Pak1 as a Novel Therapeutic Target for Antihypertrophic Treatment in the Heart

Wei Liu, MD, PhD; Min Zi, MD; Ronald Naumann, MSc; Susanne Ulm, MSc; Jiawei Jin, PhD; Domenico M. Taglieri, MD; Sukhpal Prehar, MSc; Junhong Gui, MD, PhD; Hoyee Tsui, Bsc; Rui-Ping Xiao, MD, PhD; Ludwig Neyses, MD; R. John Solaro, PhD; Yunbo Ke, PhD; Elizabeth J. Cartwright, PhD; Ming Lei, MD, PhD; Xin Wang, MD, PhD

Background—Stress-induced hypertrophic remodeling is a critical pathogenetic process leading to heart failure. Although 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 that 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 antihypertrophic role. To investigate the in vivo role of Pak1 in the heart, we generated mice with a cardiomyocyte-specific deletion of Pak1 (Pak1cko). When subjected to 2 weeks of pressure overload, Pak1cko mice developed greater cardiac hypertrophy with attendant blunting of JNK activation compared with controls, and these knockout mice underwent the transition into heart failure when prolonged stress was applied. Chronic angiotensin II infusion also caused increased cardiac hypertrophy in Pak1cko mice. Moreover, we discovered that the Pak1 activator FTY720, a sphingosine-like analog, was able to prevent pressure overload-induced hypertrophy in wild-type mice without compromising their cardiac functions. Meanwhile, FTY720 failed to exert such an effect on Pak1cko mice, suggesting that the antihypertrophic effect of FTY720 likely acts through Pak1 activation.

Conclusions—These results, for the first time, establish Pak1 as a novel antihypertrophic regulator and suggest that it may be a potential therapeutic target for the treatment of cardiac hypertrophy and heart failure. (Circulation. 2011;124:2702-2715.)

Key Words: cardiac hypertrophy ■ heart failure ■ signal transduction ■ stress

Cardiac hypertrophy is a critical pathogenetic process leading to heart failure, with an incidence and prevalence that is rapidly increasing worldwide. The lifetime risk of heart failure is 1 in 5 among both men and women. Cardiac hypertrophy is characterized as proliferation-independent cardiomyocyte growth, which bears some similarity to tumor 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.

Clinical Perspective on p 2715

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). There is provocative evidence for a role in of Pak1 tumor formation,3,4 yet its role in cardiac hypertrophy signaling remains largely unexplored. The Pak family is a group of evolutionarily conserved serine/threonine protein kinases consisting of 6 isoforms subdivided into 2 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, 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. 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. 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 antihypertrophic treatment.

Using both primary cardiomyocytes and Pak1cko mice, we discovered that Pak1 acts as a novel signaling hub relaying antihypertrophic and survival signals from small GTPases to the JNK cascade in the heart. Furthermore, we observed that application of FTY720, a sphingosine-like synthetic analog with antihypertrophic 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 antihypertrophic treatment.

Methods

More details are available in the online-only Data Supplement Methods.

Adenoviral Infection of NRCMs

Adenovirus-mediated gene transfection of NRCMs was performed by methods described previously. Adenovirus expressing shPak1 (CTGTCTGGATGTGTTGGAAT) was generated using the BLOCK-iT adenoviral RNAi expression system (In Vitrogen). Adenoviruses expressing caPak1 (constitutively active Pak1) or NFAT-luciferase reporter gene (Ad-NFAT-Luc) are described elsewhere. Ad-caMKK7 (constitutively active MKK7) was a kind gift from Dr. Hiroki Aoki (Kurume University, Japan). After infection, NRCMs were subjected to immunocytochemistry, quantitative reverse transcriptase polymerase chain reaction (RT-PCR), immunoblotting, and immunofluorescence staining to investigate the role of Pak1 in cardiac hypertrophy.

Generation of Pak1FT and Pak1ko Mice

Pak1 genomic DNA was used for constructing a gene-targeting vector with 2 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. 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 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 1 μg·g⁻¹·d⁻¹ for 14 days via osmotic mini-pumps (Alzet) implanted subcutaneously in 8- to 10-week-old male Pak1 disease mice and their littermates (Pak1FT mice), or by transverse aortic constriction (TAC) as previously described. 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 Pak1cko mice (C57BL/6 background, 8- to 10-week-old male) were randomized into different groups for intraperitoneal injection of FTY720 (10 μg·g⁻¹·d⁻¹, Cayman Chemical) or vehicle (saline) for 5 days. FTY720-LD50 (50% lethal dose) is 300 μg/g. Seven 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 Bonferroni corrected post hoc t test was used for comparisons among multiple groups. Comparisons between 2 groups were performed using Student t test. Probability 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 conclusions were the same. For simplicity we present only the parametric results.

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 minutes with angiotensin II (Ang II, 10 μmol/L), phenylephrine (PE, 30 μmol/L), or isoproterenol (ISO, 10 μmol/L) 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 TAC for 2 weeks (Figure 1A). We next assessed whether Pak1 exerts a prohypertrophic or an antihypertrophic effect in response to hypertrophic stimuli in NRCMs. NRCMs were infected with the Ad-caPak1 (constitutively active Pak1) or control adenovirus Ad-GFP 48 hours before PE treatment. Unexpectedly, Ad-caPak1 abrogated the prohypertrophic effect of PE, showing a significantly smaller cell surface area concomitant with a nearly 2-fold downregulation of ANP mRNA expression (Figure 1B and 1C). 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 after 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, in which Pak1 expression was deleted by 85% after infection with Ad-shPak1; expression of Pak2 and Pak3 (close Pak family isoforms) remained unchanged (Figure 2A). Compared with 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 and 2C).

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 (Pak1cko). Mice
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 20 μm). 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.
with a germline modification in the pak1 gene with 2 LoxP elements flanking exon 3 (Pak1<sup>f/f</sup>) were generated (online-only Data Supplement Figure IA and IB). Pak1<sup>f/f</sup> mice were healthy and fertile, indicating that the presence of two LoxP sites did not affect Pak1 function in vivo.

To establish Pak1<sup>cko</sup> mice, Pak1<sup>f/f</sup> mice were bred with αMHC-Cre mice. Pak1<sup>cko</sup> 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<sup>f/f</sup> and Pak1<sup>cko</sup> mice confirmed the specific recombination of the pak1 gene in cardiomyocytes (online-only Data Supplement Figure IIA). The deletion of the pak1 gene product in cardiomyocytes was verified at mRNA and protein levels (online-only Data Supplement Figure IIB through IID). 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 (online-only Data Supplement Figure IID and IIE).

Disruption of Pak1 in Cardiomyocytes Exacerbates Pressure Overload-Induced Hypertrophy

We next determined whether Pak1 is involved in regulating cardiac hypertrophy. Pressure overload by TAC was applied to 8-week-old Pak1<sup>f/f</sup> and Pak1<sup>cko</sup> mice. Following 2 weeks of TAC, Pak1<sup>f/f</sup> mice developed a moderate 19% increase in heart weight/tibia length (HW/TL) ratio, whereas Pak1<sup>cko</sup> mice showed a 53% increase in HW/TL ratio (Figure 3A). Consistent with this result, there was a significant increase in the cross-sectional area of Pak1<sup>cko</sup>-TAC cardiomyocytes (338.7±2.74 μm²) compared with Pak1<sup>f/f</sup>-TAC cardiomyocytes (242.43±4.54 μm²) (Figure 3B).

Sirius Red staining to determine collagen deposition (Figure 3C) showed more interstitial fibrosis in Pak1<sup>cko</sup>-TAC compared with Pak1<sup>f/f</sup>-TAC myocardium (61.1% fibrotic area compared with 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<sup>cko</sup>-TAC myocardium compared with Pak1<sup>f/f</sup> 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<sup>cko</sup> 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<sup>cko</sup> 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 upregulated in the Pak1<sup>cko</sup> myocardium.

Although greater hypertrophy and salient remodeling occurred in the Pak1<sup>cko</sup>-TAC mice, their contractile performance remained normal, as indicated by similar fractional shortening between the 2 groups after TAC (online-only Data 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<sup>cko</sup> 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<sup>f/f</sup> and Pak1<sup>cko</sup> mice to 5 weeks. Indeed, Pak1<sup>cko</sup> mice showed characteristics of heart failure after TAC. Lung weight to tibia length (LW/TL) ratio was substantially higher in Pak1<sup>cko</sup>-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<sup>f/f</sup> mice exhibited preserved contractility (Figure 4B). The increases in HW/TL ratio (83%) and in the myocyte cross-sectional area (419.83±2.0 μm²) became even more prominent in Pak1<sup>cko</sup> mice after prolonged TAC stress (Figure 4C and 4D). In addition, increased collagen deposition (9.1%) was scattered over the working myocardium of Pak1<sup>cko</sup>-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.

Enhanced Hypertrophic Remodeling Is Induced in Pak1<sup>cko</sup> 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 a 2-week infusion of Ang II (1 μg/g/d), Pak1<sup>cko</sup> mice demonstrated significantly increased hypertrophy, as reflected by a 35% enhancement in HW/TL and enlarged cardiomyocytes (292.61±3.51 μm² versus 190.69±2.96 μm² of Pak1<sup>f/f</sup> myocytes) (online-only Data Supplement Figure IIIA and IIIB). Ventricular fibrosis was more visible in the knockouts (online-only Data Supplement Figure IIC). We measured ROS production by DHE staining; there were no significant differences detected between the two genotypes (online-only Data Supplement Figure IID). Also, cardiac function in Pak1<sup>cko</sup> mice was comparable to that in the control group (online-only Data Supplement Figure IIIE). 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<sup>cko</sup> myocardium, whereas activation of p38, ERK1/2, and PKB, as well as PP2A activity (phosphorylation of Y307), remained the same between the 2 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<sup>cko</sup>-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
Figure 3. Exacerbated cardiac hypertrophy in Pak1<sup>cko</sup> mice after 2 weeks of transverse aortic constriction. A, HW/TL ratios of Pak1<sup>f/f</sup> and Pak1<sup>cko</sup> mice. B, Measurements of mean cross-sectional area. C, Histological view of cardiac fibrosis detected by Sirius Red staining (scale bar 50 μm). Quantification of the relative area of fibrosis is expressed as percentage fibrosis. D, Increased apoptosis in Pak1<sup>cko</sup> 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).
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 antihypertrophic treatment. First, we demonstrated that 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 (200 nmol/L, 48 hours) 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. It is noteworthy that, using trypan blue staining to check cell viability, we found that FTY720 at a dose of 200 nmol/L 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 antihypertrophic therapy in vivo.

To test this hypothesis, we applied FTY720 (10 μg · g⁻¹ · d⁻¹ 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 had an HW/TL ratio (6.01 ± 0.22 mg/mm) and cardiomyocyte cross-sectional areas (196.73 ± 3.06 μm²) comparable to the FTY720-treated sham mice (HW/TL 5.61 ± 0.14 mg/mm, cross-sectional areas 192.63 ± 3.65 μm²) or vehicle-treated sham mice (HW/TL 5.6 ± 0.11 mg/mm, cross-sectional areas 193.75 ± 2.35 μm²) (Figure 7A and 7B). Accordingly, echocardiography also demonstrated that cardiac structure and function of the FTY720-treated TAC mice were similar to the sham groups (Figure 7C and 7D). In contrast to the FTY720-treated TAC mice, the TAC mice...
treated with vehicle developed hypertrophy (Figure 7A through 7D). 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 with the vehicle treated groups (Figure 7E and 7F).

Next, we determined whether the antihypertrophic effect of FTY720 was due to Pak1 activation; therefore, the same FTY720 treatment protocol was applied to Pak1cko mice subjected to either TAC or sham operation. Interestingly, despite FTY720 treatment, TAC was still able to induce hypertrophy in the hearts of Pak1cko 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 μm² [FTY720] versus 313.8 ± 1.72 μm² [vehicle]) (Figure 8A and 8B).

Echocardiography and hemodynamic analysis demonstrated comparable cardiac structure and function between the FTY720-treated TAC mice and the sham groups (Figure 8C through 8F).

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 Pak1cko 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 antagonizing cardiac hypertrophy because hearts of Pak1<sup>cko</sup> 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 antihypertrophic treatment.
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 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 prohypertrophic effects in the adult heart.22,23 Thus, we...
predicted that Pak1 may exert a function similar to that of the Ras pathway in facilitating cardiac hypertrophy. However, to our surprise, our studies revealed the antihypertrophic 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. 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

Figure 8. FTY720 failed to block increased cardiac hypertrophy in Pak1-$^{cre}$- transverse aortic constriction (TAC) mice. Heart weight to tibia length (HW/TL) ratio (A) and mean cross-sectional areas (B) showed no difference between vehicle- and FTY720-treated Pak1-$^{cre}$-TAC mice, although TAC induced increased hypertrophy in both groups, compared with sham groups. Echocardiographic parameters of (C) ventricular posterior wall thickness (dPW) and (D) interventricular septal wall thickness (dIVS) are comparable between vehicle- and FTY720-treated Pak1-$^{cre}$-TAC mice. Fractional shortening (FS%) (E) and dP/dt$_{max}$ and dP/dt$_{min}$ (F) show normal cardiac functions in FTY720-treated Pak1-$^{cre}$-TAC mice ($n=4–6$ mice per group). *$P<0.03$, **$P<0.04$ versus vehicle-treated sham mice.
are downstream effectors of heterotrimeric G-proteins.\textsuperscript{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.\textsuperscript{28} When the heart is exposed to various hypertrophic stimuli, prohypertrophic and antihypertrophic signaling pathways are concurrently activated to regulate the hypertrophic process. We discovered that Pak1 was activated by a number of neuroendocrine factor and by pressure overload to antagonize cardiac hypertrophy.

The Cdc42-Pak1-JNK Axis Is a Critical Pathway Relaying Antihypertrophic 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\textsuperscript{29} 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. Their data agree with previous investigations in mammalian cells showing that Cdc42 induces JNK activation.\textsuperscript{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 regulates the activity of the ERK cascade.\textsuperscript{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 were detected when Pak1 was scarce. MKK4 and MKK7 are upstream kinases for JNKs, which are implicated in the progression of heart failure.\textsuperscript{35,36} JNKs have been shown to antagonize cardiac hypertrophy through inhibition of NFAT activity.\textsuperscript{37} Our recent studies using mice with cardiomyocyte-specific deletion of MKK4 or MKK7 support this mechanism.\textsuperscript{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 antihypertrophic signaling.

It is interesting to note that a recent study by Higuchi et al\textsuperscript{38} described a novel property of Pak1; it not only has catalytic functions, but can also act as a scaffolding protein for priming Akt activation. 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).\textsuperscript{10} It was proposed that p38 seemed to be an intermediate for Pak1-mediated PP2A activity.\textsuperscript{10} As such, we 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 that, at least in the model we employed, 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 downregulation of Cdc42,\textsuperscript{29} downregulation of Rac1 inhibits the development of cardiac hypertrophy in response to Ang II infusion via decreased activity of NADPH.\textsuperscript{30} Subsequent studies by Custodis et al\textsuperscript{40} 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. Rac1 overexpression in myocardium induced hypertrophy in juvenile transgenic mice concurrent with altered intracellular distribution of Pak from the cytosol to cytoskeletal fraction.\textsuperscript{41} Yet, in this study by Sussman et al,\textsuperscript{41} no information was provided as to which isoform of Pak was involved in the translocation. It is known that other Pak isoforms, such as Pak2 and Pak3, which share substantial sequence homology with Pak1,\textsuperscript{32} are also expressed in cardiomyocytes. We have demonstrated that Pak1\textsuperscript{ko} 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.\textsuperscript{39} Taking this evidence into account, it is plausible that Pak1 is a primary effector of Cdc42 rather than Rac1. Pak1 is an indispensable component of the Cdc42-Pak1-JNK axis serving as a critical antihypertrophic regulatory pathway.

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

FTY720 is a sphingosine-like analog approved by the Food and Drug Administration for treating relapsing multiple sclerosis. We have previously reported that FTY720 prevents arrhythmias in an ex vivo rat heart subjected to ischemia/reperfusion injury.\textsuperscript{14} In the ischemia/reperfusion model, Pak1 activation was suggested to be involved in an FTY720-induced protective effect.\textsuperscript{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 (10 μg·g\textsuperscript{-1}·d\textsuperscript{-1}) 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\textsuperscript{ko} mice provides further support that FTY720 induces its antihypertrophic 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.\textsuperscript{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 its ability to prevent hypertrophy without deteriorating cardiac function. Furthermore, FTY720, which is derived from myriocin, a component of the natural product Isaria sinclairii, represents a nontoxic sphingosine-like derivative with oral bioavailability that may be useful in the 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 that limits excessive hypertrophic remodeling. Pak1 most likely acts downstream of Cdc42 to convey both antihypertrophic 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 antihypertrophic treatment.

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Disclosures
None.

References
36. Fliesch M, Margules KB, Mochmann HC, Engel D, Sivasubramanian N, Mann DL. Differential regulation of mitogen-activated protein kinase in...
Heart failure (HF) is one of the most devastating diseases. The lifetime risk of heart failure is 1 in 5 among 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. On the basis of 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 indispensable component of the antihypertrophic 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 analog), a Food and Drug Administration–approved drug for treating relapsing multiple sclerosis, is able to limit cardiac hypertrophy of murine hearts. This antihypertrophic effect of FTY720 is likely to function through the activation of Pak1. Overall, our findings provide the evidence to establish Pak1 as a novel antihypertrophic regulator and a potential therapeutic target for the treatment of cardiac hypertrophy and heart failure.
Pak1 as a Novel Therapeutic Target for Antihypertrophic Treatment in the Heart
Wei Liu, Min Zi, Ronald Naumann, Susanne Ulm, Jiawei Jin, Domenico M. Taglieri, Sukhpal Prehar, Junhong Gui, Hoyee Tsui, Rui-Ping Xiao, Ludwig Neyses, R. John Solaro, Yunbo Ke, Elizabeth J. Cartwright, Ming Lei and Xin Wang

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SUPPLEMENTAL MATERIAL

Methods

Adenoviral Infection of NRCMs

The sequence of the scrambled shRNA was TTCTCCGAACGTGTCACGTTT (Ad-shC2 was kindly provided by Dr. Delvac Oceandy, University of Manchester, UK), while the sequence of shPak1 was CTGTTCTGGATGTGTTGGAAAT. Adenovirus expressing shPak1 was generated using the BLOCK-iT adenoviral RNAi expression system according to the manufacturer’s protocol (Invitrogen). Adenoviruses expressing caPak1 (constitutively active Pak1), GFP, LacZ, or NFAT-luciferase reporter gene (Ad-NFAT-Luc) have been described elsewhere1-3. Ad-caMKK7 (constitutively active MKK7) was a kind gift from Dr. Hiroki Aoki (Kurume University, Japan). Primary cultures of neonatal rat cardiomyocytes (NRCMs) were prepared as previously described4. To determine the efficiency of gene knockdown, immunoblot analyses were performed at 72 h after adenoviral shRNA infection. To evaluate hypertrophic responses, NRCMs were infected with various adenoviruses at 25 MOI in serum-free medium for 48 h, followed by additional 48 h of PE treatment (30 µM, Sigma). Thereafter NRCMs were subjected to immunocytochemistry or quantitative real-time PCR analyses. To screen downstream candidates, NRCMs were infected with either Ad-shC2 or Ad-shPak1 for 72 h prior to PE stimulation (30 µM, 30 minutes). After PE treatment, NRCMs were lysed for immunoblot analyses.

Immunocytochemistry and Trypan Blue Staining

NRCMs were plated onto laminin-coated coverslips prior to infection of Ad-caPak1 or Ad-shPak1 for 48 h, followed by PE stimulation (48 h). Thereafter, NRCMs were subjected to immunocytochemistry using the primary α-actinin antibody (1:100, Sigma, A7811) and the secondary anti-mouse antibody conjugated to Alexa Fluoro
NRCMs were counterstained with DAPI to visualize their nuclei. Images of 150 visible cells were collected and their surface area measured using Image J software. To examine the effect of FTY720 on reversing PE-induced hypertrophy in NRCMs, NRCMs were infected with Ad-shC2 or Ad-shPak1 for 48h, followed by incubation of vehicle, or PE (30 µM), or FTY720 (200nM), or PE plus FTY270 for further 48h. Thereafter, NRCMs were subjected to triple immunostaining using anti-ANP antibody (1:500, Peninsula Laboratories, T4015), anti-α-actinin antibody (1:100, Sigma, A7811) and DAPI (for visualizing the nuclei). Total of 500 cardiomyocytes from random fields per experimental group were selected to count ANP-expressing NRCMs. To evaluate cell viability after various treatments, NRCMs were fixed in 4% paraformaldehyde for 30 minutes prior to being stained in 0.4% trypan blue solution (Invitrogen) for 5 seconds, then total of 1000 cardiomyocytes from random fields per experimental group were selected to count trypan blue stained-cells.

**Luciferase Reporter Assay**

To investigate whether activated Pak1 affects the NFAT reporter activity, NRCMs were co-infected with Ad-caPak1 and Ad-NFAT-Luc (25 MOI) for 24h followed by 24h treatment of PE (30µM). Ad-LacZ is a control virus. To measure the NFAT reporter activity when Pak1 is knocked down, NRCMs were firstly infected with Ad-shC2, or Ad-shPak1, or Ad-shPak1 plus Ad-caMKK7, then infected with Ad-NFAT-Luc for a further 24 h, followed by PE treatment (30 µM, 24 h). After the treatment, aliquots of NRCM lysates were assayed for NFAT luciferase activity using the luciferase assay kit (Promega).
Immunoblot Analyses

Protein extracts (50 µg) prepared from tissues or NRCMs were subjected to immunoblot analyses with antibodies against MEKK1 (sc-49449), phospho-MEKK1 (sc-130202), JNK (sc-571), p38 (sc-535), phospho-PP2A (sc-271904), p53 (sc-6243) and Bax (sc-493) (Santa Cruz); Bim (202000) (Calbiochem); M KK4 (612637), Bad (610391) and Cdc42 (610928) (BD Transduction); GAPDH (ab9482) (Abcam); Rac1 (07-1464) (R&D systems); Pak1(2602), Pak2 (2608), Pak3 (2609), M KK7 (4172), ERK1/2 (9102), PKB (9272), PP2A (2038), Bcl-2 (2876), phospho-Pak1 (2601, Thr 423), phospho-MKK4 (9151), phospho-MKK7 (4171), phospho-JNK (9251), phospho-p38 (9211), phospho-ERK1/2 (9101), phospho-PKB (9271) and phospho-Bad (9291) (Cell Signaling). For antibody against MEKK1 or Phospho-MEKK1, we used 1:500 dilution, for rest antibodies, we used 1:1000 dilution. Immunocomplexes were detected by enhanced chemiluminescence with anti-mouse, anti-rabbit, or anti-goat immunoglobulin G coupled to horseradish peroxidise as the secondary antibody (Amersham-Pharmacia).

Generation of Pak1\textsuperscript{f/f} and Pak1\textsuperscript{cko} Mice

Pak1 genomic DNA cloned from an RPCI-21 PAC library (UK HGMP Resource Centre) was used for constructing a gene targeting vector with two LoxP sites flanking exon 3 of pak1. Exon 3 contains the GTPase-binding domain (PBD); the removal of this exon caused a frame shift in the open reading, resulting in the loss of Pak1 expression. The targeting vector consists of a 9.0-Kb Pst1 genomic fragment encompassing exons 3, 4 and 5 of the pak1 gene. A thymidine kinase neomycin resistance (Neo-TK) cassette containing two LoxP sites, a stop codon and a polyadenylation termination signal was inserted behind the exon 3, while a third LoxP site was placed in front of exon 3. 129Sv-derived R1 embryonic stem (ES) cell
transfection, selection and screening were performed as described previously\. Three homologously recombined ES cell clones identified by an external probe were transfected with a Cre-expressing plasmid and selected in culture medium containing 1-(2-deoxy-2-fluoro- \(\beta\)-D-arabinofuranosyl)-5-iodouracil (FIAU). The clones with two LoxP sites flanking exon 3 were identified by Southern blotting using an internal probe. Germline transmitting chimeras were generated and high-grade agouti-marked male chimeras were 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\(^{lf}\)) mice. To generate cardiomyocyte-specific Pak1 knockout mice (Pak1\(^{ko}\)), Pak1\(^{lf}\) mice were mated with mice expressing Cre under \(\alpha\) myosin heavy chain (\(\alpha\)MHC) promoter\. Specific recombination of the \(pak1\) gene in cardiomyocytes was verified by PCR of genomic DNA using forward (5’-GACCTGGGTTAAATCAC-3’) and reverse (5’-GGTCTGCCCTTTACCAGGC-3’) primers.

**Immunostaining of Isolated Mouse Cardiomyocytes**

To verify Pak1 deletion, cardiomyocytes were isolated from 8-week old mice using a modified collagenase and protease digestion method\. Dissociated cardiomyocytes were plated and fixed in 4% paraformaldehyde prior to being incubated with the Pak1 specific antibody (1:100, Cell Signaling). The secondary anti-rabbit antibody conjugated to Alexa Fluoro 568 (1:500, Invitrogen) was applied to detect the immune signals. Fluorescence images were viewed with a Nikon upright confocal microscope.

**Histology and TUNEL Assay**

5\(\mu\)m-thick paraffin embedded sections were stained with Hematoxylin & eosin or Sirius Red as described8, 9. To calculate the mean cross-sectional area approximately 200 randomly selected cardiomyocytes were measured. 50 randomly chosen frames
of Sirius Red stained sections were quantified to assess the degree of myocardial fibrosis using Image J software. TUNEL assay to detect apoptosis was performed on paraffin sections using the in situ Cell Death Detection kit (Roche). Total of 10,000 cardiomyocytes from random fields per animal were analyzed.

**Measurement of Myocardial Superoxide Production**

DHE staining was used for in situ detection of ROS production. Fresh frozen ventricular myocardium (10µm sections) was incubated in the dark for 30 minutes at 37°C with dihydroethidium (2x10^{-6} mol/L, Invitrogen). The staining was examined and fluorescence intensities in ventricular sections were quantified by the Image J analysis system.

**Echocardiography and Hemodynamic Analysis**

Mice were anesthetized with Avertin (Sigma, 200 mg/kg) via intraperitoneal injection. Transthoracic M-mode echocardiographic recordings were performed using an Acuson Sequoia C256 system (Siemens) following a protocol described previously1–8. Three measurements taken at end-systole and end-diastole were averaged to calculate intraventricular septal thickness (IVS), left ventricular posterior wall thickness (LVPW), 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 previously1. Maximal derived pressures were obtained during systole (dP/dt_{max}) and diastole (dP/dt_{min}) as indices representing cardiac contractile functions.
Quantitative Real-time PCR

Total RNA was extracted from tissues or NRCMs using Trizol reagent, followed by the synthesis of cDNA. The primers for ANP, BNP, Myh7, Col1α2 and Col3α1 and GAPDH were obtained from Qiagen. The primers used for RCAN1.4 have been described previously. The primers (forward: 5’-CAGCTTTTGATTCCCTGCAT-3’ and reverse: 5’-GCCAGCCATGTGCTAAAAGT-3’) were designed for Pak1. PCR products were detected in the ABI-PRISM 7700 sequence detection system (Applied Biosystems), and the results were analyzed using the 2-ΔΔCT method. The level of expression of mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

References


Supplement Table 1. Echocardiographic assessment of Pak1^{f/f} and Pak1^{cko} mice after 2 weeks of TAC. dPW, end-diastolic left ventricular posterior wall thickness; dIVS, end-diastolic interventricular septal wall thickness; LVEDD, diastolic left ventricular internal dimension; LVESD, systolic left ventricular internal dimension; FS, fraction shortening. n=5-7 per group, Data are presented as means ± SEM, A^P=0.01, B^P=0.04, C^P=0.03, D^P=0.02 versus Pak1^{f/f}-TAC mice.

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<tr>
<td></td>
<td>Pak1^{f/f}</td>
<td>Pak1^{cko}</td>
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<tr>
<td>dPW (mm)</td>
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<td>LVESD (mm)</td>
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<td>FS (%)</td>
<td>37.8 ± 0.69</td>
<td>38.6 ± 1.16</td>
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Supplement Figure I. Targeted disruption of the *pak1* gene in the mouse genome. (A) Schematic diagram illustrates the *pak1* wild-type locus, *pak1* targeting vector, predicted structure of the mutated *pak1* locus, together with the selected flox allele and the knockout allele, obtained by Cre enzyme-mediated recombination. Restriction enzyme sites (S, SpeI; X, XhoI; P, PstI; H, HindIII; B, BamHI), exons (E3, E4, E5), external and internal probes as well as the LoxP sites (black arrowheads) are indicated on the *pak1* locus. (B) Southern blotting analyses using random-primed 32P-labeled mouse *pak1* genomic external and internal probes demonstrate the presence of all expected genotypes. For the 1st targeting, genomic DNA extracted from the ES cell clones were digested by SpeI and XhoI, followed by blotting with the external probe (left panel), *pak1* wild-type allele: *pak1*+; mutated allele: *pak1m*. After the digestion with HindIII and BamHI, genomic DNA was detected by the internal probe for the 2nd targeting (right panel), knockout allele: *pak1−*; flox allele: *pak1f*.
Supplement Figure II. Generation of Pak1\textsuperscript{cko} mice. (A) Genomic DNA isolated from ventricular cardiomyocytes (CM), brain, liver and skeletal muscle (SM) of 8-week old Pak1\textsuperscript{f/f} and Pak1\textsuperscript{cko} mice were amplified by PCR with primers specific for the pak1 gene, confirming the specific recombination of the pak1 gene in cardiomyocytes. (B) Quantitative RT-PCR analyses of the mRNA levels of Pak1 in the left ventricle (LV), brain and liver demonstrate a 84% decrease in Pak1 mRNA in LV. Data are means ± SEM (n=5 per group). (C) Immunostaining of isolated single cardiomyocyte to examine the deletion of Pak1 (scale bar: 10 \mu m). Fluorescence intensity of Pak1-labeling are quantified and expressed in the bar graphs. 60 randomly selected cardiomyocytes from each group were stained with a Pak1 specific antibody (n=6 per group). (D) Immunoblot analyses demonstrate the deletion of Pak1 in Pak1\textsuperscript{cko} myocardium. Pak2 and Pak3 protein levels were also examined to determine the specificity of Pak1 deletion. (E) Immunoblot analyses show similar protein levels of Cdc42, Rac1, ERK1/2, JNK and p38 in Pak1\textsuperscript{cko} and Pak1\textsuperscript{f/f} controls. GAPDH expression is the protein loading control.
Supplement Figure III. Pak1 deletion in cardiomyocytes promoted Ang II-induced cardiac hypertrophy. 2 weeks infusion of Ang II induced greater hypertrophy in Pak1\textsuperscript{-/-} mice, indicated by increased HW/TL ratios (A) and larger cardiomyocytes (B). (C) Ang II treatment resulted in more cardiac fibrosis in the Pak1\textsuperscript{-/-} heart. (D) ROS production in ventricular myocardium was measured by DHE staining, and showed no increased ROS level in the Pak1\textsuperscript{-/-} heart. (E) Echocardiographic analyses show preserved cardiac contractility in Ang II-infused Pak1\textsuperscript{-/-} heart. Data are presented as means ± SEM, n=5-7 per group.