Acta Biochim Biophys Sin, 2015, 47(9), 687–695
doi: 10.1093/abbs/gmv067
Advance Access Publication Date: 22 July 2015
Original Article

Original Article

Protein kinase Cα inhibits myocardin-induced cardiomyocyte hypertrophy through the promotion of myocardin phosphorylation

Weizong Li[1,†], Nan Wang[1,*,†], Man Li[1], Huiqin Gong[1], Xinghua Liao[1,2], Xiaolong Yang[1], and Tongcun Zhang[1,2,*]

[1]Key Laboratory of Industrial Microbiology, Ministry of Education and Tianjin City, College of Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, China, and [2]Department of Biochemistry, Medical College, Wuhan University of Science and Technology, Wuhan 430081, China

[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-22-60602099; Fax: +86-22-60602298; E-mail: tony@tust.edu.cn (T.Z.)/wn929@tust.edu.cn (N.W.)

Received 24 February 2015; Accepted 27 April 2015

Abstract

Myocardin plays a key role in the development of cardiac hypertrophy. However, the upstream signals that control the stability and transactivity of myocardin remain to be fully understood. The expression of protein kinase Cα (PKCα) also induces cardiac hypertrophy. An essential downstream molecule of PKCα, extracellular signal-regulated kinase 1/2, was reported to negatively regulate the activities of myocardin. But, the effect of cooperation between PKCα and myocardin and the potential molecular mechanism by which PKCα regulates myocardin-mediated cardiac hypertrophy are unclear. In this study, a luciferase assay was performed using H9C2 cells transfected with expression plasmids for PKCα and myocardin. Surprisingly, the results showed that PKCα inhibited the transcriptional activity of myocardin. PKCα inhibited myocardin-induced cardiomyocyte hypertrophy, demonstrated by the decrease in cell surface area and fetal gene expression, in cardiomyocyte cells overexpressing PKCα and myocardin. The potential mechanism underlying the inhibition effect of PKCα on the function of myocardin is further explored. PKCα directly promoted the basal phosphorylation of endogenous myocardin at serine and threonine residues. In myocardin-overexpressing cardiomyocyte cells, PKCα induced the excessive phosphorylation of myocardin, resulting in the degradation of myocardin and a transcriptional suppression of hypertrophic genes. These results demonstrated that PKCα inhibits myocardin-induced cardiomyocyte hypertrophy through the promotion of myocardin phosphorylation.

Key words: PKCα, myocardin, cardiomyocyte hypertrophy, phosphorylation

Introduction

Cardiac hypertrophy, an essential adaptive or maladaptive response of myocytes to diverse pathophysiological stimuli, is characterized by increased cardiomyocyte size, elevated protein synthesis, and altered gene expression [1–3]. Numerous transcription factors, such as GATA-binding protein 4, myocyte enhancer factor 2, nuclear factor of activated T cells, and serum response factor (SRF), have been identified as transcriptional regulators that regulate the expressions of a series of hypertrophic genes through the recruitment of cofactors [4,5].

Myocardin, an SRF coactivator belonging to the secreted aspartyl proteinase superfamily, is specifically expressed in cardiomyocytes and smooth muscle cells. Myocardin potently transactivates CArG

box (CC[A/T]$_6$GG)-containing cardiac and smooth muscle target genes through an association with SRF [6–10]. Previous studies have confirmed that the overexpression of myocardin induces hypertrophy and fetal cardiac gene expression [9,11,12]. However, the upstream signals that control the stability and transactivity of myocardin remain to be fully understood.

Protein kinase C (PKC), a serine/threonine kinase, plays a key role in the regulation of cardiac contraction, hypertrophy, injury, failure, etc. [13–15]. It has been shown that PKCα is a key regulator of cardiomyocyte hypertrophic growth [16]. Using adenovirus-mediated transfection of wild-type or dominant inhibitory forms of PKCα, -β2, -δ, and -ε in neonatal rat cardiomyocytes, Braz *et al.* [17] demonstrated that the inhibition of PKCα reduces agonist-induced hypertrophy. Similarly, dominant negative PKCα suppresses agonist-induced cardiac hypertrophy [16].

As an essential downstream molecule of PKCα, extracellular signal-regulated kinase 1/2 (ERK1/2) was reported to negatively regulate the activities of myocardin [18]. But, the effect of cooperation between PKCα and myocardin on cardiac hypertrophy and the exact molecular mechanism by which PKCα regulates myocardin-mediated cardiac hypertrophy are unclear. In this study, the relationship between myocardin and PKCα was explored. Our results showed that PKCα inhibits myocardin-induced cardiomyocyte hypertrophy in H9C2 cells overexpressing PKCα and myocardin. Furthermore, PKCα promotes the myocardin phosphorylation, resulting in myocardin degradation and transcriptional activity decrease.

Materials and Methods

Cell culture

Neonatal rat cardiomyocytes were isolated from day 1 to day 3 Sprague-Dawley rat pups, as described previously [19]. The isolated cells were cultured in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12; Gibco, Gaithersburg, USA) supplemented with 20% fetal bovine serum (FBS) at 37°C in humidified air with 5% CO_2.

The rat cardiomyocyte-derived cell line H9C2 was obtained from American Type Culture Collection (Manassas, USA) and cultured in high glucose DMEM supplemented with 10% FBS.

Cell transfection and luciferase reporter assay

The pcDNA3.1-myocardin expression plasmid encoding mouse myocardin was constructed, as described previously [19]. PKCα cDNA was amplified by polymerase chain reaction (PCR) and subsequently cloned into the pCMV vector. The promoter regions of atrial natriuretic factor (ANF) (−957/+37) were amplified by PCR and cloned into pGL3 luciferase reporter vector. The primers used to generate PKCα-flag and ANF-luc are shown as follows: *ANF*: forward 5′-AGG AACGCGTGCCTTCACGGATCACTTC-3′, reverse 5′-TCTGCTCG AGGCTGTCTCGGCTCACTCT-3′ and *PKCα*: forward 5′-GGTACT CGAGATGGCTGACGTTTTCCCG-3′, reverse 5′- CCGCGGTACC CATACTGCACTCTGTAAGAT-3′. The luciferase reporter gene plasmid containing the 4×CArG box was a gift from Professor Eric N. Olson of the Department of Molecular Biology at the University of Texas Southwestern Medical Center (Dallas, USA).

For the transfection experiments, neonatal rat cardiomyocytes were transfected using TurboFect transfection reagent (Thermo, Waltham, USA), and H9C2 cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, USA). After 6 h of incubation, the medium was replaced with normal culture medium. The luciferase reporter assay was performed in 24-well plates. Twenty-four hours after transfection, the cells were harvested, and luciferase activity was measured using a luciferase assay system (Promega, Madison, USA).

Immunofluorescence

The cardiomyocytes were fixed with 4% paraformaldehyde for 15 min and treated with 0.25% Triton X-100 for 20 min at room temperature, followed by blocking with normal goat serum for 20 min at room temperature. After overnight incubation with mouse anti-α-actinin (Sigma, St Louis, USA), the cells were subsequently incubated with the fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA) for 30 min at 37°C. After washing with phosphate-buffered saline (PBS), the samples were observed under a laser scanning confocal microscope (OLYMPUS, Tokyo, Japan). The fluorescent stain 4′,6′-diamidino-2-phenylindole was used to stain the nuclei, and the relative area was analyzed using iPP 6.0 software.

Reverse transcriptase–PCR and quantitative real-time PCR

Reverse transcriptase (RT)–PCR and quantitative real-time PCR (qRT-PCR) analyses were carried out as described previously [19]. qRT-PCR was performed using Biosystems StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions. Fast SYBR® Green Master Mix was obtained from Applied Biosystems. The data were expressed as the relative expression levels after normalization to GAPDH. The PCR primers used in the experiment are as follows: ANF for RT–PCR: forward 5′-GAAGTCAACCCGTCTCA-3′, reverse 5′-ATCCTGTCA ATCCTACCC-3′; brain natriuretic peptide (BNP) for RT–PCR: forward 5′-TTCTGCTCCTGCTTTTCCTT-3′, reverse 5′-CGGTCTAT CTTCTGCCCAAA-3′; *GAPDH* for RT–PCR and qRT-PCR: forward 5′-ATTCAACGGCACAGTCAAGG-3′, reverse 5′-GCAGAA GGGGCGGAGTGA-3′; and qRT-PCR primers: ANF for qRT-PCR: forward 5′-GGGCTTCTTCCTCTTCCTG-3′, reverse 5′-CGCTTCA TCGGTCTGCTC-3′ and BNP for qRT-PCR: forward 5′-TTCTGC TCCTGCTTTTCC-3′, reverse 5′-CTTTTGTAGGGCCTTGGT-3′.

Western blot analysis

After treatment, cells were washed twice with ice-cold PBS and lysed in RIPA buffer (CW BioTech, Beijing, China). The proteins were subsequently electrophoresed on a 12% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE), followed by transferring onto a nitrocellulose (NC) membrane and blocking with PBS containing 5% skimmed milk powder (w/v) for 60 min at room temperature. The membranes were immunoblotted with mouse anti-myocardin (Sigma), rabbit anti-PKCα (Beyotime Biotechnology, Haimen, China), rabbit anti-ANF (Abcam, Cambridge, UK), rabbit anti-BNP (Abcam), anti-phos-ERK1/2 (Beyotime Biotechnology), anti-ERK1/2 (Cell Signaling, Beverly, USA), and anti-β-actin (Santa Cruz) antibodies overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies (Li-COR Biosciences, Lincoln, USA) for 1 h at room temperature. The specific proteins were visualized using the Odyssey Infrared Imaging System (Gene Company Limited, Beijing, China). The relative protein expression levels were analyzed using ImageJ software.

Total protein/DNA content ratio analysis

Protein and DNA from cardiomyocytes were measured with a published method described previously [20]. Myocytes were collected

using trypsin and then split into two equal aliquots for protein and DNA measurements. For protein assays, one aliquot was resuspended in protein lysis buffer (50 mM Tris–HCl, pH 7.4, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 100 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride). The total cell protein was measured using a BCA protein assay kit (Solarbio, Beijing, China) using bovine serum albumin as the standard. The other aliquot was resuspended in Tris–HCl, EDTA, NaCl (TEN) buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, pH 8.0, and 150 mM NaCl). DNA was measured by using Hoechst 33258 (Solarbio) using Salmon sperm DNA (Solarbio) as the standard. The DNA was analyzed at 365 nm excitation and 460 nm emission using a Synergy4 system (BioTek, Winooski, USAS). Protein-to-DNA ratio was calculated in the extracts obtained from individual wells of each culture tray.

Immunoprecipitation and co-immunoprecipitation analyses

The immunoprecipitation (IP) experiments were performed to detect the phosphorylation of myocardin in H9C2 cells. The protein extracts were isolated from cells using RIPA buffer. The tagged proteins were immunoprecipitated overnight at 4°C using an anti-Myc antibody, followed by the addition of protein A/G agarose (CW0349) and further incubation for 3 h. All complexes were pelleted at 1400 *g* for 3 min. Proteins bound to the beads were fractionated through 12% SDS–PAGE and transferred to an NC membrane. The membranes were immunoblotted with anti-Myc-tag (Proteintech, Chicago, USA), anti-phospho-threonine (Cell Signaling), anti-phospho-serine (IMMUNECHEM, Burnaby, Canada), and anti-β-actin (Santa Cruz) antibodies overnight at 4°C, followed by incubation with corresponding HRP-conjugated secondary antibodies (Li-COR Biosciences) for 1 h at room temperature. The specific proteins were visualized using the Odyssey Infrared Imaging System (Gene Company Limited).

For Co-IP assay, a similar protocol was followed using COS7 cells to confirm the interaction between myocardin and PKCα. IP was performed using anti-Myc or anti-flag antibodies (Proteintech). The myc- or flag-precipitated proteins were immunoblotted using anti-flag and anti-myc antibodies, respectively.

Statistical analysis

Data were expressed as the mean ± SEM, and the number of experiments performed independently was also shown. Data were analyzed using Student's *t*-test. Differences at $P < 0.05$ were considered statistically significant.

Results

PKCα decreases the transcriptional activity of myocardin

To determine the relationship between myocardin and PKCα, H9C2 cells were transfected with expression plasmids for myocardin, ANF-luc, and increasing amounts of PKCα, and then luciferase assays were performed. The overexpression of myocardin significantly increased ANF promoter transcriptional activity, whereas co-transfection with expression plasmids for myocardin and PKCα decreased the transcriptional levels of ANF in a dose-dependent manner (**Fig. 1A,B**).

To further determine whether PKCα represses the transcriptional activity of myocardin, H9C2 cells were transfected with expression plasmids for myocardin and ANF-luc/4×CArG-luc and then stimulated with increasing amounts of PMA, an activator of PKCα, followed by analysis using luciferase assay. The stimulation of myocardin-transfected H9C2 cells with increasing amounts of PMA for 12 h decreased ANF promoter activity in a dose-dependent manner (**Fig. 1C**). As shown in **Fig. 1D,E**, the co-transfection of PKCα and stimulation with 50 nM PMA significantly inhibited the transcriptional activity of myocardin. These results demonstrated that PKCα negatively regulates the transcriptional activity of myocardin.

PKCα inhibits myocardin-induced cardiomyocyte hypertrophy

To investigate the role of PKCα in myocardin-induced cardiac hypertrophy, neonatal rat cardiomyocytes were transfected with expression plasmids for myocardin and/or PKCα, followed by starvation for 6 h and stimulation with or without 50 nM PMA for an additional 24 h. The overexpression of myocardin or PKCα increased the cell surface area, whereas co-transfection with the expression plasmids for myocardin and PKCα strongly decreased the cell size (**Fig. 2A**). The protein/DNA ratio was determined to demonstrate cellular hypertrophy (**Fig. 2B**). The overexpression of myocardin or PKCα resulted in a significant increase in the protein/DNA ratio. Myocardin-induced increase in the protein/DNA ratio was attenuated by the overexpression of PKCα. As shown in **Fig. 2B**, myocardin-activated ANF and BNP mRNA levels were significantly inhibited by PKCα. PKCα also markedly reduced myocardin-stimulated ANF and BNP expressions (**Fig. 2C, D**). Interestingly, co-transfection with the expression plasmid for PKCα also suppressed the myocardin level (**Fig. 2E,F**). Thus, these results showed that PKCα inhibits myocardin-induced cardiomyocyte hypertrophy.

PKCα inhibitor recovers the PKCα-mediated repression of myocardin transcriptional activity and enhances the expression of the myocardin target genes ANF and BNP

To further determine whether PKCα inhibits myocardin-induced cardiomyocyte hypertrophy, H9c2 cells were co-transfected with expression plasmids for myocardin and PKCα. In addition, cells were pre-treated for 1 h with 100 nM Calphostin C, an inhibitor of PKCα, followed by stimulation with 50 nM PMA for 24 h. As shown in **Fig. 3A,B**, compared with cardiomyocytes overexpressing both myocardin and PKCα, cells pre-treated with the PKCα inhibitor showed significantly increased surface area and protein/DNA ratio with a hypertrophic growth phenotype. Furthermore, PKCα strongly inhibited myocardin-mediated ANF and BNP expressions, whereas pre-treatment with the PKCα inhibitor dramatically increased the levels of these markers in the presence of myocardin and PKCα (**Fig. 3C,D**). The PKCα inhibitor also partially restored the myocardin levels (**Fig. 3E,F**). When pre-treated with Calphostin C for 1 h followed by stimulation with PMA for 12 h, co-transfected cardiomyocytes showed a significant increase in ANF and 4×CArG promoter activities (**Fig. 3G**).

PKCα promotes phosphorylation of myocardin

To confirm the effect of Calphostin C on the function of PKCα, the nuclear translocation of PKCα was tested by immunofluorescent assays. As shown in **Fig. 4A**, PMA promoted the nuclear translocation of PKCα, whereas Calphostin C inhibited the nuclear translocation of PKCα. To confirm the effect of the ERK inhibitor, U0126, on the phosphorylation of ERK1/2, the phosphorylation of ERK1/2 was detected with anti-phos-ERK1/2 antibodies, and the total expression of ERK1/2 was detected with anti-ERK1/2 antibodies by western blot analysis. As shown in **Fig. 4B**, U0126 completely counteracted PMA-induced ERK phosphorylation.

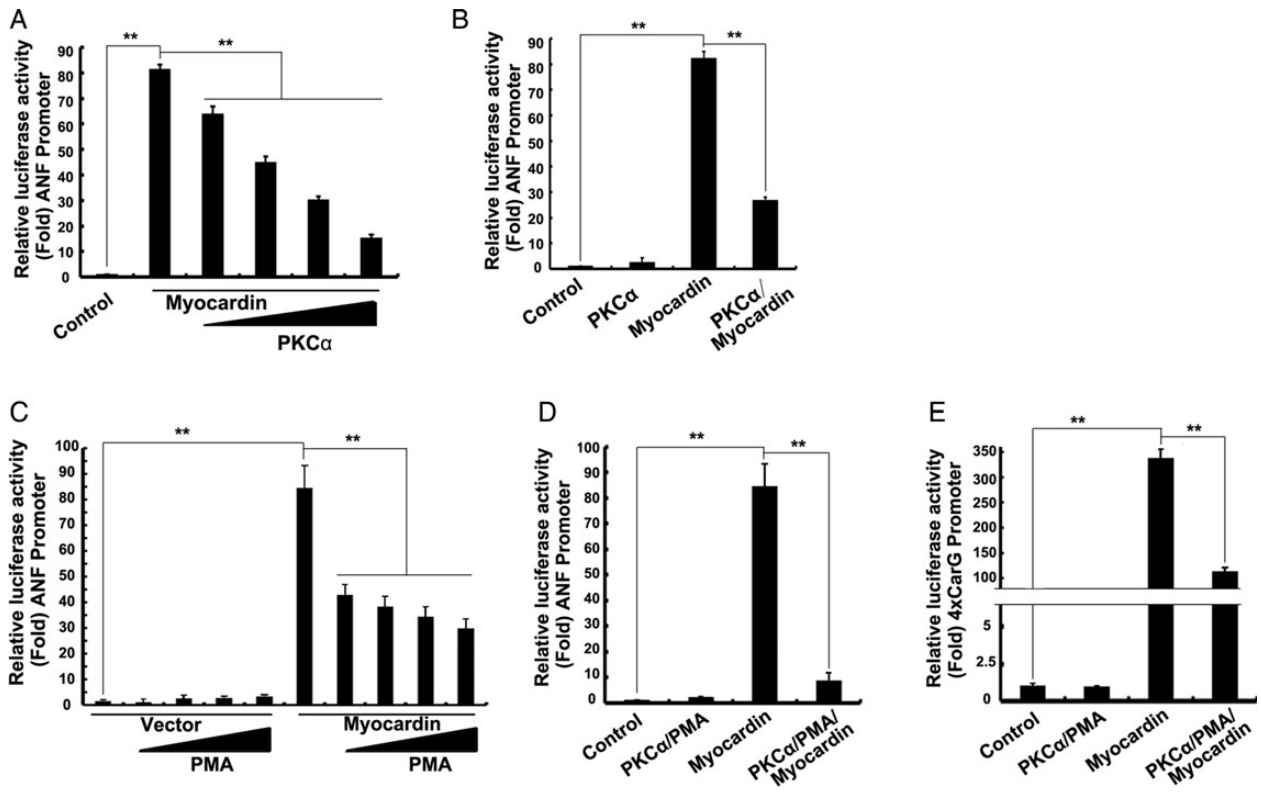

Figure 1. PKCα decreases the transcriptional activity of myocardin (A,B) H9C2 cells were transfected with expression plasmids for myocardin, ANF-luc, and increasing amounts of PKCα (0.3, 0.4, 0.5, and 0.6 μg). Subsequently, luciferase assays were performed. (C) H9C2 cells were transfected with expression plasmids for myocardin and ANF-luc, starved for 6 h, and stimulated with increasing amounts of PMA (0, 50, 100, 150, and 200 nM) for 12 h, followed by analysis using luciferase assays. (D,E) After co-transfection with expression plasmids for myocardin and/or PKCα, the starved cells were treated with 50 nM PMA for 12 h. The promoter activities of ANF and 4×CArG were analyzed in H9C2 cells. $^{**}P < 0.01$, $n = 3$.

Myocardin is phosphorylated through either ERK1/2 or glycogen synthase kinase 3β (GSK-3β), and phosphorylation reduces the transcriptional activity of this protein, resulting in the significant attenuation of cardiac hypertrophy [21,22]. In this study, PKCα was also found to inhibit myocardin transcriptional activity. Thus, we investigated whether PKCα promotes myocardin phosphorylation. First, the phosphorylation of endogenous myocardin was examined in the presence of PMA. As shown in **Fig. 4C**, endogenous myocardin was phosphorylated at serine and threonine residues, whereas the inhibition of PKCα/MAPK/ERK through Calphostin C or U0126 impaired myocardin phosphorylation. Next, the phosphorylation of myocardin was detected in H9C2 cells overexpressing myocardin-myc and PKCα-flag. As shown in **Fig. 4D**, PKCα induced excessive myocardin phosphorylation in cardiomyocytes overexpressing myocardin, and this increased phosphorylation was inhibited by the PKCα-specific inhibitor Calphostin C. Interestingly, U0126, which almost completely inhibits the PMA-induced phosphorylation of myocardin, partially suppressed PKCα-induced phosphorylation at serine residues, but was ineffective for the inhibition of phosphorylation at threonine residues (**Fig. 4D**). This result suggested that myocardin can be phosphorylated by PKCα directly and by ERK1/2 as well. To further determine whether PKCα interacts with myocardin, H9C2 cells were transfected with expression plasmids for PKCα-flag and/or myocardin-myc, followed by the Co-IP assay. The nuclear proteins were immunoprecipitated using anti-flag or anti-myc antibody. Western blot analysis of the Co-IP experiments showed that PKCα directly interacts with myocardin (**Fig. 4E**).

Phosphorylation of myocardin promotes the degradation of myocardin through PKCα

It has been reported that myocardin phosphorylation leads to the degradation of myocardin [23]. We also found that PKCα activation decreased the levels of myocardin protein in cells co-transfected with expression plasmids for myocardin and PKCα in the presence of PMA. However, the myocardin mRNA levels remained unaffected, as shown in **Fig. 2B,D**. Thus, we hypothesized that myocardin phosphorylation results in the degradation of myocardin through PKCα. To confirm this hypothesis, cardiomyocytes were co-transfected with expression plasmids for myocardin and PKCα, followed by treatment with the proteasome inhibitor MG-132 (1 μM) and 50 nM PMA for 24 h. As shown in **Fig. 5A,B**, PKCα overexpression did not influence myocardin mRNA levels in H9C2 cells, but decreased myocardin protein levels. When co-transfected cells were incubated with the proteasome inhibitor MG-132, the myocardin protein levels were strongly up-regulated, and myocardin degradation was significantly inhibited (**Fig. 5C**). These data suggested that PKCα mediates the degradation of myocardin, thereby inhibiting the expression of myocardin.

Discussion

As a coactivator of SRF, myocardin is required to induce cardiac hypertrophy and fetal cardiac gene program [11]. In addition, PKCα regulates the hypertrophic growth of cardiomyocytes through ERK1/2 [18]. Consistent with the results of previous studies [11,24], we also

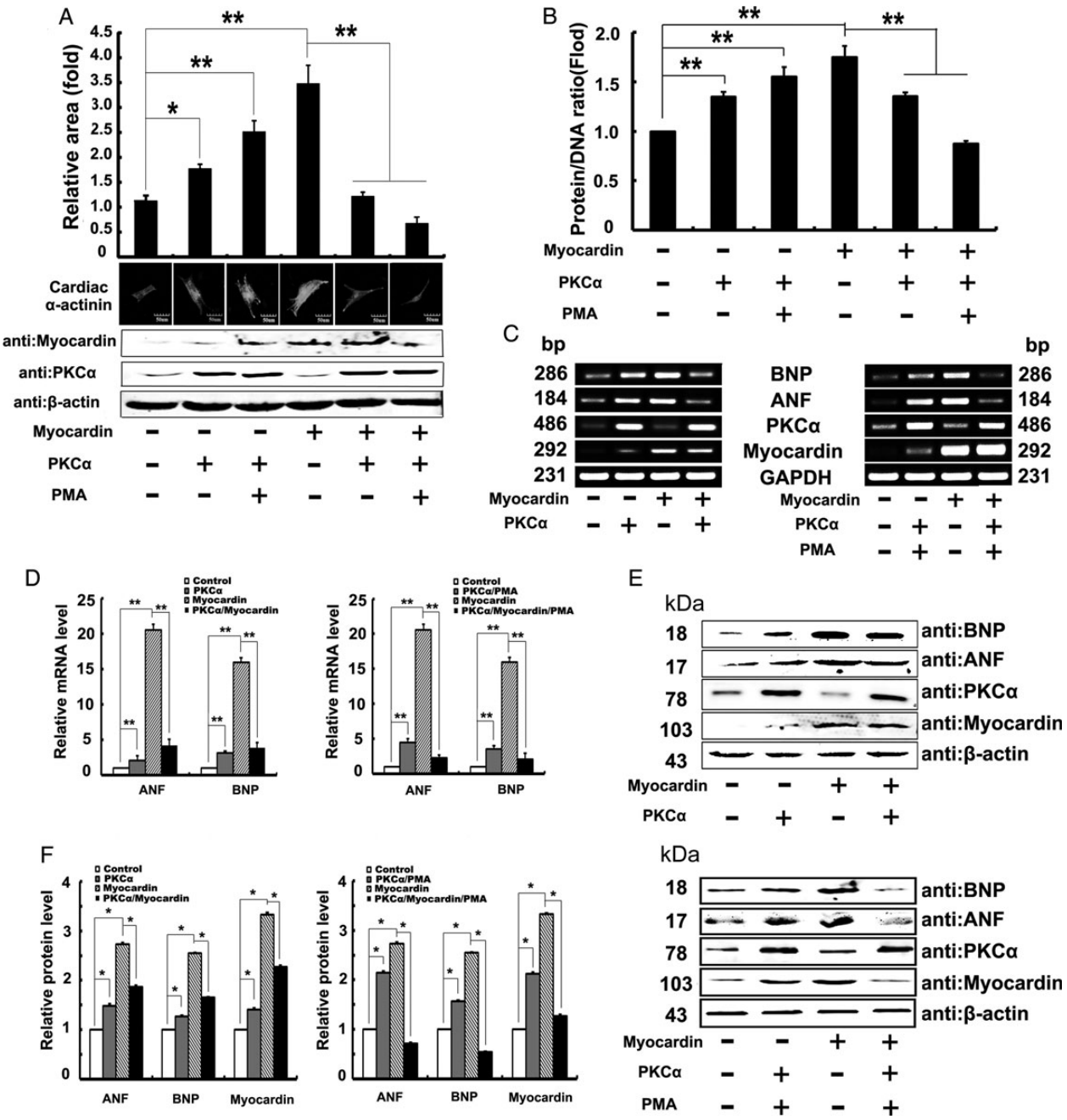

Figure 2. PKCα inhibits myocardin-induced cardiomyocyte hypertrophy (A) Neonatal rat cardiomyocytes were transfected with myocardin and/or PKCα expression plasmids, starved for 6 h, and subsequently stimulated with or without 50 nM PMA for 24 h. The morphological changes of cardiomyocytes were observed under a phase-contrast microscope. The relative area of cardiomyocytes was analyzed using iPP 6.0 software, and the results were normalized to the control. $^{**}P<0.01$, $n=3$. (B) Cellular hypertrophy was determined by measuring the protein/DNA ratio. (C,D) The left panel shows the results of the qRT-PCR analysis of ANF and BNP mRNA levels in H9C2 cells co-transfected with expression plasmids for myocardin and/or PKCα. The right panel shows the results of the qRT-PCR analysis of ANF and BNP mRNA levels in co-transfected H9C2 cells with PMA stimulation. ANF and BNP mRNA levels were analyzed using ImageJ software, and the results were normalized to GAPDH. $^{**}P<0.01$, $n=3$. (E,F) Western blot analysis of BNP, ANF, PKCα, and myocardin expressions after co-transfection with expression plasmids for myocardin and PKCα with (upper panel) or without PMA stimulation (lower panel). The relative protein expression levels were analyzed using ImageJ software, and the results were normalized to β-actin. $^{*}P<0.05$; $^{**}P<0.01$, $n=3$.

found that the overexpression of either myocardin or PKCα increased the cell surface area and induced cardiac hypertrophy. Furthermore, we examined the relationship between two transcription factors, myocardin and PKCα, in the regulation of cardiomyocyte hypertrophy. Co-transfection with expression plasmids for PKCα strongly decreased the cell size and reduced cardiac hypertrophy induced by myocardin. These findings suggested that PKCα can inhibit myocardin function. Luciferase reporter assays also confirmed the inhibitory effect of PKCα on the transcriptional activity of myocardin using a luciferase reporter gene plasmid containing 4×CArG box. The results showed that PKCα overexpression suppressed the transcriptional activity of myocardin (**Fig. 1**E). Furthermore, the PKCα inhibitor

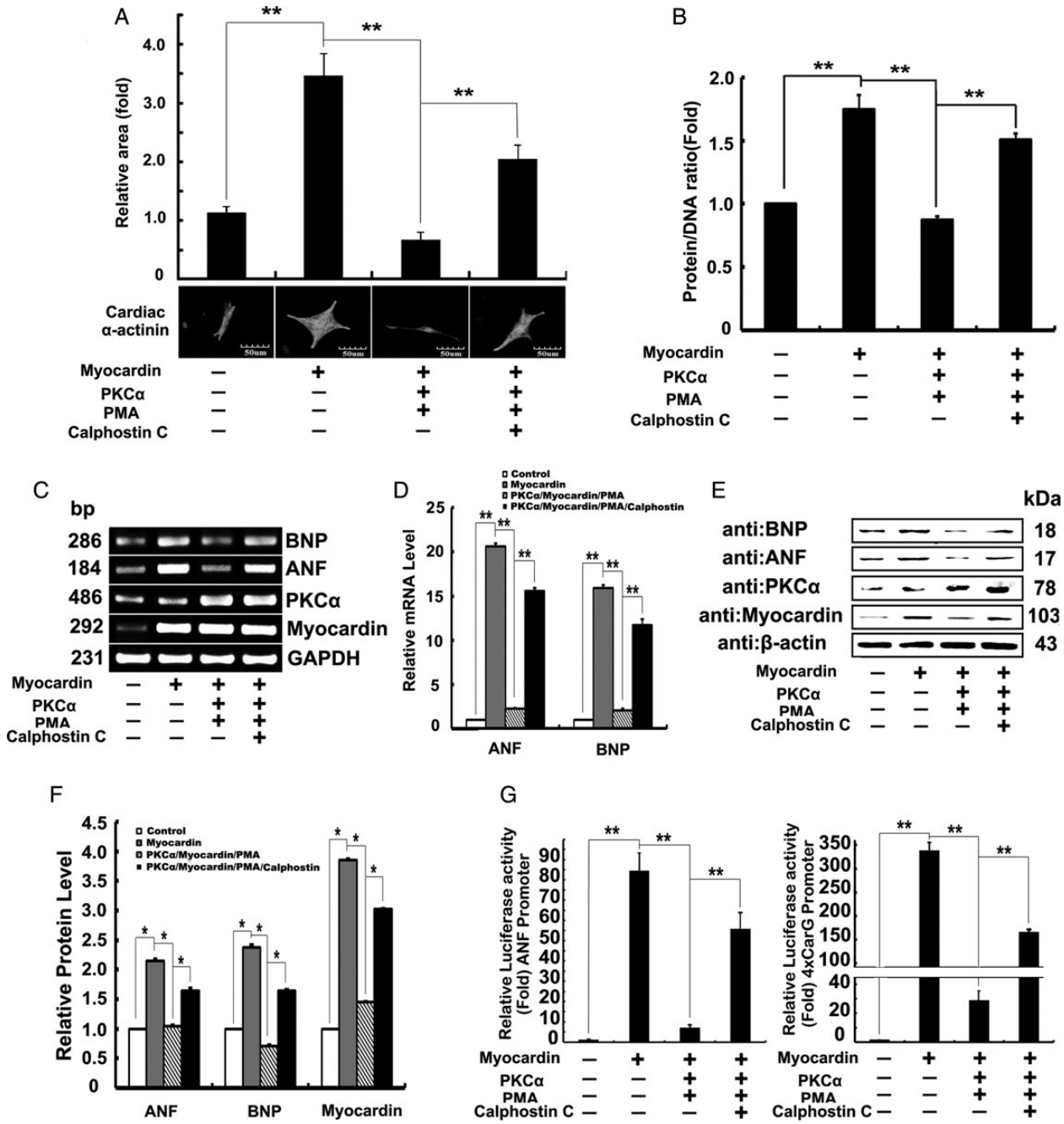

Figure 3. PKCα inhibitor recovers the PKCα-mediated repression of myocardin transcriptional activity and enhances the expression of myocardin target genes ANF and BNP (A) Neonatal cardiomyocytes were co-transfected with expression plasmids for myocardin and/or PKCα, starved for 6 h, pre-treated with 100 nM Calphostin C for 1 h, and subsequently incubated with 50 nM PMA for 24 h. The morphological changes of cardiomyocytes were observed under a phase-contrast microscope. The relative area of cardiomyocytes was analyzed using iPP 6.0 software, and the results were normalized to the control. $^{**}P < 0.01$, $n = 3$. (B) Cellular hypertrophy was determined by measuring the protein/DNA ratio. $^{**}P < 0.01$, $n = 3$. (C,D) The mRNA levels of ANF and BNP were examined in H9C2 cells using qRT-PCR. ANF and BNP mRNA levels were analyzed using ImageJ software, and the results were normalized to GAPDH. $^{**}P < 0.01$, $n = 3$. (E,F) Western blot analysis of myocardin, PKCα, ANF, and BNP expressions. The relative protein expression levels were analyzed using ImageJ software, and the results were normalized to β-actin. $^{*}P < 0.05$, $n = 3$. (G) A luciferase assay was performed after transfection with ANF-luc (left panel) or 4×CArG-luc (right panel) and indicated combinations of expression vectors and stimulatory factors as described in (A). $^{**}P < 0.01$, $n = 3$.

recovered the PKCα-mediated repression of myocardin transcriptional activity and enhanced the expression of the myocardin target genes ANF and BNP (**Fig. 3**). In addition, myocardin overexpression did not affect the expression of PKCα in cardiomyocytes (**Figs. 2D** and **3D**). These results suggested that the overexpression of PKCα reduced myocardin-induced cardiac hypertrophy.

The impaired myocardin function induced by PKCα was restored after pre-treatment with Calphostin C. Similarly, the activities of myocardin and myocardin-related transcription factors (MRTFs) are also negatively regulated through ERK1/2, an essential downstream molecule of PKCα [21,22]. Purcell *et al.* [25] reported that p65, which could promote the development of cardiac hypertrophy, inhibited

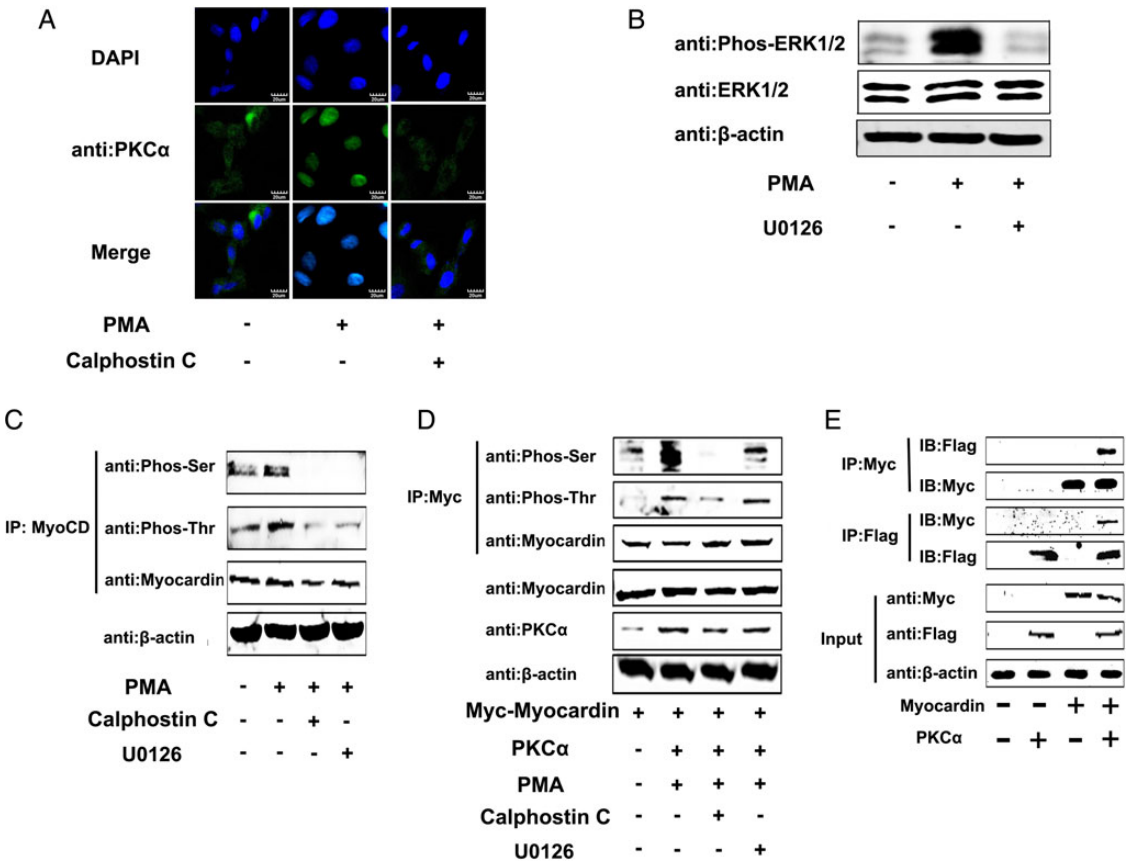

Figure 4. PKCα promotes phosphorylation of myocardin (A,B) H9C2 cells were pre-treated with 100 nM Calphostin C or 10 μM U0126 for 1 h and subsequently stimulated with 50 nM PMA for 24 h. The nuclear translocation of PKCα was detected by immunofluorescent assay and the phosphorylation of ERK1/2 was detected by western blot analysis, respectively. (C) H9C2 cells were pre-treated with 100 nM Calphostin C or 10 μM U0126 for 1 h and then stimulated with 50 nM PMA for 24 h. The proteins were immunoprecipitated with the anti-myocardin antibody, and myocardin phosphorylation was detected by western blot analysis using anti-phos-Ser or anti-phos-Thr antibodies. (D) H9C2 cells were co-transfected with expression plasmids for myocardin-myc and PKCα-flag, starved for 6 h, pre-treated with 100 nM Calphostin C or 10 μM U0126 for 1 h, and subsequently stimulated with 50 nM PMA for 24 h; then myocardin phosphorylation was detected. (E) COS7 cells were transfected with expression plasmids for PKCα-flag and/or myocardin-myc, followed by the Co-IP assay.

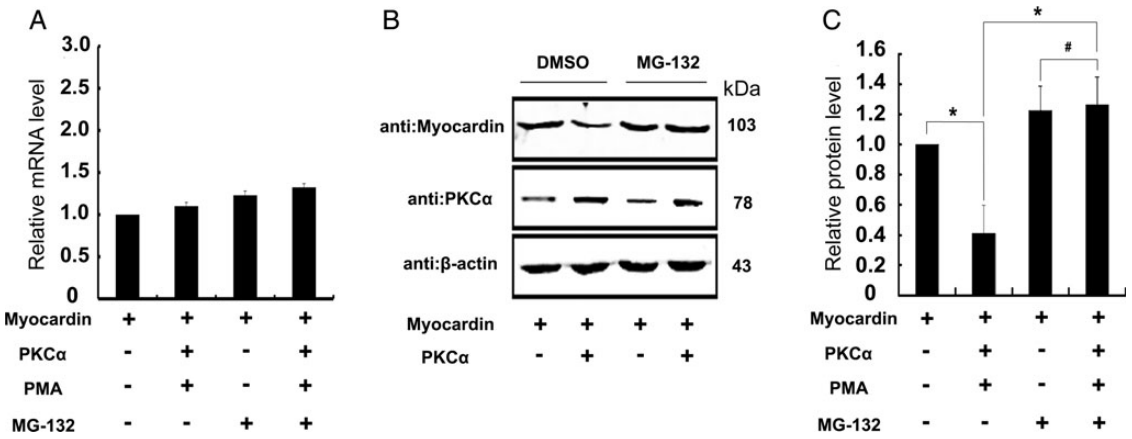

Figure 5. Phosphorylation of myocardin through PKCα promotes the degradation of myocardin (A,B) H9C2 cells were co-transfected with expression plasmids for myocardin and PKCα, and subsequently the cells were treated with a combination of 50 nM PMA and 1 μM MG-132 for 24 h. Myocardin expression was analyzed by qRT-PCR and western blot analysis. (C) The relative levels of myocardin protein were analyzed using ImageJ software, and the results were normalized to β-actin. $^{*}P < 0.05$, $^{\#}P > 0.05$, $n = 3$.

myocardin transactivity by physically interacting with it. Liao *et al.* [19] showed that p65 inhibited myocardin-mediated cardiomyocyte hypertrophy by epigenetic modifications.

The activity of myocardin is regulated through post-translational modifications such as phosphorylation, ubiquitination, etc. For example, myocardin phosphorylation inhibits its transcriptional activity.

The phosphorylation of myocardin through ERK1/2 reduces smooth muscle gene transcription [18], and the phosphorylation of myocardin through GSK-3β suppresses cardiac hypertrophy through the reduced transcriptional activation of myocardin [26]. MRTF-A is phosphorylated through ERK1/2, leading to the inhibition of MRTF-A activity [21,22]. In this study, we demonstrated that endogenous myocardin is slightly phosphorylated through PMA. PKCα might promote myocardin phosphorylation through the activation of ERK1/2 [18]. Consistent with the results of previous studies [21,22], we demonstrated that specific inhibitors of PKCα or ERK1/2 reduced the phosphorylation of endogenous myocardin (**Fig. 4A**). Similarly, co-transfection with expression plasmids for PKCα in cardiomyocytes significantly increased myocardin serine phosphorylation. This increased phosphorylation is almost completely inhibited by Calphostin C, but partially inhibited by U0126. The effect of U0126 suggested that myocardin can be phosphorylated by PKCα directly and by ERK1/2 as well. Zhang *et al.* [27] have revealed that PKCα can be transferred to the nucleus after being activated by PMA. Thus PKCα may be activated and located into the nucleus, where PKCα can directly phosphorylate myocardin. Our Co-IP assay results showed that PKCα directly interacts with myocardin. These results suggested that PKCα directly suppresses the transcriptional activity of myocardin through promoting the phosphorylation of myocardin.

Furthermore, the phosphorylation of myocardin through GSK-3β was observed, and E3 ligase C terminus of Hsc70-interacting protein (CHIP) revealed the ubiquitin-mediated degradation of myocardin in the proteasome [23]. ERK1/2 phosphorylates myocardin, but does not cause myocardin degradation [18]. The results obtained in the present study showed that PKCα phosphorylates and promotes the degradation of myocardin, suggesting that PKCα and ERK1/2 phosphorylate myocardin at different sites. All these data suggested that phosphorylation through PKCα attenuates the transcriptional activity and promotes the degradation of myocardin.

Yoshida *et al.* [28] reported the role of Kruppel-like factor 4 (KLF4) in myocardin-induced cardiomyocyte hypertrophy using KLF4 knockout mice and cell line H9C2. Overexpression of KLF4 inhibits myocardin-induced cellular enlargement by decreasing the expression and activity of myocardin in cardiac cells. In this study, our results showed that PKCα could inhibit myocardin-induced cardiomyocyte hypertrophy by directly promoting myocardin phosphorylation and degradation.

In summary, these results demonstrated that PKCα inhibits myocardin-induced cardiomyocyte hypertrophy. PKCα directly phosphorylates and promotes the degradation of myocardin, thereby decreasing its transcriptional activity. These findings provide new insight into the interaction between PKCα and myocardin in cardiac hypertrophy.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 31171303, 31171297, and 31270837) and the Program for Changjiang Scholars and Innovative Research Team at the University of Ministry of Education of China (No. IRT1166).

References

1. Nakayama H, Bodi I, Maillet M, DeSantiago J, Domeier TL, Mikoshiba K, Lorenz JN, *et al.* The IP3 receptor regulates cardiac hypertrophy in response to select stimuli. *Circ Res* 2010, 107: 659–666.
2. Callis TE, Pandya K, Seok HY, Tang RH, Tatsuguchi M, Huang ZP, Chen JF, *et al.* MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. *J Clin Invest* 2009, 119: 2772–2786.
3. Li H, He C, Feng J, Zhang Y, Tang Q, Bian Z, Bai X, *et al.* Regulator of G protein signaling 5 protects against cardiac hypertrophy and fibrosis during biomechanical stress of pressure overload. *Proc Natl Acad Sci USA* 2010, 107: 13818–13823.
4. Akazawa H, Komuro I. Roles of cardiac transcription factors in cardiac hypertrophy. *Circ Res* 2003, 92: 1079–1088.
5. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 2006, 7: 589–600.
6. Kawai-Kowase K, Owens GK. Multiple repressor pathways contribute to phenotypic switching of vascular smooth muscle cells. *Am J Physiol Cell Physiol* 2007, 292: C59–C69.
7. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* 2004, 84: 767–801.
8. Wang DZ, Olson EN. Control of smooth muscle development by the myocardin family of transcriptional coactivators. *Curr Opin Genet Dev* 2004, 14: 558–566.
9. Wang D, Chang PS, Wang Z, Sutherland L, Richardson JA, Small E, Krieg PA, *et al.* Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell* 2001, 105: 851–862.
10. Pipes GC, Creemers EE, Olson EN. The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. *Genes Dev* 2006, 20: 1545–1556.
11. Xing W, Zhang TC, Cao D, Wang Z, Antos CL, Li S, Wang Y, *et al.* Myocardin induces cardiomyocyte hypertrophy. *Circ Res* 2006, 98: 1089–1097.
12. Huang J, Min Lu M, Cheng L, Yuan LJ, Zhu X, Stout AL, Chen M, *et al.* Myocardin is required for cardiomyocyte survival and maintenance of heart function. *Proc Natl Acad Sci USA* 2009, 106: 18734–18739.
13. Rybin VO, Steinberg SF. Protein kinase C isoform expression and regulation in the developing rat heart. *Circ Res* 1994, 74: 299–309.
14. Steinberg SF. Cardiac actions of protein kinase C isoforms. *Physiology (Bethesda)* 2012, 27: 130–139.
15. Bowling N, Walsh RA, Song G, Estridge T, Sandusky GE, Fouts RL, Mintze K, *et al.* Increased protein kinase C activity and expression of Ca^{2+}-sensitive isoforms in the failing human heart. *Circulation* 1999, 99: 384–391.
16. Braz JC, Bueno OF, De Windt LJ, Molkentin JD. PKC alpha regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase1/2 (ERK1/2). *J Cell Biol* 2002, 156: 905–919.
17. Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevitsky R, Kimball TF, *et al.* PKC-alpha regulates cardiac contractility and propensity toward heart failure. *Nat Med* 2004, 10: 248–254.
18. Taurin S, Sandbo N, Yau DM, Sethakorn N, Kach J, Dulin NO. Phosphorylation of myocardin by extracellular signal-regulated kinase. *J Biol Chem* 2009, 284: 33789–33794.
19. Liao XH, Wang N, Zhao DW, Zheng DL, Zheng L, Xing WJ, Zhou H, *et al.* NF-κB (p65) negatively regulates myocardin-induced cardiomyocyte hypertrophy through multiple mechanisms. *Cell Signal* 2014, 26: 2738–2748.
20. Giordano A, Romano S, Mallardo M, D'Angelillo A, Calì G, Corcione N, Ferraro P, *et al.* Romano MF: FK506 can activate transforming growth factor-beta signalling in vascular smooth muscle cells and promote proliferation. *Cardiovasc Res* 2008, 79: 519–526.
21. Wang Z, Wang DZ, Hockemeyer D, McAnally J, Nordheim A, Olson EN. Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature* 2004, 428: 185–189.
22. Yoshida T, Gan Q, Shang Y, Owens GK. Platelet-derived growth factor-BB represses smooth muscle cell marker genes via changes in binding of MKL factors and histone deacetylases to their promoters. *Am J Physiol Cell Physiol* 2007, 292: C886–C895.
23. Xie P, Fan Y, Zhang H, Zhang Y, She M, Gu D, Patterson C, *et al.* CHIP represses myocardin-induced smooth muscle cell differentiation via ubiquitin-mediated proteasomal degradation. *Mol Cell Biol* 2009, 29: 2398–2408.

24. Vijayan K, Szotek EL, Martin JL, Samarel AM. Protein kinase C-α-induced hypertrophy of neonatal rat ventricular myocytes. *Am J Physiol Heart Circ Physiol* 2004, 287: H2777–H2789.
25. Purcell NH, Tang G, Yu C, Mercurio F, DiDonato JA, Lin A. Activation of NF-κB is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. *Proc Natl Acad Sci USA* 2001, 98: 6668–6673.
26. Badorff C, Seeger FH, Zeiher AM, Dimmeler S. Glycogen synthase kinase 3β inhibits myocardin-dependent transcription and hypertrophy induction through site-specific phosphorylation. *Circ Res* 2005, 97: 645–654.
27. Zhang Y, Matkovich SJ, Duan X, Diwan A, Kang MY, Dorn GW II. Receptor-independent protein kinase Cα (PKCα) signaling by calpain-generated free catalytic domains induces HDAC5 nuclear export and regulates cardiac transcription. *J Biol Chem* 2011, 286: 26943–26951.
28. Yoshida T, Yamashita M, Horimai C, Hayashi M. Kruppel-like factor 4 protein regulates isoproterenol-induced cardiac hypertrophy by modulating myocardin expression and activity. *J Biol Chem* 2014, 289: 26107–26118.