell size and in *ANP* mRNA level in NRCMs infected with Ad-shPak1 (Figure 2B-C).

To investigate the potential mechanism whereby Pak1 deficiency promoted hypertrophy, we screened a range of hypertrophic regulators. Our data demonstrate a prominent defect in JNK phosphorylation in shPak1-infected NRCMs after PE stimulation (Figure 2D). Furthermore, MKK4 and MKK7 (upstream activators of JNK) were found not to respond to PE stimulation in the absence of Pak1 (Figure 2D). However, phosphorylation levels of MEKK1, p38, ERK1/2 and PKB were similar in the two groups following PE treatment (Figure 2D).

Finally, NFAT transcriptional activity was examined when Pak1 was knocked down. We discovered that PE stimulation of shPak1-infected NRCMs resulted in enhanced NFAT activity. However, this increase in NFAT activity was mitigated by infection with constitutively active MKK7 (Ad-caMKK7), indicating that loss of Pak1 induces greater cardiomyocyte hypertrophy by promoting increased NFAT activity, which is likely to occur via the JNK pathway (Figure 2E).

Generation and Characterization of Cardiomyocyte-Specific Pak1 Knockout Mice

Prompted by our results showing that Pak1 might be a critical signaling nexus limiting hypertrophy, we moved on to studies addressing our hypothesis in the intact heart. To precisely ascertain the *in vivo* role of Pak1 in the heart, we generated cardiomyocyte-specific Pak1 deletion mice (Pak1^{cko}). Mice with a germ-line modification in the *pak1* gene with two LoxP elements flanking exon 3 (Pak1$^{f/f}$) were generated (Supplement Figure I A-B). Pak1$^{f/f}$ mice were healthy and fertile, indicating that the presence of two LoxP sites did not affect Pak1 function *in vivo*.

To establish Pak1^{cko} mice, Pak1$^{f/f}$ mice were bred with αMHC-Cre mice. Pak1^{cko} mice developed to term, and were viable and fertile in adulthood. PCR amplification of genomic

DNA prepared from cardiomyocytes, brain, liver and skeletal muscle of 8-week old $Pak1^{f/f}$ and $Pak1^{cko}$ mice confirmed the specific recombination of the *pak1* gene in cardiomyocytes (Supplement Figure II A). The deletion of *pak1* gene product in cardiomyocytes was verified at mRNA and protein levels (Supplement Figure II B-D). Notably, loss of Pak1 in cardiomyocytes did not induce any compensatory changes in the protein levels of its activators, Cdc42 and Rac1, as well as its close family members Pak2 and Pak3, and potential effectors, such as ERK1/2, JNK and p38 (Supplement Figure II D-E).

Disruption of Pak1 in Cardiomyocytes Exacerbates Pressure Overload-Induced Hypertrophy

Following on, we determined whether Pak1 is involved in regulating cardiac hypertrophy. Pressure overload by TAC was applied to 8-week old $Pak1^{f/f}$ and $Pak1^{cko}$ mice. Following 2 weeks of TAC, $Pak1^{f/f}$ mice developed a moderate 19% increase in heart weight/tibia length (HW/TL) ratio, whereas $Pak1^{cko}$ mice showed a 53% increase in HW/TL ratio (Figure 3A). Consistent with this result there was a significant increase in cross-sectional area of $Pak1^{cko}$-TAC cardiomyocytes (338.7±2.74 μm^2), compared to 242.43±4.54 μm^2 of $Pak1^{f/f}$-TAC cardiomyocytes (Figure 3B).

Sirius Red staining to determine collagen deposition (Figure 3C) showed more interstitial fibrosis in $Pak1^{cko}$-TAC myocardium (6.1% fibrotic area compared to 1.6% in the controls). Loss of Pak1 also induced cardiomyocyte apoptosis indicated by a 5-fold increase in the number of TUNEL-positive nuclei in $Pak1^{cko}$-TAC myocardium compared to $Pak1^{f/f}$ hearts (Figure 3D). Reactivation of the fetal gene program was measured by quantitative RT-PCR; expression of *ANP*, brain natriuretic peptide (*BNP*) and β myosin heavy polypeptide (*Myh7*) mRNA was significantly elevated in the hypertrophied $Pak1^{cko}$ myocardium (Figure 3E). Regulator of calcineurin 1 variant 4 (RCAN1.4) is a target gene of NFAT transcription factors. Increased *RCAN1.4* mRNA expression was detected in TAC stressed $Pak1^{cko}$ hearts, indicating enhanced NFAT signaling in the knockout mice (Figure 3E). Moreover, as illustrated in Figure 3E, mRNA levels of procollagen type I, α2 (*Col1α2*) and procollagen type III, α1 (*Col3α1*) were markedly up-regulated in the $Pak1^{cko}$ myocardium.

Although greater hypertrophy and salient remodeling occurred in the $Pak1^{cko}$-TAC mice, their contractile performance remained normal, as indicated by similar fractional shortening (FS %) between the two groups after TAC (Supplement Table I). Thus we conclude that ablation of Pak1 in cardiomyocytes promotes hypertrophic remodeling in response to TAC stress.

Prolonged Load Stress Sensitizes $Pak1^{cko}$ Mice to Heart Failure

To further determine whether loss of Pak1 in cardiomyocytes predisposes mice to heart failure, we extended the TAC stress imposed on $Pak1^{f/f}$ and $Pak1^{cko}$ mice to 5 weeks. Indeed, $Pak1^{cko}$ mice showed characteristics of heart failure after TAC. Lung weight to tibia length (LW/TL) ratio was substantially higher in $Pak1^{cko}$-TAC mice, indicating pulmonary edema due to contractile insufficiency (Figure 4A). A significant reduction in FS (19.89±1.8%) in the knockouts confirmed heart failure, whereas $Pak1^{f/f}$ mice exhibited preserved contractility (Figure 4B). The increases in HW/TL ratio (83%) and in myocyte cross-sectional area (419.83±2.0 μm^2) became even more prominent in $Pak1^{cko}$ mice after prolonged TAC stress (Figure 4C-D). In addition, increased collagen deposition (9.1%) was scattered over the working myocardium of $Pak1^{cko}$-TAC mice (Figure 4E). These results demonstrated that mice were more vulnerable to longer pressure overload stress, and more readily made the transition into heart failure when Pak1 was absent.

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Enhanced Hypertrophic Remodeling is Induced in Pak1^{cko} Mice Responding to Ang II Infusion

To determine the general significance of our findings, we investigated whether Pak1 resists hypertrophy induced by neuroendocrine stimuli. When subjected to 2-week infusion of Ang II (1μg/g/day), Pak1^{cko} mice demonstrated significantly increased hypertrophy, as reflected by a 35% enhancement in HW/TL and enlarged cardiomyocytes (292.61±3.51μm^2 versus 190.69±2.96μm^2 of Pak1$^{f/f}$ myocytes) (Supplement Figure III A-B). Ventricular fibrosis was more visible in the knockouts (Supplement Figure III C). We measured ROS production by DHE staining; there were no significant differences detected between the two genotypes (Supplement Figure III D). Also, cardiac function in Pak1^{cko} mice was comparable to the control group (Supplement Figure III E). Together, these results illustrate that Pak1 antagonizes cardiac hypertrophy not only by mechanical stress-induced membrane receptor activation, but also by neuroendocrine agonist stimulation.

The JNK Cascade acts Downstream of Pak1 in Cardiac Hypertrophic Remodeling

To obtain *in vivo* evidence of the regulatory mechanism whereby Pak1 modulates hypertrophic responses, we surveyed downstream candidates. Consistent with data that we obtained from NRCMs, TAC treatment did not induce the activation of MKK4/MKK7-JNK pathway in the Pak1^{cko} myocardium, whereas activation of p38, ERK1/2 and PKB, as well as PP2A activity (phosphorylation of Y307) remained the same between the two groups (Figure 5A).

We also examined apoptotic molecules that might be responsible for the higher rate of cardiomyocyte death in the knockout hearts. Interestingly, we found augmented protein levels of p53, Bax and Bad in the Pak1^{cko}-TAC myocardium. However, there were no significant differences observed in either the expression of Bim and Bcl-2, or phosphorylation of Bad at Ser 112 (Figure 5B), which is a known site for Pak1-mediated phosphorylation[20]. Thus, these data demonstrate that the MKK4/MKK7-JNK pathway acts downstream of Pak1 in protecting the heart from hypertrophic stress.

FTY720 Induces Pak1 Activation and Prevents Cardiac Hypertrophy

Led by the results above, we tested whether Pak1 is a potential therapeutic target for anti-hypertrophic treatment. Firstly, we demonstrated FTY720 was able to induce Pak1 phosphorylation in NRCMs and in wild-type mouse myocardium (Figure 6A). Then, we discovered that treatment of NRCMs with FTY720 (200nM, 48h) significantly reduced PE-induced hypertrophic responses, indicated by a significantly smaller cell surface area together with markedly decreased ANP expression (Figure 6B). Interestingly, Pak1-knockdown NRCMs treated with or without FTY720 showed no significant differences in PE-induced increases in cell surface area and ANP expression (Figure 6B), suggesting FTY720 likely functions via Pak1 activation to block hypertrophic responses. Noteworthy, using trypan blue staining to check cell viability, we found that FTY720 at a dose of 200nM was sufficient to restrain hypertrophic responses, but did not exhibit a toxic effect on NRCMs (Figure 6C), indicating FTY720 may be a suitable agent for anti-hypertrophic therapy *in vivo*.

To test this hypothesis, we applied FTY720 (10μg/g/day of body weight) to wild-type mice for 5 days commencing on the second day after TAC or sham operation. Treatment with vehicle (saline) was given to the control groups following the same protocol. Notably, after 5 days of treatment with FTY720, TAC-mice showed comparable HW/TL ratio (6.01±0.22 mg/mm) and cardiomyocyte cross-sectional areas (196.73±3.06 μm^2) to the FTY720-treated sham-mice (HW/TL: 5.61±0.14 mg/mm; cross-sectional areas: 192.63±3.65 μm^2) or vehicle-treated sham-mice (HW/TL: 5.6±0.11 mg/mm; cross-sectional areas: 193.75±2.35

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μm^2) (Figure 7A-B). Accordingly, echocardiography also demonstrated cardiac structure and function of the FTY720-treated TAC-mice were similar to the sham groups (Figure 7C-D). In contrast to the FTY720-treated TAC-mice, the TAC-mice treated with vehicle developed hypertrophy (Figure 7A-D). Consistent with *in vitro* data, FTY720 used in this protocol did not exhibit cardiac toxicity in the mice, as FS, dP/dt_{max} (contractile response) and dP/dt_{min} (lusitropic response) in the FTY720 treated groups remained normal, compared to the vehicle treated groups (Figure 7 E-F).

Next, we determined whether the anti-hypertrophic effect of FTY720 was due to Pak1 activation, therefore, the same FTY720 treatment protocol was applied to Pak1^{cko} mice subjected to either TAC or sham operation. Interestingly, despite FTY720 treatment, TAC was still able to induce hypertrophy in the hearts of Pak1^{cko} mice (HW/TL: 7.92±0.22 mg/mm [FTY720] versus 8.06±0.2 mg/mm [vehicle]; cross-sectional areas: 310.76±3.02 μm^2 [FTY720] versus 313.8±1.72 μm^2 [vehicle]) (Figure 8A-B). Echocardiography and hemodynamic analysis demonstrated comparable cardiac structure and function between the FTY720 treated TAC-mice and the sham groups (Figure 8C-F).

Together, these data suggest that the activation of Pak1 by FTY720 is able to prevent the development of cardiac hypertrophy.

Discussion

With the use of cultured rat cardiomyocytes and Pak1^{cko} mice, we have identified a novel cardioprotective role of Pak1 in attenuating cardiac hypertrophy and halting the transition to heart failure. The major findings of this study are: 1) Pak1 is activated by both mechanical stress and neuroendocrine agonists in the heart; 2) Pak1 is an indispensable upstream activator for the JNK pathway in response to hypertrophic challenges; 3) Pak1 plays a critical role in antagonising cardiac hypertrophy, since hearts of Pak1^{cko} mice are vulnerable to cardiac hypertrophy and readily progress to failure with application of sustained pressure overload; 4) the activation of Pak1 by FTY720 is able to prevent the development of cardiac hypertrophy, suggesting Pak1 may be a potentially important therapeutic target for anti-hypertrophic treatment.

Pak1 is an Anti-Hypertrophic Regulator in the Heart

This study, for the first time, demonstrates a differing role for Pak1 in cardiomyocyte growth. During the past decade, growing evidence has suggested that Pak1 activation is frequently associated with cell proliferation, survival of cancer cells and increased invasiveness. In fact, more than half of human breast cancers exhibit hyperactivation and/or overexpression of Pak1[21]. In cancers, Pak1 activation is inextricably linked with aberrant Ras/Raf/ERK signaling[8]. In the heart, mature cardiomyocytes are terminally differentiated; growth signals do not lead to proliferation but rather to hypertrophy, which explains why many oncogenes display pro-hypertrophic effects in the adult heart[22, 23]. Thus, we predicted that Pak1 may exert a similar function to that of the Ras pathway in facilitating cardiac hypertrophy. However, to our surprise, our studies revealed the anti-hypertrophic property of Pak1 in the heart.

The initiation of cardiac hypertrophy involves neuroendocrine factors, such as angiotensin II, endothelin-1 and phenylephrine, all of which act on G-protein-coupled receptors (GPCRs), in turn; these receptors stimulate heterotrimeric G-proteins such as $G_{q/11}$, $G_{12/13}$, and G_i for signal transduction[24-26]. However, small GTPases do not bind with GPCRs, but become activated through exchange of GDP for GTP. The dynamic GTP-binding and GDP-hydrolysis cycle of small GTPases is tightly regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), which are downstream effectors of

heterotrimeric G-proteins[6, 27]. Pressure overload is a potent hypertrophic inducer in the heart. It not only induces the release of neuroendocrine factors to stimulate GPCRs, but also activates stretch sensors or receptor tyrosine kinases for the development of cardiac hypertrophy[28]. When the heart is exposed to various hypertrophic stimuli, pro-hypertrophic and anti-hypertrophic signaling pathways are concurrently activated to regulate the hypertrophic process. We discovered that Pak1 was activated by a number of neuroendocrine factors, and by pressure overload to antagonize cardiac hypertrophy.

The Cdc42-Pak1-JNK Axis is a Critical Pathway Relaying Anti-Hypertrophic Signals

Results of studies investigating the role of Cdc42, a key regulator of Pak1 activation, in hypertrophic signaling are consistent with our findings. Maillet et al. reported that diverse hypertrophic stimuli increased the activated GTP-bound form of Cdc42. Loss of Cdc42 in cardiomyocytes rendered mice more capable of cardiac hypertrophic growth, and Cdc42 is required for JNK activation in response to hypertrophic stress[29]. Their data agree with previous investigations in mammalian cells showing that Cdc42 induces JNK activation[30-32]. Interestingly, Maillet et al. also showed that Cdc42 deficiency in NRCMs led to blunted phosphorylation of MEKK1. MEKK1 is a MAP3K; which preferentially activates the JNK pathway through MKK4 and MKK7, and also regulates the activity of the ERK cascade[33, 34]. However, we did not observe altered MEKK1 phosphorylation due to Pak1 deficiency; whereas blunted activation of the MKK4/MKK7-JNK pathway and increased NFAT activity was detected when Pak1 was scarce. MKK4 and MKK7 are upstream kinases for JNKs, which are implicated in the progression of heart failure[35, 36]. JNKs have been shown to antagonize cardiac hypertrophy through inhibition of NFAT activity[37]. Our recent studies employing mice with cardiomyocyte-specific deletion of MKK4 or MKK7 support this mechanism[16, 19]. Considering the above evidence, we believe that Pak1 acts upstream of the JNK pathway in hypertrophic signaling, and MEKK1 might not be a direct effector downstream of Pak1-mediated anti-hypertrophic signaling.

It is interesting to note that a recent study by Higuchi et al. described a novel property of Pak1; it not only has catalytic function, but can also act as a scaffolding protein for priming Akt activation[38]. Whether Pak1 is able to directly phosphorylate MKK4/MKK7 or aid recruitment of MKK4/MKK7 to specific MAP3Ks in response to hypertrophic stimuli thus remains to be determined.

Our previous study has shown that Pak1 is involved in modulating cardiac contractility through PP2A-mediated dephosphorylation of cardiac troponin I (cTnI)[10]. It was proposed that p38 seemed to be an intermediate for Pak1-mediated PP2A activity[10]. As such, we have examined p38 activation and PP2A phosphorylation of Y307 (indicating the catalytic activity of PP2A), however, no alteration in p38 activation or PP2A activity was observed in our experimental setting due to Pak1 deficiency in cardiomyocytes under TAC stress. These results suggest at least in the model we have employed, that PP2A or p38 is unlikely to be downstream of Pak1 and responsible for the development of cardiac hypertrophy.

Current knowledge of Cdc42 and Rac1, both of which activate Pak1, suggests differing roles for these small G proteins in hypertrophic signaling in the heart. In contrast to the promotion of cardiac hypertrophy by down-regulation of Cdc42[29], down-regulation of Rac1 inhibits the development of cardiac hypertrophy in response to Ang II infusion via decreased activity of NADPH[39]. Subsequent studies by Custodis et al. indicated that Rac1 binding to Rho guanine dissociation inhibitor α may be a mechanism by which Rac1 mediates hypertrophy in a mouse pressure overload model[40]. Rac1 overexpression in myocardium induced hypertrophy in juvenile transgenic mice concurrent with altered intracellular distribution of Pak from the cytosol to cytoskeletal fraction[41]. Yet in this study by Sussman et al. no information was provided as to which isoform of Pak was involved in the translocation[41]. It

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is known that other Pak isoforms, such as Pak2 and Pak3, which share substantial sequence homology with Pak1[42], are also expressed in cardiomyocytes. We have demonstrated that Pak1^{cko} mice exhibited greater hypertrophy with no increase in ROS production after 2 weeks of Ang II infusion, which is in stark contrast to phenotypes reported in Rac1 cardiomyocyte-specific knockouts[39]. Taking this evidence into account it is plausible that Pak1 is a primary effector of Cdc42 rather than Rac1. Pak1 is an indispensible component of the Cdc42-Pak1-JNK axis serving as a critical anti-hypertrophic regulatory pathway.

Pak1 Activation by FTY720 Exerts a Beneficial Effect for Restraining Cardiac Hypertrophy

FTY720 is a sphingosine-like analogue, approved by the FDA for treating relapsing multiple sclerosis. We have previously reported that FTY720 prevents arrhythmias in an *ex vivo* rat heart subjected to ischemia/reperfusion injury[14]. In the ischemia/reperfusion model, Pak1 activation was suggested to be involved in an FTY720-induced protective effect[14]. Our test to determine whether FTY720 activation of Pak1 extended to the induction of cardiac hypertrophy demonstrated that administration of a pharmacological dose of FTY720 (10μg/g/day) was sufficient and effective to limit TAC-induced cardiac hypertrophy in wild-type mice. Meanwhile, the observation that FTY720 failed to block increased cardiac hypertrophy in TAC stressed-Pak1^{cko} mice provides further support that FTY720 induces its anti-hypertrophic effect through the activation of Pak1.

Cardiac hypertrophy is traditionally regarded as an adaptive response to normalize ventricular wall stress. According to Laplace's law, FTY720 treatment might cause deterioration in cardiac function and chamber dilation in TAC stressed mice due to limited cardiac hypertrophy; however, none of these were observed in our study. Similarly, in response to pressure overload, preserved cardiac function with no or little hypertrophy was reported by a number of investigations, including studies in which NFAT signaling was inhibited[43-47]. These findings suggest that hypertrophy may not always be a necessary compensatory response; increased wall stress per se does not cause cardiac dysfunction. Therefore, FTY720 treatment could be of clinical interest given the fact of its ability to prevent hypertrophy, without deteriorating cardiac function. Furthermore, FTY720, which is derived from myriocin[48], a component of the natural product *Isaria sinclairii*, represents a non-toxic sphingosine-like derivative with oral bioavailability that may be useful in treatment and/or prevention of cardiac disorders in high-risk patients.

In conclusion, we have discovered a novel role for Pak1 as a critical signaling hub in cardioprotection which limits excessive hypertrophic remodeling. Pak1 most likely acts downstream of Cdc42 to convey both anti-hypertrophic and survival signals to the JNK pathway in cardiomyocytes. Our demonstration of prevention of cardiac hypertrophy by administration of FTY720 provides convincing evidence for the identification of Pak1 as a potential therapeutic target for anti-hypertrophic treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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CLINICAL PERSPECTIVE

Heart failure (HF) is one of the most devastating diseases. The life time risk of heart failure is one in five amongst both men and women. Despite advances in diagnostic and therapeutic technology during past decades, the survival rate after the onset of HF still remains significantly high. There is general agreement that cardiac hypertrophy is a determinant of the clinical course of HF. Therefore, understanding molecular mechanisms of hypertrophic remodeling is a key step in deciphering the pathogenesis of HF. Cardiac hypertrophy is characterized as proliferation-independent cardiomyocyte growth. The signaling programs regulating cell proliferation may be closely related to the programs that control growth of postmitotic adult cardiomyocytes. Based on this premise, we identified p21-activated kinase 1 (Pak1), which is important in cell proliferation, as a novel regulator antagonising cardiac hypertrophy. We have also elucidated that Pak1 is an indispensible component of the anti-hypertrophic signaling, in which Cdc42 (small GTPases) is its upstream activator, and JNK (MAP kinase) works downstream of Pak1. Most interestingly, we discovered that FTY720 (a sphingosine-like analogue), a FDA approved drug for treating relapsing multiple sclerosis, is able to limit cardiac hypertrophy of murine hearts. This anti-hypertrophic effect of FTY720 is likely to function through the activation of Pak1. Overall, our findings provide the evidence to establish Pak1 as a novel anti-hypertrophic regulator and a potential therapeutic target for the treatment of cardiac hypertrophy and heart failure.

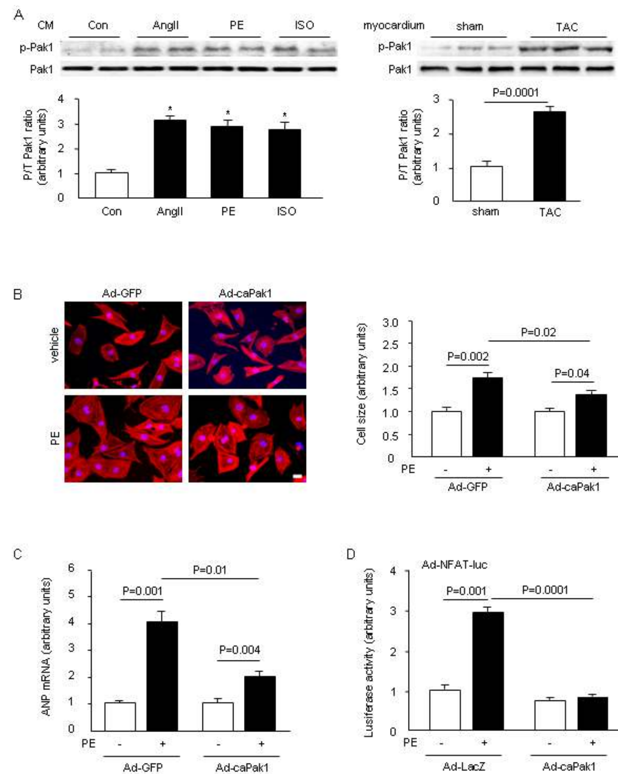

Figure 1.

Activated Pak1 attenuated hypertrophic responses in NRCMs. (A) Immunoblotting analyses of Pak1 phosphorylation at Thr 423 indicate that Pak1 activation was induced by angiotensin II (Ang II), phenylephrine (PE), and isoproterenol (ISO) in NRCMs (n=3), and by TAC stress in myocardium (n=5 mice per group) (Western blot images: upper panel; the ratios of P/T Pak1 are represented by the bar graphs: lower panel, *P=0.001 versus untreated NRCMs). (B) Representative images of α-actinin immunostaining of NRCMs infected with Ad-caPak1 or Ad-GFP (scale bar: 20μm). Quantification of cell surface area is presented in the bar graph (n=3 independent experiments, 50 cells counted per experiment). (C) qPCR analyses show decreased *ANP* mRNA expression in Ad-caPak1-infected NRCMs after PE stimulation. The data are derived from 5 independent experiments performed in triplicate and are normalized to the GAPDH content (n=5). (D) The luciferase reporter assays showed increased NFAT transcriptional activity in control NRCMs (infected with Ad-LacZ) after PE stimulation, whilst activated Pak1 (Ad-caPak1) diminished this increased NFAT transcriptional activity. Data are means ± SEM, n=3 independent experiments.

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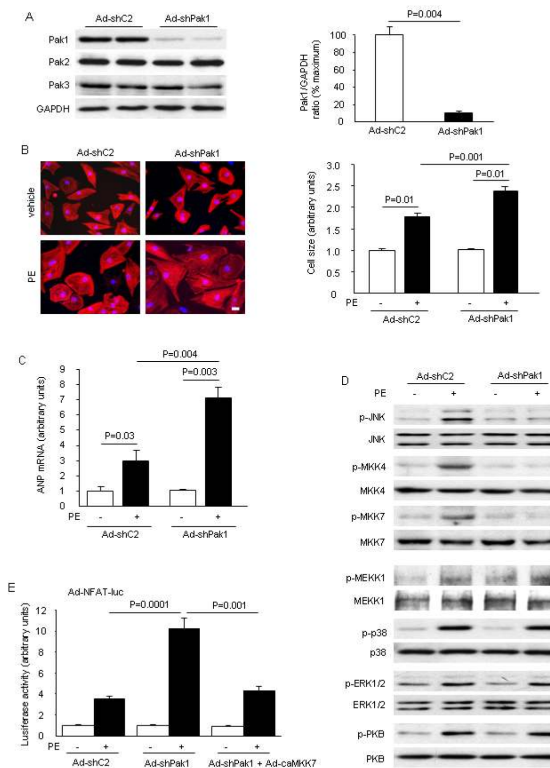

Figure 2.

Knockdown of Pak1 in NRCMs augmented hypertrophic responses. (A) NRCMs were infected with Ad-shC2 or Ad-shPak1 for 72 hours prior to immunoblot analysis of Pak1 protein level. Pak2 and Pak3 protein levels were determined to examine the specificity of Pak1 knockdown. GAPDH expression is the protein loading control. (B) Representative images of α-actinin immunostaining of NRCMs infected with Ad-shC2 or Ad-shPak1 (scale bar: 20μm). Quantification of cell surface area is presented in the bar graph (n=3 independent experiments, 50 cells counted per experiment). (C) qPCR analyses of *ANP* mRNA expression (n=5). (D) Immunoblot analyses of expression and phosphorylation levels of JNK, MKK4, MKK7, MEKK1, p38, ERK1/2 and PKB in response to PE for 30 minutes. (E) The luciferase reporter assays showed substantially increased NFAT transcriptional activity in Ad-shPak1-infected NRCMs after PE stimulation, and activated MKK7 repressed Pak1 deficiency-induced higher NFAT transcriptional activity. Data are means ± SEM, n=4 independent experiments.

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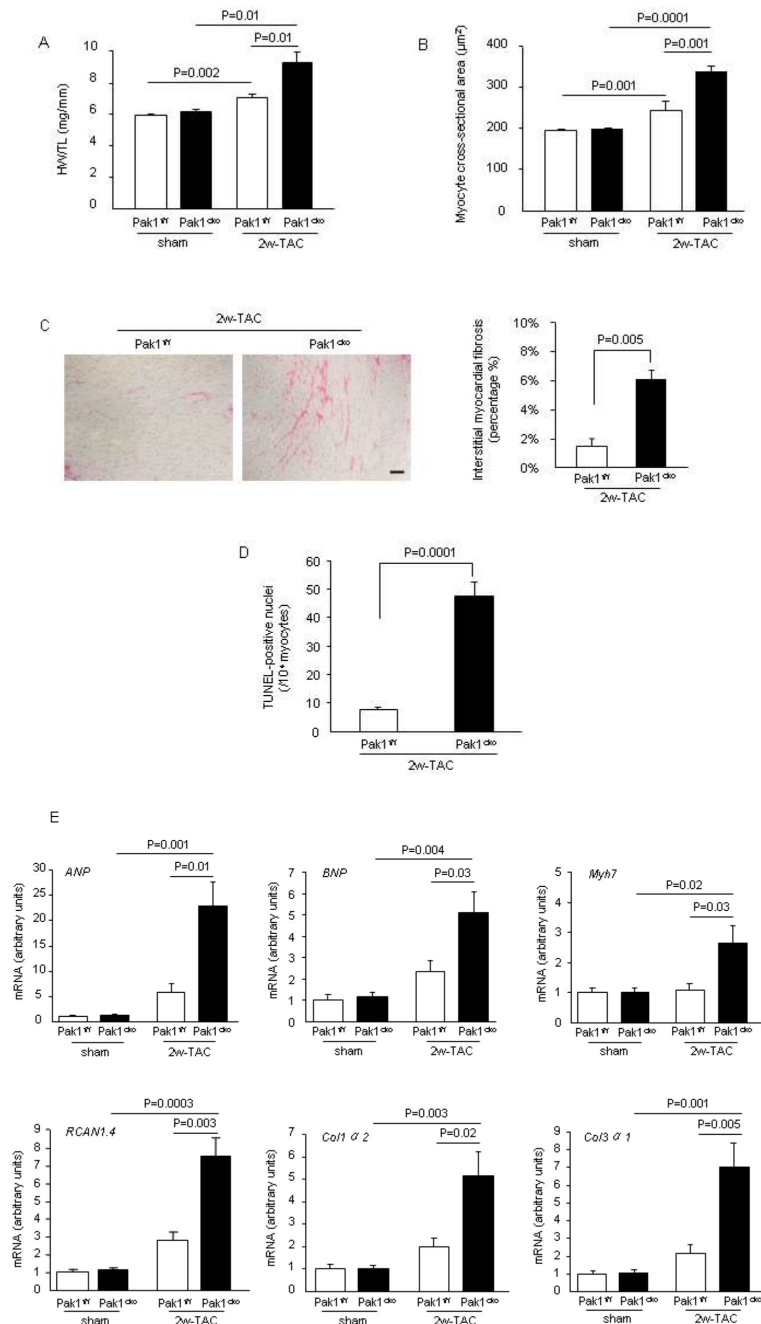

Figure 3.

Exacerbated cardiac hypertrophy in Pak1^{cko} mice after 2 weeks of TAC. (A) HW/TL ratios of Pak1$^{f/f}$ and Pak1^{cko} mice. (B) Measurements of mean cross-sectional area. (C) Histological view of cardiac fibrosis detected by Sirius Red staining (scale bar: 50µm). Quantification of the relative area of fibrosis is expressed as percentage fibrosis. (D) Increased apoptosis in Pak1^{cko} ventricular myocardium was detected by TUNEL assay. TUNEL-positive nuclei are represented in the bar graph (n=5-7 mice per group). (E) qPCR analyses of *ANP, BNP, Myh7, RCAN1.4, Col1α2* and *Col3α1*. The data are normalized to the GAPDH content (n=5-7 mice per group).

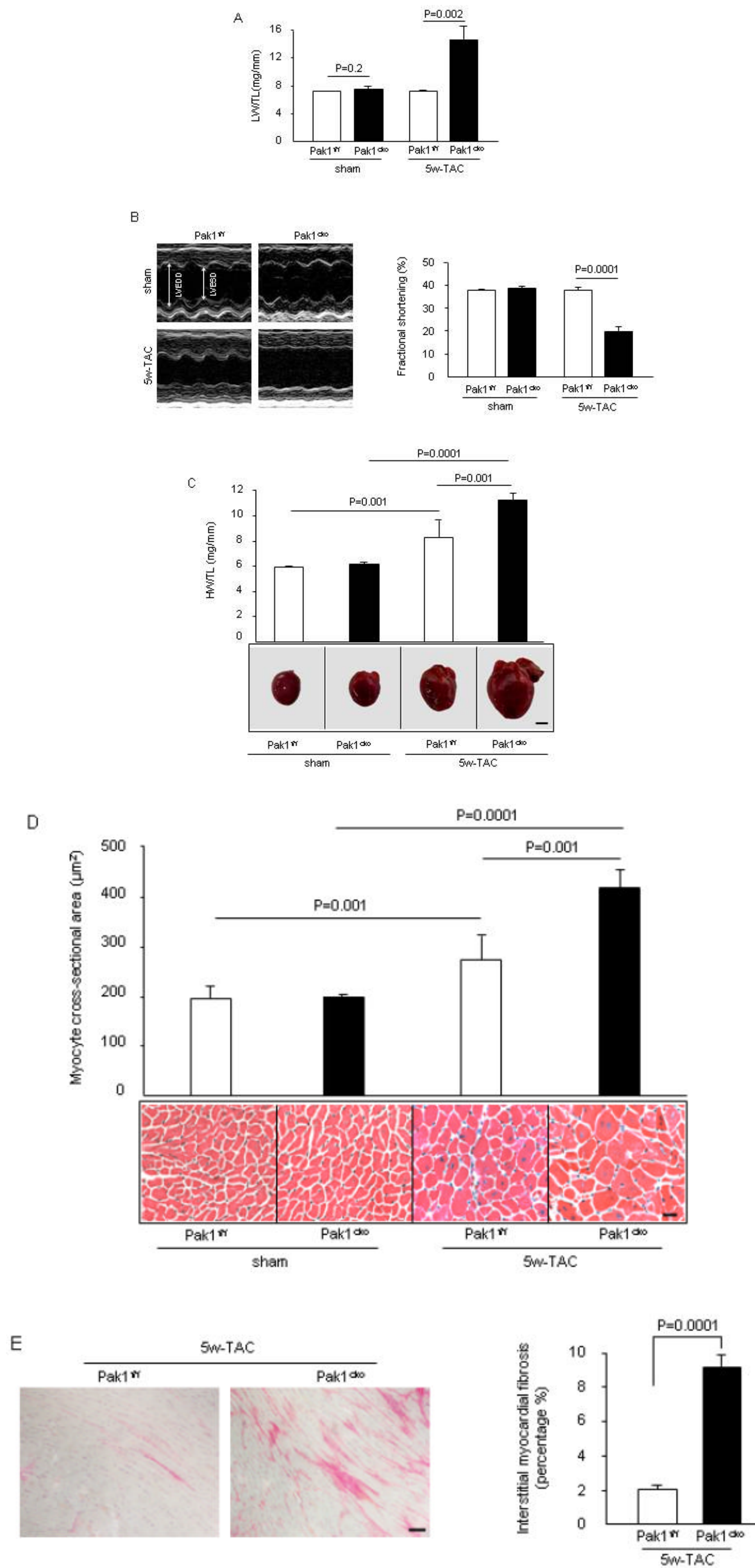

Figure 4.

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Pak1^{cko} mice exhibit signs of heart failure when exposed to prolonged TAC. (A) 5 weeks of TAC caused a significant increase in lung weight relative to tibia length in Pak1^{cko} mice. (B) M-mode echocardiography show dilated ventricular chamber (left panel), and reduced FS% (right panel) in Pak1^{cko}-TAC mice. (C) HW/TL ratios of Pak1$^{f/f}$ and Pak1^{cko} mice (upper panel). Morphometry demonstrates greater hypertrophy in Pak1^{cko}-TAC mice (lower panel, scale bar: 2mm). (D) Measurements of mean cross-sectional areas (upper panel), and hematoxylin & eosin staining of heart cross-sections (lower panel, scale bar: 20μm). (E) Massive interstitial fibrosis in the Pak1^{cko} heart (scale bar: 50μm). Quantification of fibrotic area is presented in the bar graph (n=7 mice per group).

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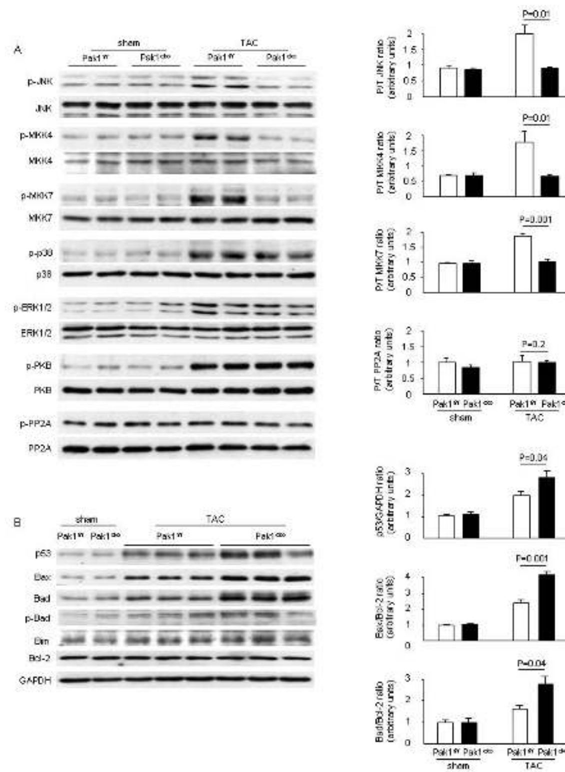

Figure 5.

Pak1 is an upstream activator for the JNK pathway in response to TAC stress. (A) Protein extracts from Pak1$^{f/f}$ and Pak1^{cko} hearts were examined by immunoblotting for total JNK, MKK4, MKK7, p38, ERK1/2, PKB and PP2A expression, as well as their phosphorylation levels. The ratios of P/T JNK, P/T MKK4, P/T MKK7 and P/T PP2A are represented by the bar graphs. (B) Immunoblot analyses of protein levels of p53, Bax, Bad, Bim and Bcl-2, as well as phosphorylated Bad. GAPDH expression is the protein loading control. The ratios of p53/GAPDH, Bax/Bcl-2 and Bad/Bcl-2 are represented by the bar graphs (n=6 mice per group).

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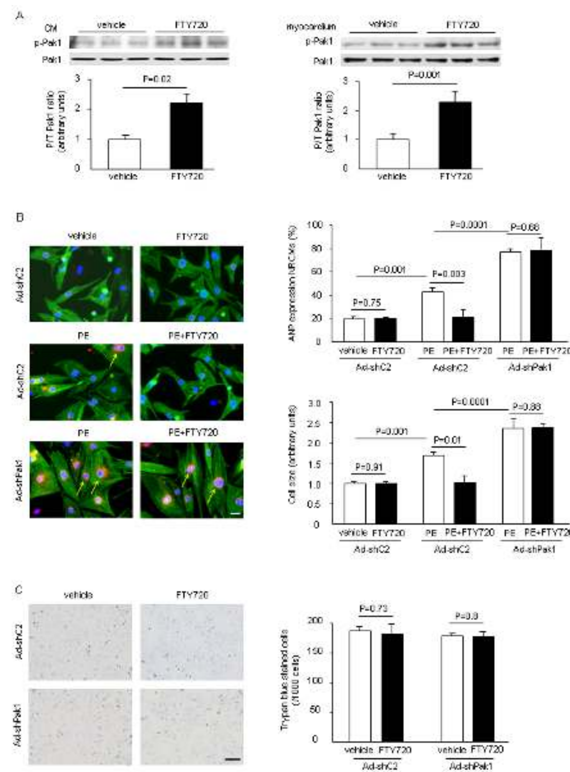

Figure 6.

FTY720 induced Pak1 activation and prevented hypertrophic responses in NRCMs. (A) FTY720-induced Pak1 phosphorylation was examined by immunoblot analyses in NRCMs (100 nM, 1h), and in myocardium (10μg/g, 1h). Western blot images: upper panel; the ratios of P/T Pak1 are represented by the bar graphs: lower panel; n=5. (B) Representative images of triple staining of NRCMs (red staining for ANP, pointed by arrows; green for α-actinin; blue for DAPI, scale bar: 20μm). Quantification of ANP-expressing cells or cell size is presented by the bar graphs (n=5). (C) Trypan blue staining to detect cell viability after FTY720 treatment in NRCMs infected with Ad-shPak1 or Ad-shC2 (scale bar: 100μm, n=4 independent experiments, 250 cells counted per experiment). .

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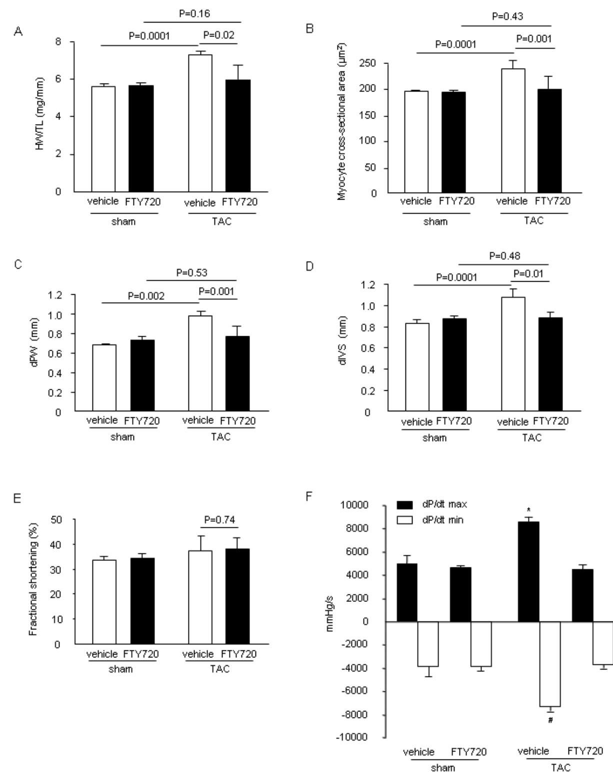

Figure 7.

FTY720 treatment prevented the induction of cardiac hypertrophy in the wild type TAC-hearts, demonstrated by significant decreases in (A) HW/TL ratio, and in (B) mean cross-sectional areas, compared to vehicle-treated TAC-hearts. Echocardiography showed (C) increased ventricular posterior wall thickness (dPW) and (D) interventricular septal wall thickness (dIVS) in vehicle-treated TAC-mice, but not in FTY720-treated TAC-mice. (E) FS % and (F) *In vivo* hemodynamic analysis (dP/dt_{max} - contractile response and dP/dt_{min} - lusitropic response) demonstrated that cardiac functions were preserved in FTY720-treated groups (n=5-8 mice per group). * P=0.02, # P=0.01 versus vehicle-treated sham-mice, respectively.

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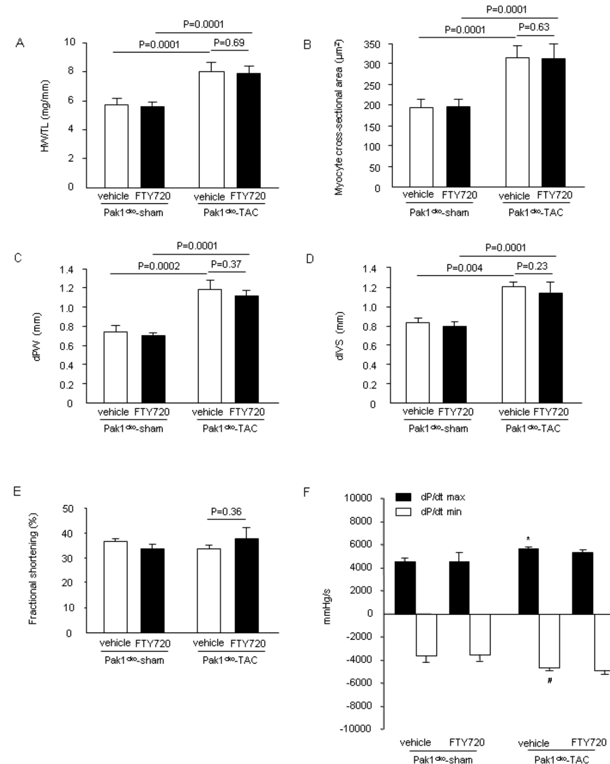

Figure 8.

FTY720 failed to block increased cardiac hypertrophy in Pak1^{cko}-TAC mice. (A) HW/TL ratio, and (B) mean cross-sectional areas, showed no difference between vehicle- and FTY720-treated Pak1^{cko}-TAC mice, although TAC induced increased hypertrophy in both groups, compared to sham-groups. Echocardiographic parameters of (C) dPW and (D) dIVS are comparable between vehicle- and FTY720-treated Pak1^{cko}-TAC mice. (E) FS% and (F) dP/dt_{max} and dP/dt_{min}, show normal cardiac functions in FTY720-treated Pak1^{cko}-TAC mice (n=4-6 mice per group). * P= 0.03, # P=0.04 versus vehicle-treated sham-mice, respectively.