

## Original Article

# Cardiomyocyte specific deletion of PP2A causes cardiac hypertrophy

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Received March 8, 2016; Accepted April 12, 2016; Epub April 15, 2016; Published April 30, 2016

**Abstract:** Cardiac hypertrophy is a common pathological alteration in heart disease, which has been reported to be connected with serine/threonine protein phosphatases that control the dephosphorylation of a variety of cardiac proteins. Herein, we generated protein phosphatase type 2A knockout expressing a tamoxifen-inducible Cre recombinase protein fused to two mutant estrogen-receptor ligand-binding domains (MerCreMer) under the control of the  $\alpha$ -myosin heavy chain promoter. Cardiac function of mice was determined by echocardiography. Decrease in PP2A activity leads to increased cardiomyocyte hypertrophy and fibrosis. Loss of PP2A $\alpha$  leads to the heart failure, including the changes of EF, FS, LV, ANP and BNP. On the molecular level, knockout mice shows increased expression of B55a and B56e at 60 days after tamoxifen injection. Additionally, the regulation of the Akt/GSK3 $\beta$ / $\beta$ -catenin pathway is severely disturbed in knockout mice. In conclusion, cardiomyocyte specific deletion of PP2A gene causes the cardiac hypertrophy. We will use the knockout mice to generate a type of cardiomyocyte hypertrophy mouse model with myocardial fibrosis.

**Keywords:** PP2A, hypertrophy, knockout mouse model

## Introduction

In the pathology of Cardiac hypertrophy, most cardiac proteins have a dynamic balance between their phosphorylation state and dephosphorylation state through the action of multiple kinases and phosphatases. These kinases and phosphatases control multiple physiological processes and play important roles in cardiac pathophysiology. Protein phosphatase type 2A (PP2A), which constitutes a large family of serine/threonine protein phosphatases, controls the dephosphorylation of a variety of cardiac proteins in cardiac pathophysiology, thereby fine-tuning cardiac electrophysiology and function [1]. PP2A Heterotrimeric PP2A holoenzymes are constituted with a catalytic C subunit, a structural A subunit, and a regulatory B-type subunit [2, 3], which accounts for greater than 90% of protein dephosphorylation activity in the heart together with PP1 [4]. The A structure subunit recruits the C catalytic

subunit to form the core dimer, which acts as a scaffold for B subunits of the enzyme [5]. PP2A activity are due to the tyrosine or serine/threonine phosphorylation of not only the catalytic C subunit, but also the regulatory B subunit, especially those of the B' family [6, 7]. The regulatory B subunits play key roles in controlling PP2A substrate specificity, cellular localization, and enzymatic activity [8]. Four B subunit families have been identified (PR55 or B, PR61 or B', PR72/130 or B'' and PR93/PR110 or B'''). Each family of B subunits contains several isoforms that can bind the AC dimer in a mutually exclusive manner [9].

Total myocardial serine/threonine protein phosphatase activity is mainly the result of both PP1 and PP2A activity [10]. PP2A is a Ser/Thr phosphatase that exists across many eukaryotic cell types. Transgenic mouse model overexpressing PP2A $\alpha$  under control of the  $\alpha$ -myosin heavy chain promoter show necrotic isles within the

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myocardium, cardiac hypertrophy, and left ventricular fibrosis [11]. PP2A overexpressing mice develop dilated cardiomyopathy which is associated with the disruption of the signaling cascade of Akt/GSK3 $\beta$ / $\beta$ -catenin [12]. However, PP2A $\alpha$  deficiency in mice resulted in embryonic lethality by embryonic day 6.5 [13].

In this study, the Cre/loxP system is used to achieve cardiac-specific PP2A $\alpha$  exon 2 knockouts in adult mice. In the Myh6-MerCreMer (MCM) model, the Cre fusion protein is expressed and controlled by the human  $\alpha$ -myosin heavy chain gene promoter [14]. As determined by the palindromic core sequence of the loxP site, an important coding exon 2 is flanked (floxed) by loxP sites ppp2ca<sup>fllox/fllox</sup> mice that are in the same orientation and thus inactivate PP2A $\alpha$ . While Cre recombinase is expressed, the region between the two loxP sites is deleted and the PP2A $\alpha$  exon2 gene is temporally inactivated.

Cardiac-specific induction of Cre recombinase in Myh6-MerCreMer mice, employing a widely used protocol (5 days of injection of TAM at a dose of 40 mg/kg), results in severe, transient systolic dysfunction associated with depletion of cardiac mitochondrial ATP content [15]. The PP2A $\alpha$  knockout mice were treated with tamoxifen by intraperitoneal injection once a day for 3 days at a dosage of 20 mg/kg per day. Ppp2ca<sup>fllox/+</sup>; Myh6-MerCreMer mice were used as control group to avoid any potential nonspecific effects of Cre activation in the heart. In knockout mice heart, B55a and B56e express increased with significant difference compared with the control group. The regulation of the Akt/GSK3 $\beta$ / $\beta$ -catenin pathway is severely disturbed in knockout mice hearts.

These types of knockout mice have experience cardiac hypertrophy and myocardial fibrosis as adults; therefore, we generated a unique mouse model to study the role of PP2A $\alpha$  in cardiac hypertrophy. Moreover, this mouse model can be used to further research of PP2A as a potential therapeutic target for cardiac hypertrophy.

### Materials and methods

#### Animals

All animal protocols were approved by the Animal Care and Use Committee of the Model

Animal Research Center of Nanjing Medical University. This study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (Number: NAJMU-IACRCUC-20100601001). All mice were fed a standard diet and housed in specific pathogen-free barrier facilities. To induce Cre recombination, cardiac specific knockout mice as well as wild control mice at 3 month, were treated with tamoxifen (Sigma, USA) by intraperitoneal injection once a day for 3 days at a dosage of 20 mg/kg per day. Mice were killed two months after the end of tamoxifen administration.

#### Generation of knockout and control group mice

Genomic DNA was extracted from a 2 mm tail clip using the PBDN extraction method ([http://www.jax.org/imr/tail\\_nonorg.html](http://www.jax.org/imr/tail_nonorg.html)). In brief, mouse tails were digested with 40 mg Proteinase K (Invitrogen) in 200 ml PBDN buffer at 55°C for 16 h. After heating at 96°C for 10 min to inactivate Proteinase K, and the tail digest was used as template DNA for PCR analysis. To detect expression of the CD11b-cre transgene, the following primers were used: CreF (5'-CGTACTGACGGTGGGAGAAT-3') and CreR (5'-TGCATGATCTCCGGTAT TGA-3'). Flox was detected by ppp2ca-loxp1-F1 (5'-TACTTC-AGTGTTACT GACACAGACTC-3') And ppp2ca-loxp1-R1 (5'-GTGAAAACAGGTCATTCC CAT-3'). PCR reactions were performed in PCR buffer (25 ml) containing 0.2 mM dNTP (Promega), forward and reverse primer (0.2 mM each), template DNA (1 ml), DMSO (5%; v/v, Merck), MgCl<sub>2</sub> (4 mM), and 0.25 ml Ampli Taq Gold TM (Roche). Amplification was performed as follows: initial denaturation, 94°C for 5 min; 35 PCR cycles, 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; and final extension, 72°C for 10 min. Amplification of the Myh6-MerCreMer transgene generated a 374 bp DNA fragment.

#### RT-PCR and quantitative real-time PCR analyses

Total RNA were isolated from mice heart and cardiomyocytes were dissolving in Trizol reagent (Invitrogen, USA). RT-PCR was performed using PrimeScript<sup>TM</sup> (TAKATA, Japan). The cDNA of PP2A $\alpha$  was detected by primers Mpp2ca-P5F and Mpp2ca-P5R. The PCR products were 623-bp (control) and 423-bp (knockout). Expression

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**Table 1.** Sequences of the specific primers used for PCR

Genes	Primers	Sequences
Mpp2ca-P5	Forwards	5'-GGTCAAGAGCCTCTGCGAGAA-3'
	Reverse	5'-CCGGTCATGGCACCAGTTAT-3'
$\beta$ -actin	Forwards	5'-GAGAAGATCTGGCACCACACC-3'
	Reverse	5'-GCATACAGGGACAGCACAGC-3'
PP2A-C $\alpha$	Forwards	5'-ATGGACGAGAAGTTGTTACC-3'
	Reverse	5'-CAGTGACTGGACATCGAACCT-3'
PP2A-C $\beta$	Forwards	5'-GAGGGTACTACTCTGTGGAGAC-3'
	Reverse	5'-CCGGCTTTCGTGATTCTCT-3'
PP2A-B55 $\alpha$	Forwards	5'-AGGAGGGAATGATATTCAGTGGT-3'
	Reverse	5'-CCTGTGGCTAGTAATTCTCCAGA-3'
PP2A-B55 $\beta$	Forwards	5'-TGCCTTATATCTTCAGACCTCCA-3'
	Reverse	5'-AATGTCAGCTTCAGTATGGCAG-3'
PP2A-B55 $\delta$	Forwards	5'-GGAAGCCGACATCATCTCCAC-3'
	Reverse	5'-GTGAGCGCGCCTTTATTCT-3'
PP2A-B56 $\alpha$	Forwards	5'-ATTGAAGAGCCGCTTTTAAAGCA-3'
	Reverse	5'-TGAGGGTTTCAGCACATTGT-3'
PP2A-B56 $\beta$	Forwards	5'-GGGCCTACATCCGCAAACA-3'
	Reverse	5'-GGATCAGGACTCGAACCCAGG-3'
PP2A-B56 $\gamma$	Forwards	5'-CCCCTGGTCTCTCCATATT-3'
	Reverse	5'-GCAACACTGACGTAACCTCTGG-3'
PP2A-B56 $\gamma$ 2	Forwards	5'-TTCTCCACATTCGAGATGTTCTCT-3'
	Reverse	5'-GCGCTTACTTCTCTCCACTT-3'
PP2A-B56 $\delta$	Forwards	5'-CCCAGTCTCAGTCACCATCAT-3'
	Reverse	5'-GCGTCGTTCTTCTTGACAATC-3'
PP2A-B56 $\epsilon$	Forwards	5'-GACGGATTTCTCGGAAGTCC-3'
	Reverse	5'-GAGGTTGGAACGTCTTTCAGC-3'
ANF	Forwards	5'-AGGAGAAGATGCCGGTAGAAGA-3'
	Reverse	5'-GCTTCTCAGTCTGCTCACTCA-3'
BNP	Forwards	5'-AAGCTGCTGGAGCTGATAAGA-3'
	Reverse	5'-GTTACAGCCCAAACGACTGAC-3'
$\alpha$ -MHC	Forwards	5'-CCAATTCTCCTTGTCCTACTATG-3'
	Reverse	5'-ACAAACCCACCACCGTCTCA-3'
$\beta$ -MHC	Forwards	5'-CCTCCTCACATCTTCTCCATCTCT-3'
	Reverse	5'-CTCCGGATTCTCCGGTGAT-3'

levels of mRNA of ANP (atrial natriuretic peptide), BNP (Brain natriuretic peptide)  $\alpha$ -MHC ( $\alpha$ -myosin heavy chain) and  $\beta$ -MHC ( $\beta$ -myosin heavy chain) were determined using quantitative real-time PCR and  $\beta$ -actin was used for normalization. The sequences of the specific primers used for PCR are listed in **Table 1**.

### Protein phosphatase assay

Protein phosphatase 2A activity was measured in whole heart homogenate with Serine/Threo-

nine Phosphatase Assay System (Product number: V2460). The non-radioactive Serine/Threonine Phosphatase Assay System provides a fast, convenient and flexible method for measuring protein serine/threonine phosphatase activity. This system determines the amount of free phosphate generated in a reaction by measuring the absorbance of a molybdate: malachite green: phosphate complex. The system allows the use of a variety of buffer conditions and substrates, including naturally phosphorylated proteins or synthetic phosphopeptides.

### Western blot

Protein extraction was quantified according to standard procedures, and proteins underwent electrophoretic separation (20  $\mu$ g protein/lane) and electroblotting onto PVDF membranes. Hearts were homogenized in hypotonic solution. A mix of protease inhibitors (Roche) were also added to the buffer. For the present studies, the following primary antibodies were used: anti-PP2AC, GAPDH, AKT, P-AKT(S473), P-AKT (T308), phospho-GSK3 $\beta$  (ser9),  $\beta$ -catenin, P- $\beta$ -catenin (Ser552) (Cell signaling).

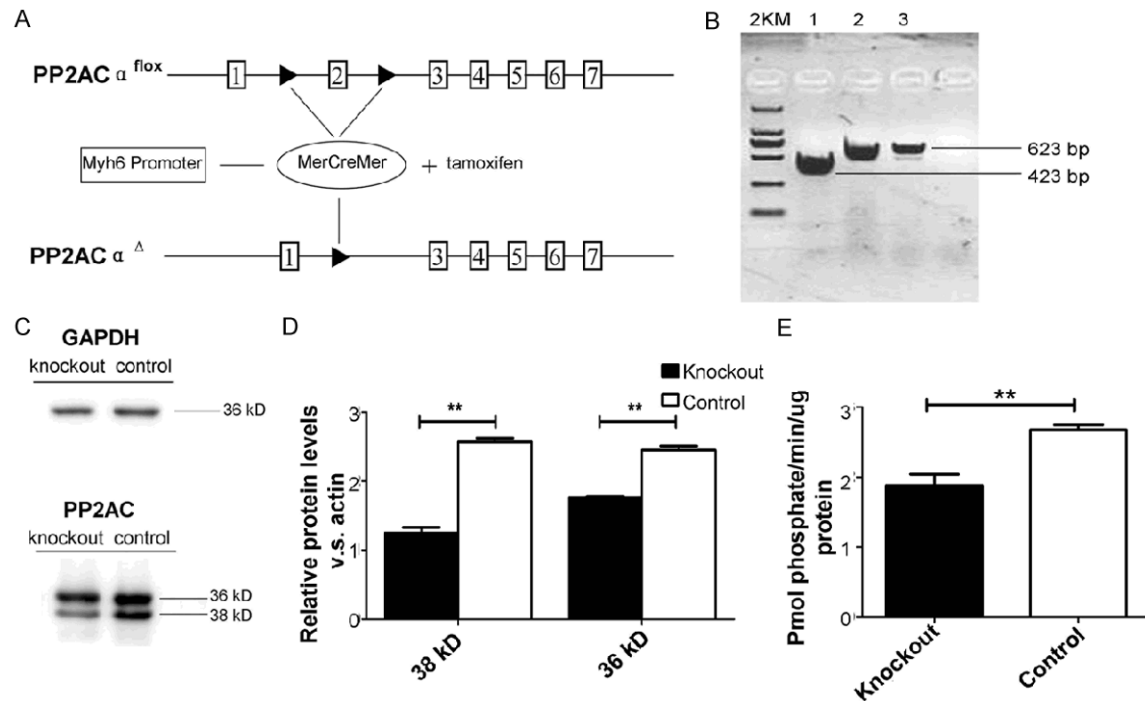
### Echocardiography

Adult animals were lightly anesthetized before undergoing echocardiography or hemodynamic analysis. Echocardiography of neonates was performed while conscious. Mice were anesthetized with tribromoethanol (0.18 mL/10 g of body weight) and echocardiographic images were obtained using a VisualSonics Vevo 2100 system (Canada). M-mode echocardiography of the left ventricle was recorded at the tip of the mitral valve apparatus using a 30-MHz transducer (Vevo 2100, Canada).

### Tissue preparation and histological analyses

Hearts were excised and washed in cold PBS carefully, then freed of connective tissue. Each heart was weighted and heart weight was normalized by body weight (HW/BW ratio). For collagen quantification, paraffin sections (6  $\mu$ m) of myocardial sections were also stained with Masson's trichrome. Normal cardiac myocytes

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**Figure 1.** Construction and identification of PP2A $\alpha$  knockout mice. A. The recombination process and the loxP sites used for PP2A $\alpha$  knockout. B. Prim PP2A-P5F and PP2A-P5R were used to detect PP2A $\alpha$  cDNA. The solid line represents detecting PP2A $\alpha$  cDNA was 200 bp shorter than the control. Quantitative real-time PCR assay was carried out to examine the expression levels of PP2A $\alpha$  gene. C. Western blot demonstrates PP2AC protein levels (36 and 38 kDa) of mice heart protein extracts from the knockout group and the control group 10 days after tamoxifen injection. D. Statistical analysis for the western blot. E. Phosphatase activity was elevated both in knockout and control groups.  $n \geq 6$  in each group, \* $P < 0.05$ , \*\* $P < 0.01$ .

were stained dark red, and fibrotic areas were stained blue.

### Statistical analysis

Significance was determined using an unpaired two-way Student's *t*-test (GraphPad Prism 5). Statistical comparisons between two different groups were performed with unpaired student's *t*-test, ANOVA followed by post-hoc testing (LSD) was used for all comparisons between multiple groups. \* $P < 0.05$ , \*\* $P < 0.01$  was considered significant.

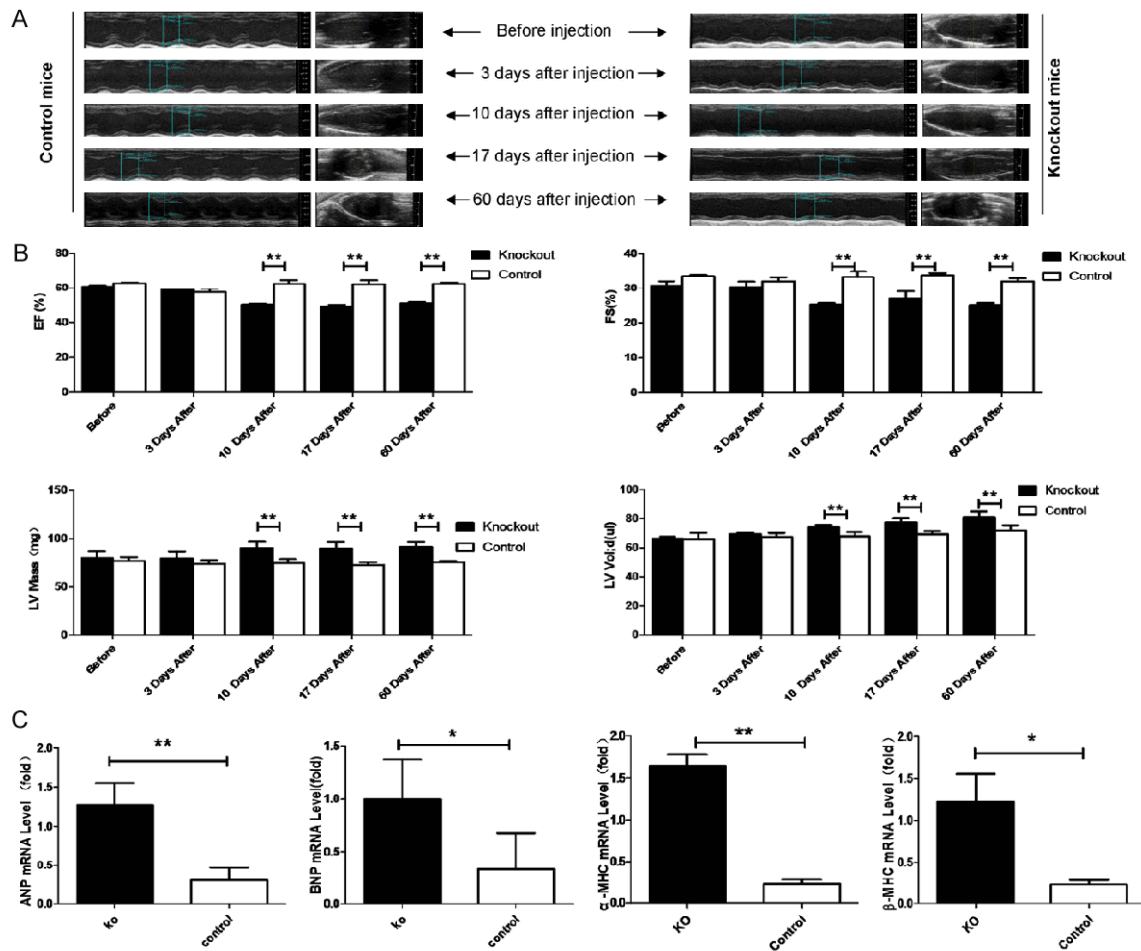
### Result

#### Induced PP2A $\alpha$ knockout in the working myocardium of adult hearts

A tamoxifen (Tam)-inducible, myocardial cell-specific PP2A $\alpha$  (exon 2, 200 bp) knockout mouse model was used to investigate the role of PP2A in myocardial fibrosis introduced heart failure. PP2A $\alpha$  knockout mice were obtained by crossing homozygous PP2A $\alpha^{\text{lox/lox}}$  mice carrying the loxP sites across exon 2 with heterozy-

gous Myh6-MerCreMer (MCM) mice carrying the Cre recombinase sequence downstream of the cardiac-specific  $\alpha$ -myosin heavy chain promoter (**Figure 1A**). In our study, knockout and control groups consisted of PP2A $\alpha^{\text{lox/+}}$ , Myh6-MerCreMer transgenic mice treated with tamoxifen at 3 month (**Figure 1B**). Total RNA were isolated from mice's hearts and the mutant form of PP2A $\alpha$  knockout mRNA in these mice was verified by RT-PCR, which was 200 bp shorter than control wild type, PP2A $\alpha$  gene level was decreased in knockout mice by quantitative real-time PCR examine (**Figure 1C**). To examine PP2A $\alpha$  at the protein level, western blot was performed to detect the catalytic subunits of PP2A. **Figure 1D** shows that the protein levels of PP2AC decrease significantly in knockout mice. Phosphatase activity was found elevated in knockout in comparison to control controls (**Figure 1E**), PP2A activity was reduced significantly. These results indicate that PP2A activity impaired knockout mice were constructed by breeding the PP2A $\alpha^{\text{lox/lox}}$  mice with Myh6-MerCreMer mice.

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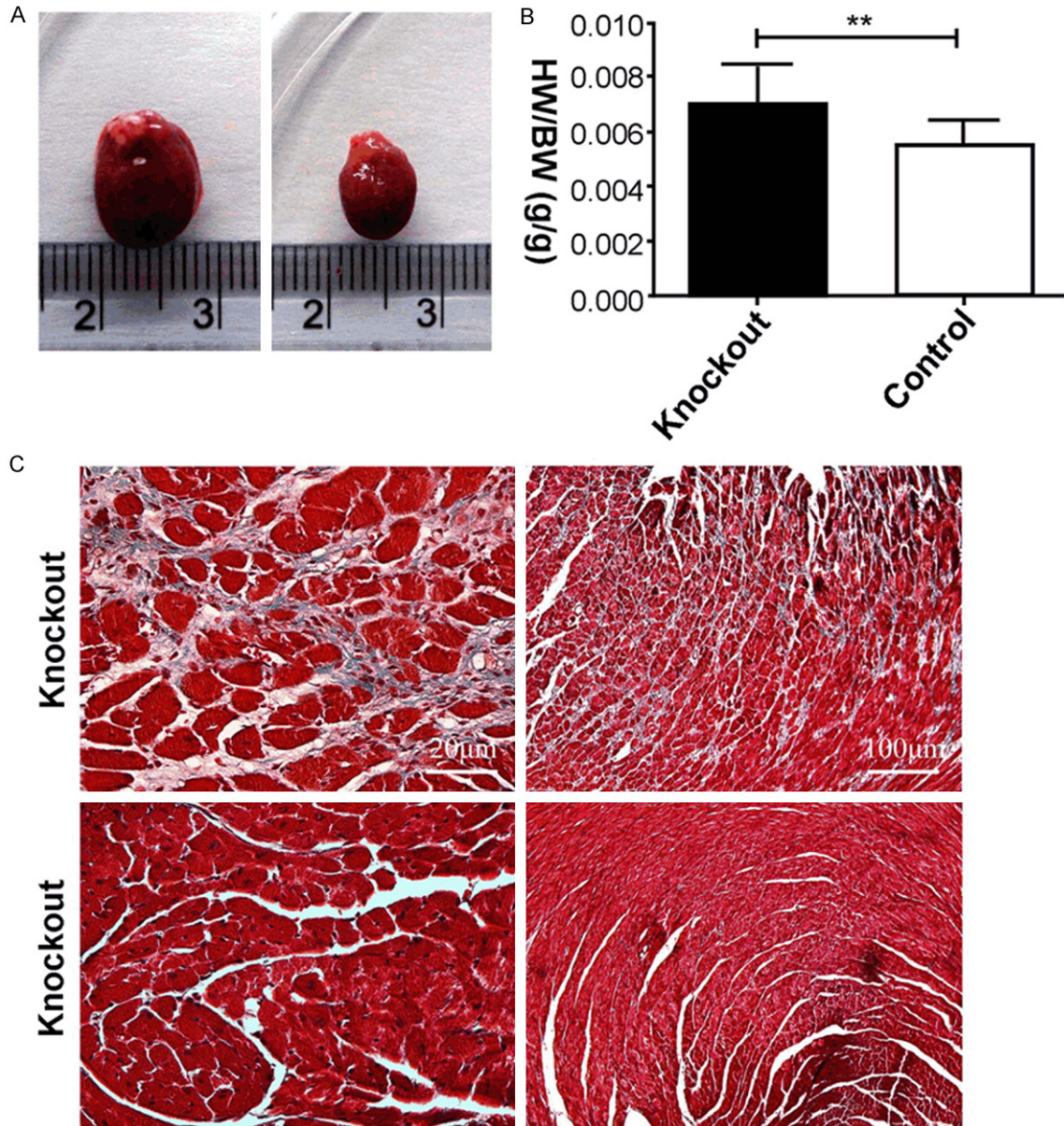
**Figure 2.** Echocardiographic assessment of knockout and control wild type mice. A. Echocardiography was performed before tamoxifen injection, 3 days after injection, 10 days after injection, 17 days after injection, 60 days after injection. B. Ejection fraction (EF%), fraction shortening (FS%), left ventricular mass (LV Mass [mg]), left ventricular end-diastolic volumes (LV vol [ $\mu$ l]) were calculated. C. Quantitative real-time PCR assay was carried out to examine the expression levels of fetal genes including A. ANF, B. BNP, and C.  $\beta$ MHC.  $n \geq 6$  in each group, \* $P < 0.05$ , \*\* $P < 0.01$ .

### Loss of PP2A $\alpha$ leads to heart failure

We next investigated the cardiac phenotype of both knockout and control group mice at 3 month. Echocardiography was used to evaluate cardiac chamber size and function in vivo among knockout and control mice before tamoxifen injection, 3, 10, 17 and 60 days after injection. The wall motion in knockout hearts was drastically attenuated and the blood flow in the left ventricular chamber was not properly maintained start at about 10 days after injection (**Figure 2A**). Calculated ejection fraction (EF%), fractional shortening (FS%) and a measure of systolic function indicated that the cardiac function in PP2A $\alpha$  knockout mice was impaired gradually after tamoxifen injection

and eventually went into a decompensation stage also start at about 10 days after injection. We found that left ventricular mass (LV Mass) and left ventricular end-diastolic volumes (LV Vol), important descriptors of cardiac status, in knockout mice were larger than in control mice since 10 days after injection (**Figure 2B**). Cardiac hypertrophy is frequently associated with gene re-expression during fetal and perinatal development, and with the upregulation of some cardiac proteins, such as ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide),  $\alpha$ -MHC ( $\alpha$ -myosin heavy chain) and  $\beta$ -MHC ( $\beta$ -myosin heavy chain). Quantitative real-time PCR of myocardial cell RNA showed that these genes were significantly upregulated in the knockout mice hearts compared with

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**Figure 3.** Morphological features of PP2A $\alpha$  knockout mice at 60 days after injection. **A.** Overall appearance of knockout and control mice. **B.** Heart to body ratio (H/B) of knockout and control mice. **C.** Myocardial fibrosis induced by PP2A $\alpha$  knockout and evaluated by Masson trichrome staining; indicative heart section from knockout mice and the control group;  $n \geq 6$  in each group,  $*P < 0.05$ ,  $**P < 0.01$ .

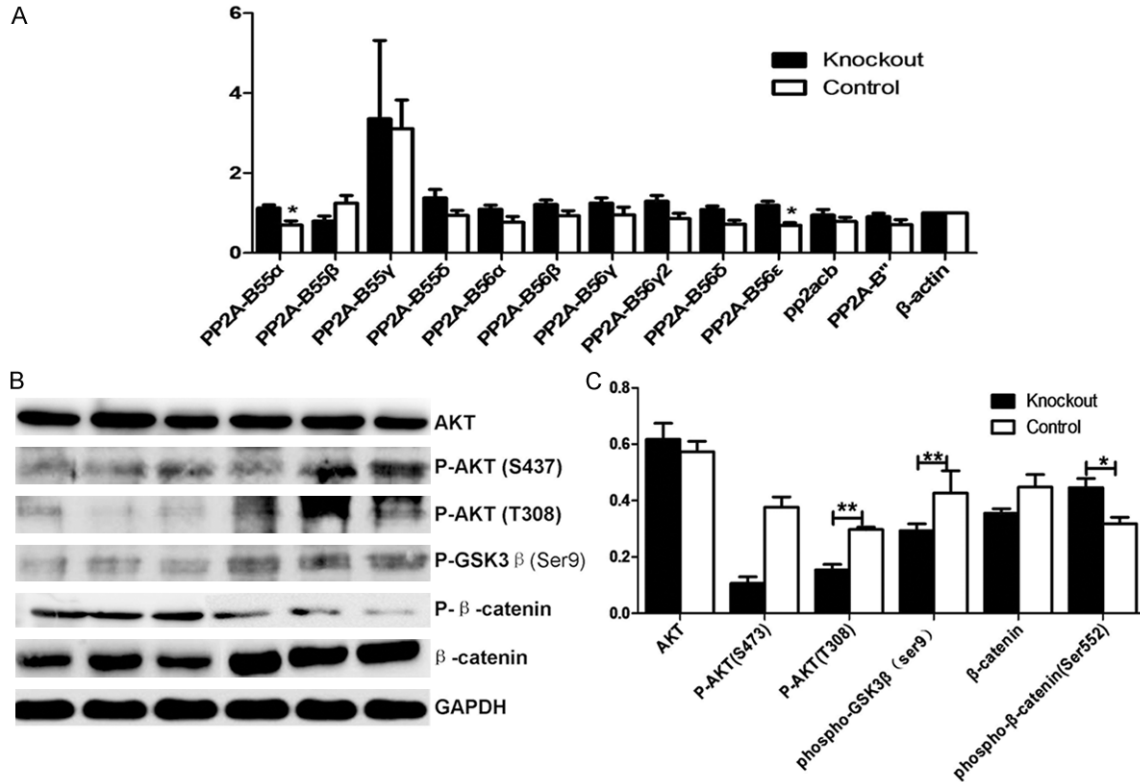
controls at 60 days after injection. Hypertrophy markers were highly expressed in knockout heart (Figure 2C).

### *Morphological features of PP2A $\alpha$ knockout mice*

Mice were killed at 60 days after tamoxifen injection. Knockout mouse heart volume increased significantly compared with control

mice (Figure 3A). Compared with knockout and control mice, heart to body ratio (H/B) of both mice (Figure 3B) was in accordance with LV Mass and LV vol (Figure 2B). Figure 3C shows Masson's trichrome staining of a representative heart tissue section from a mouse. The amount of collagen fibrils in knockout mice was significantly higher compared to control mice (Figure 3C). Pathologic cardiac remodeling of myocardial fibers in knockout mice is caused by

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**Figure 4.** PP2A B subunits changed and Akt/GSK3 $\beta$ / $\beta$ -catenin pathway disrupted. A. Quantitative real-time PCR assay was carried out to examine the expression levels of PP2ACb genes. B. Western blot of AKT, P-AKT (S473), P-AKT (T308), phospho-GSK3 $\beta$  (Ser9),  $\beta$ -catenin, P- $\beta$ -catenin (Ser552) from mice heart protein extracts from the knockout group and the control group 10 days after tamoxifen injection. C. Statistical analysis for the western blot.  $n \geq 6$  in each group, \* $P < 0.05$ , \*\* $P < 0.01$ .

compensatory response to long-term cardiac function defecting.

*PP2A activity decreased leads to B subunits changed and Akt/GSK3 $\beta$ / $\beta$ -catenin pathway disrupted*

PP2A protein B subunits expression were detected from adult mice hearts. RNA extracted from mice heart, was reverse transcribed into cDNA. PP2A-B subunits expression were tested by qPCR with  $\beta$ -actin as a reference. The results showed in knockout mice heart, B55a and B56e express increased with significant difference compared with the control group. B55b, B56a, B56b, B56c1, B56c2, B56d and B56e, also express higher, while the expression of B55b decreased (**Figure 4A**).

The B55a subunit directly binds to the  $\beta$ -catenin destruction complex, where PP2A dephosphorylates  $\beta$ -catenin and induces its degradation [16, 17]. Hence, we tried to explore the involve-

ment of members of the Wnt signaling pathway in our infarction model (**Figure 4B**). While we found no differences regarding the total amount of Akt between the different groups, there was a significant decrease of Akt-phosphorylation in knockout mice (**Figure 4C**). GSK-3 $\beta$ , the  $\beta$ -catenin negative regulator, is activated both directly and through AKT inhibition by PP2A. In knockout mice, no significant difference of phosphorylated GSK3 $\beta$  was detected. Controversially, we found significantly lower concentrations of  $\beta$ -catenin in PP2A knockout mice with an increase of  $\beta$ -catenin phosphorylated on Ser552. These findings indicate that the otherwise close regulation of  $\beta$ -catenin by Akt and GSK3 $\beta$  is lost under lower activity of PP2A.

### Discussion

PP2A is ubiquitously expressed in almost every tissue, is globular in structure. The present study highlights the importance of PP2A for proper cardiac function in adult mice. Protein

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phosphatases contribute to cardiac structural remodeling, most notably to hypertrophy. Former research of heart-specific PP2A overexpression using a mutant PP2A scaffolding subunit mouse model showed that it was unable to bind regulatory subunits, and the mice then experienced cardiac hypertrophy, dilatation, and contractile dysfunction [18-20]. L-type  $\text{Ca}^{2+}$  currents decreased in this model, but arrhythmogenesis was not assessed. Other effects of PP2A modulation on cardiac disease have been less extensively characterized [21, 22]. For instance, expression of casein kinase-2 interacting protein-1 (CKIP-1), a modulator of PP2A and PP1, can reduce PP2A-mediated dephosphorylation of histone deacetylase type-4, and then promote cardiac hypertrophy and fibrosis in mice [23]. Reduced PP2A activity *in vivo* may contribute to heart failure-related structural remodeling, but the precise roles of PP1/PP2A in atrial hypertrophy and fibrosis remain largely unknown [24].

We chose to induce Cre recombinase by tamoxifen at 3 month in knockout mice to avoid mice dying during the postnatal period [25]. The generation of mice with cardiac PP2A activity decreased was initially driven by the intention to further understand the causal relationship between increased phosphatase expression and the development of heart failure. PP2A transgenic mice are characterized by an impaired cardiac pump function indicating that the development of dilated cardiomyopathy. Next, we measured calculated EF%, FS%, left ventricular mass (LV Mass) and left ventricular end-diastolic volumes (LV Vol) in mice using M-mode echocardiography before tamoxifen injection, 3 days after injection, 10 days after injection, 17 days after injection, 60 days after injection. The cardiomyocyte-specific PP2A $\alpha$  knockout induced cardiac hypertrophy since 10 days after injection, as indicated by the significant increase in absolute H/B weight ratio at 60 days after injection. Myocardial hypertrophy in this model was characterized by increased size of cardiomyocytes and expression of ANP, BNP,  $\alpha$ -MHC and  $\beta$ -MHC genes [26].

Quantitative real-time PCR of myocardial cell RNA showed that these genes were significantly upregulated in the knockout mice hearts compared with controls at 60 days after injection. Hypertrophy markers were highly expres-

sed in knockout heart. In the heart, PP1, PP2A and calcineurin make up almost 90% of protein phosphatase activity [24, 27]. PP2A $\alpha$  knockout mice experienced cardiac hypertrophy and myocardial fibrosis as adults, thus heart dysfunction observed in our mouse model was caused by decreased PP2A activity. We used Masson's trichrome staining to test the presence of collagen fibrils inside the myocardium and particularly between the capillaries and the myocardium cell. Induced hypertrophy, may be the cause of the perivascular myocardial fibrosis that we noted.

Different B subunits interact via the same or overlapping sites within the A subunit of the core dimer. The association of these B subunits with the core AC dimer is mutually exclusive [28-30]. The PP2A holoenzyme's substrate specificity, enzymatic activity, and/or cellular localization can be modulated by the B regulatory subunit [31].

PP2A-B subunits expression were tested by qPCR with  $\beta$ -actin as a reference. The results showed in knockout mice heart, B55a and B56e express increased with significant difference compared with the control group. B55b, B56a, B56b, B56c1, B56c2, B56d, B56e, B''also express higher without significant, while the expression of B55b decreased. Each family of B subunits contains several isoforms that can bind the AC dimer in a mutually exclusive manner [32, 33]. Overexpression of B56 family members inhibits secondary axis induced by Wnt and impairs endogenous axis specification during *Xenopus* embryonic development. Based on epistasis analysis, B56s were placed downstream of Casein Kinase I, GSK3, and Axin, but upstream of  $\beta$ -catenin in the Wnt pathway. Axin interacts with B56-containing PP2As [34, 35]. The B55a subunit directly binds to the  $\beta$ -catenin destruction complex, where PP2A dephosphorylates  $\beta$ -catenin and induces its degradation [36].

Total amount of Akt were at the same level of the PP2A knockout mice and the control. But phosphorylated Akt and phosphorylated GSK3 $\beta$  decreased, and  $\beta$ -catenin was hyperphosphorylated and the total amount of  $\beta$ -catenin was reduced in PP2A knockout mice. Wnt signaling, by inhibiting GSK3 $\beta$  mediated phosphorylation and cytoplasmic  $\beta$ -catenin degradation to introduce EMT (Epithelial-Mesenchy-



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mal Transition), has a fundamental role in cardiac remodeling and is unquestionable dependent on  $\beta$ -catenin balance [37]. Former research suggested a link between Wnt signaling and PP2A activity.  $\beta$ -catenin stability and activity are controlled at various phosphorylation sites: Phosphorylation at Ser45 through casein kinase I promotes  $\beta$ -catenin degradation [38]. Phosphorylation at Ser33/Ser37/Thr41 through GSK3 $\beta$  also promotes degradation of  $\beta$ -catenin [39]. Phosphorylation of  $\beta$ -catenin at Ser552 through protein kinase A, Akt or cAMP dependent protein kinase does not alter  $\beta$ -catenin degradation but promotes transcriptional activity [40]. GSK3 $\beta$  phosphorylation in knockout mice decreased while constitutive-active cardiac GSK3 $\beta$  is protected from adverse remodeling [41]. These findings may be related to the disruption of  $\beta$ -catenin/GSK3 $\beta$  balance and the downstream effectors of Wnt signaling.

During the postnatal stage, PP2A $\alpha$  ablation in mouse hearts causes premature death via heart failure; mice died on postnatal day 13 [42]. One major advantage of our PP2A $\alpha$  knockout mouse model is that the activity of the Cre recombinase can be controlled in a temporal fashion by tamoxifen administration. The adult knockout mice were observed in vivo echocardiography with significantly reduced cardiac function. Moreover, loss of PP2A $\alpha$  results in cardiac hypertrophy. We found a B55a and B56e express increased in knockout mice. While there were no differences regarding the total amount of Akt between the two different groups, and phosphorylated Akt and phosphorylated GSK3 $\beta$  decreased. It turned out that  $\beta$ -catenin was hyperphosphorylated and the total amount of  $\beta$ -catenin was reduced in PP2A knockout mice. Our PP2A $\alpha$  knockout mouse model can be used for further research investigating clinical targeting of phosphatases to treat cardiovascular diseases and related arrhythmias.

### Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 31171443).

### Disclosure of conflict of interest

None.

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### References

- [1] Ling S, Sun Q, Li Y, Zhang L, Zhang P, Wang X, Tian C, Li Q, Song J, Liu H, Kan G, Cao H, Huang Z, Nie J, Bai Y, Chen S, Li Y, He F, Zhang L, and Li Y. CKIP-1 inhibits cardiac hypertrophy by regulating class II histone deacetylase phosphorylation through recruiting PP2A. *Circulation* 2012; 126: 3028-3040.
- [2] Gu P, Qi X, Zhou Y, Wang Y, Gao X. Generation of Ppp2Ca and Ppp2Cb conditional null alleles in mouse. *Genesis* 2012; 50: 429-436.
- [3] Pan X, Chen X, Tong X, Tang C, Li J. Ppp2ca knockout in mice spermatogenesis. *Reproduction* 2015; 149: 385-391.
- [4] Luss H, Klein-Wiele O, Boknik P, Herzig S, Knapp J, Linck B, Muller FU, Scheld HH, Schmid C, Schmitz W, Neumann J. Regional expression of protein phosphatase type 1 and 2A catalytic subunit isoforms in the human heart. *J Mol Cell Cardiol* 2000; 32: 2349-2359.
- [5] Janssens V, Longin S, Goris J. PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail). *Trends Biochem Sci* 2008; 33: 1131-1121.
- [6] Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* 2001; 353: 417-439.
- [7] Janssens V, Derua R, Zwaenepoel K, Waelkens E, Goris J. Specific regulation of protein phosphatase 2A PR72/B" subunits by calpain. *Biochem Biophys Res Commun* 2009; 386: 676-681.
- [8] Cho US, Morrone S, Sablina AA, Arroyo JD, Hahn WC, Xu W. Structural basis of PP2A inhibition by small t antigen. *PLoS Biol* 2007; 5: e202.
- [9] Lechward K, Awotunde OS, Swiatek W, Muszynska G. Protein phosphatase 2A: variety of forms and diversity of functions. *Acta Biochim Pol* 2001; 48: 921-933.
- [10] Herzig S, Neumann J. Effects of serine/threonine protein phosphatases on ion channels in excitable membranes. *Physiol Rev* 2000; 80: 173-210.

## PP2A knockout causes cardiac hypertrophy

- [11] Carr AN, Schmidt AG, Suzuki Y, del Monte F, Sato Y, Lanner C, Breeden K, Jing SL, Allen PB, Greengard P, Yatani A, Hoit BD, Grupp IL, Hajjar RJ, DePaoli-Roach AA, Kranias EG. Type 1 phosphatase, a negative regulator of cardiac function. *Mol Cell Biol* 2002; 22: 4124-4135.
- [12] Hoehn M, Zhang Y, Xu J, Gergs U, Boknik P, Werdan K, Neumann J, Ebel H. Overexpression of protein phosphatase 2A in a murine model of chronic myocardial infarction leads to increased adverse remodeling but restores the regulation of  $\beta$ -catenin by glycogen synthase kinase 3 $\beta$ . *Int J Cardiol* 2015; 183: 39-46.
- [13] Dong D, Li L, Gu P, Jin T, Wen M, Yuan C, Gao X, Liu C, Zhang Z. Profiling metabolic remodeling in PP2A deficiency and chronic pressure overload mouse hearts. *FEBS Lett* 2015; 589: 3631-3639.
- [14] Hall ME, Smith G, Hall JE, Stec DE. Systolic dysfunction in cardiac-specific ligand-inducible MerCreMer transgenic mice. *Am J Physiol Heart Circ Physiol* 2011; 301: H253-260.
- [15] Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* 2011; 353: 417-439.
- [16] Lin X, Xu X. Distinct functions of Wnt/ $\beta$ -catenin signaling in KV development and cardiac asymmetry. *Development* 2009; 136: 207-217.
- [17] Logan CY, Nusse R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 2004; 20: 781-810.
- [18] Haq S, Michael A, Andreucci M, Bhattacharya K, Dotto P, Walters B, Woodgett J, Kilter H, Force T. Stabilization of  $\beta$ -catenin by a Wnt-independent mechanism regulates cardiomyocyte growth. *Proc Natl Acad Sci U S A* 2003; 100: 4610-4615.
- [19] Baruscotti M, Bucchi A, Viscomi C, Mandelli G, Consalez G, Gnecci-Rusconi T, Montano N, Casali KR, Micheloni S, Barbuti A, DiFrancesco D. Deep bradycardia and heart block caused by inducible cardiac-specific knockout of the pacemaker channel gene *Hcn4*. *Proc Natl Acad Sci U S A* 2011; 108: 1705-1710.
- [20] Nicolaou P, Hajjar RJ, Kranias EG. Role of protein phosphatase-1 inhibitor-1 in cardiac physiology and pathophysiology. *J Mol Cell Cardiol* 2009; 47: 365-371.
- [21] Hall DD, Feekes JA, Arachchige Don AS, Shi M, Hamid J, Chen L, Strack S, Zamponi GW, Horne MC, Hell JW. Binding of protein phosphatase 2A to the L-type calcium channel Cav1.2 next to Ser1928, its main PKA site, is critical for Ser1928 dephosphorylation. *Biochemistry* 2006; 45: 3448-3459.
- [22] Xu H, Ginsburg KS, Hall DD, Zimmermann M, Stein IS, Zhang M, Tandan S, Hill JA, Horne MC, Bers D, Hell JW. Targeting of protein phosphatases PP2A and PP2B to the C-terminus of the L-type calcium channel Ca v1.2. *Biochemistry* 2010; 49: 10298-10307.
- [23] Ling S, Sun Q, Li Y, Zhang L, Zhang P, Wang X, Tian C, Li Q, Song J, Liu H, Kan G, Cao H, Huang Z, Nie J, Bai Y, Chen S, Li Y, He F, Zhang L, Li Y. CKIP-1 inhibits cardiac hypertrophy by regulating class II histone deacetylase phosphorylation through recruiting PP2A. *Circulation* 2012; 126: 3028-3040.
- [24] Heijman J, Dewenter M, El-Armouche A, Dobrev D. Function and regulation of serine/threonine phosphatases in the healthy and diseased heart. *J Mol Cell Cardiol* 2013; 64: 90-98.
- [25] Takeda M, Briggs LE, Wakimoto H, Marks MH, Warren SA, Lu JT, Weinberg EO, Robertson KD, Chien KR, Kasahara H. Slow progressive conduction and contraction defects in loss in *Nkx2-5* mice after cardiomyocyte terminal differentiation. *Lab Invest* 2009; 89: 983-993.
- [26] Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, Molkenstin JD. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res* 2001; 89: 20-25.
- [27] Carr AN, Schmidt AG, Suzuki Y, del Monte F, Sato Y, Lanner C, Breeden K, Jing SL, Allen PB, Greengard P, Yatani A, Hoit BD, Grupp IL, Hajjar RJ, DePaoli-Roach AA, Kranias EG. Type 1 phosphatase, a negative regulator of cardiac function. *Mol Cell Biol* 2002; 22: 4124-4135.
- [28] Janssens V, Derua R, Zwaenepoel K, Waelkens E, Goris J. Specific regulation of protein phosphatase 2A PR72/B" subunits by calpain. *Biochem Biophys Res Commun* 2009; 386: 676-681.
- [29] Park JH, Sung HY, Lee JY, Kim HJ, Ahn JH, Jo I. B56alpha subunit of protein phosphatase 2A mediates retinoic acid-induced decreases in phosphorylation of endothelial nitric oxide synthase at serine 1179 and nitric oxide production in bovine aortic endothelial cells. *Biochem Biophys Res Commun* 2013; 430: 476-481.
- [30] Zwaenepoel K, Louis JV, Goris J, Janssens V. Diversity in genomic organisation, developmental regulation and distribution of the murine PR72/B" subunits of protein phosphatase 2A. *BMC Genomics* 2008; 9: 393.
- [31] Ahn JH, Sung JY, McAvoy T, Nishi A, Janssens V, Goris J, Greengard P, Nairn AC. The B"/PR72 subunit mediates Ca<sup>2+</sup>-dependent dephosphorylation of DARPP-32 by protein phosphatase 2A. *Proc Natl Acad Sci U S A* 2007; 104: 9876-9881.
- [32] Lechward K, Awotunde OS, Swiatek W, Muszynska G. Protein phosphatase 2A: variety of

## PP2A knockout causes cardiac hypertrophy

- forms and diversity of functions. *Acta Biochim Pol* 2001; 48:921-933.
- [33] Perrotti D, Neviani P. Protein phosphatase 2A: a target for anticancer therapy. *Lancet Oncol* 2013; 14: e229-238.
- [34] Gao ZH, Seeling JM, Hill V, Yochum A, Virshup DM. Casein kinase I phosphorylates and destabilizes the beta-catenin degradation complex. *Proc Natl Acad Sci U S A* 2002; 99: 1182-1187.
- [35] Li X, Yost HJ, Virshup DM, Seeling JM. Protein phosphatase 2A and its B56 regulatory subunit inhibit Wnt signaling in *Xenopus*. *EMBO J* 2001; 20: 4122-4131.
- [36] Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell* 2012; 149: 1192-1205.
- [37] Gao ZH, Seeling JM, Hill V, Yochum A, Virshup DM. Casein kinase I phosphorylates and destabilizes the beta-catenin degradation complex. *Proc Natl Acad Sci U S A* 2002; 99: 1182-1187.
- [38] Zhao J, Yue W, Zhu MJ, Sreeiavan N, Du M. AMP-activated protein kinase (AMPK) cross-talks with canonical Wnt signaling via phosphorylation of beta-catenin at Ser 552. *Biochem Biophys Res Commun* 2010; 395: 146-151.
- [39] Taurin S, Sandbo N, Qin Y, Browning D, Dulin NO. Phosphorylation of betacatenin by cyclic AMP-dependent protein kinase. *J Biol Chem* 2006; 281: 9971-9976.
- [40] Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, Mills GB, Kobayashi R, Hunter T, Lu Z. Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. *J Biol Chem* 2007; 282: 11221-11229.
- [41] Antos CL, McKinsey TA, Frey N, Kutschke W, McAnally J, Shelton JM, Richardson JA, Hill JA, Olson EN. Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. *Proc Natl Acad Sci U S A* 2002; 99: 907-912.
- [42] Chen W, Gu P, Jiang X, Ruan HB, Li C, Gao X. Protein phosphatase 2A catalytic subunit alpha (PP2Aalpha) maintains survival of committed erythroid cells in fetal liver erythropoiesis through the STAT5 pathway. *Am J Pathol* 2011; 178: 2333-2343.