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PARP-2 knockdown protects cardiomyocytes from hypertrophy via activation of SIRT1

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ABSTRACT

Poly(ADP-ribosyl)ation catalyzed by the poly(ADP-ribose) polymerases (PARPs) is an immediate post-translational modification of proteins with a homopolymeric chain of repeating ADP-ribose units. It is involved in various cellular processes, such as cell survival and death, transcription, DNA repair and cell division. Inhibitors of PARPs have been documented to be useful in different pathological conditions. Recently, activation of PARP-1, the founding member of PARP family, has been revealed to participate in the development and progression of cardiac hypertrophy and heart failure. However, the roles of other PARPs in cardiovascular system remain to be clarified. PARP-2 shares 69% similarity with PARP-1 in catalytic domains, but their functions do not fully overlap. In this study, we show the first evidence that PARP-2 is involved in cardiac hypertrophy. The mRNA and protein levels of PARP-2 were significantly increased in AngII-stimulated rat cardiomyocytes as well as in hearts of rats submitted to pressure overload. PARP-2 knockdown protected cardiomyocytes from hypertrophy, which may be attributed to activation of SIRT1. These findings shed new light on the understanding of PARP-2-related cardiomyopathy, and suggest the potential application of PARP-2 inhibitors in cardiac hypertrophy.

1. Introduction

Cardiac hypertrophy is an adaptive response of the heart to chronic pressure or volume overload, characterized by an increase in cell size, enhanced protein synthesis and reactivation of a fetal cardiac gene program [1–3]. Although cardiac hypertrophy may initially be compensatory, sustained pathologic hypertrophy is deleterious and may lead to heart failure. During the last decades, major strides have been made in understanding and managing cardiac hypertrophy. However, the signaling mechanisms leading to this complex clinical syndrome remain to be clarified [4].

Poly(ADP-ribose)polymerases (PARPs) constitute a family of proteins that catalyze the conversion of nicotinamide adenine dinucleotide (NAD) into long poly(ADP-ribose) (PAR) chains [5,6]. These highly negatively charged PAR chains can be attached to acceptor proteins, thereby altering their function [7,8]. The most extensively studied member of the PARP family, PARP-1, can primarily act as a DNA damage sensor in the nucleus. It becomes activated in response to DNA single-strand breaks induced by free radical and oxidant cell injury [9]. In physiological conditions, mild PARP-1 activation regulates many cellular processes, including DNA repair, gene transcription, cell survival and chromatin remodeling [5,10,11]. However, overactivation of PARP-1 depletes its substrate, NAD, slowing the rate of glycolysis, electron transport, and ATP formation, eventually leading to the functional impairment or death of the endothelial cells and cardiomyocytes [9,12]. Thus, PARP-1 has been implicated in a variety of cardiovascular diseases, such as myocardial ischemia/reperfusion injury, circulatory shock, diabetic cardiovascular complications, and atherosclerosis [13,14]. Data obtained from using PARP-1 inhibitors have also documented a role of PARP-1 activation in the development and progression of pressure-overload cardiac hypertrophy and heart failure [15,16].

Abbreviations: AAC, abdominal aortic constriction; AngII, angiotensinII; PARPs, poly(ADP-ribose) polymerases; NAD, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate; SD, Sprague–Dawley; DMEM, Dulbecco's modified Eagle's medium; ANF, atrial natriuretic factor; BNP, brain natriuretic polypeptide; β-MHC, myosin heavy chain β; qRT-PCR, quantitative real-time polymerase chain reaction.

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To date, 17 human PARP family members have been identified [17]. Among these members, PARP-1 and PARP-2 are so far the only PARPs whose catalytic activity has been shown to be induced by DNA-strand interruptions [18]. PARP-2 possesses a catalytic domain structurally similar to PARP-1, whereas it is less active than PARP-1, contributing only 5–10% of the total PARP activity in response to DNA damage [19]. Moreover, PARP-1 and PARP-2 have different DNA and protein targets, indicating specific pathophysiological functions of them, which have started to be recognized. Recently, researches have revealed that PARP-2 is involved in specific processes, such as genome surveillance, spermatogenesis, adipogenesis and T cell development [17,20]. However, the potential role of PARP-2 in cardiovascular system has not yet been determined.

In this study, we show the first evidence that PARP-2 may participate in cardiac hypertrophy. The expression of PARP-2 is obviously upregulated in AngII-treated cardiomyocytes as well as in hearts of rats submitted to pressure overload. PARP-2 deletion protects cardiomyocytes from hypertrophy which may be attributed to activation of the key member of sirtuins (SIRT) family, SIRT1. These findings shed new light on the understanding of PARP-2-mediated cardiomyopathy, and suggest the rational development and exploitation of specific PARP-2 inhibitors against cardiac hypertrophy.

2. Materials and methods

2.1. Animal experiments

Sprague–Dawley (SD) rats (male, weighing 180–200 g, SPF grade, Certification No. 0067950) were supplied by the Experimental Animal Center of Sun Yat-Sen University (Guangzhou, PR China). Pressure overload was induced in rats by abdominal aortic constriction (AAC) according to procedure reported by others [21]. Briefly, rats were anaesthetized with sodium pentobarbital (30 mg/kg, intraperitoneal injection). Fentanyl (0.16 mg/kg, subcutaneous injection) was given as an analgesic agent. Under sterile conditions, the abdominal aorta above the kidneys was exposed through a midline abdominal incision and constricted at the suprarenal level with a 4–0 silk suture tied around both the aorta and a blunted 22-gauge needle. The needle was promptly removed after constriction. Sham-operated animals underwent a similar procedure without banding the aorta. The investigation conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996), and was approved by the Institutional Ethics Review Board of Sun Yat-Sen University.

2.2. Cell culture

Primary cultures of neonatal rat cardiomyocytes were isolated from the hearts of 1- to 3-day-old SD rats as we previously described. Cells were seeded at a density of 1×10^6 cells/well onto 6-well plates in DMEM supplemented with 10% fetal bovine serum (FBS) and 5-bromodeoxyuridine (0.1 mM). Forty-eight hours later, the culture medium was replaced with DMEM containing 0.1% FBS. After incubation for 24 h, the cells were further treated with or without AngII for indicated time [22].

2.3. Echocardiography and morphometric measures

At 4 weeks after AAC surgery, two-dimensionally guided M-mode echocardiography was performed using a Technos MPX ultrasound system (ESAOTE, Italy) equipped with a 8.5-MHz imaging transducer as previously reported [23]. For morphometric measures, some rats were perfusion-fixed and sacrificed. The hearts were carefully excised, and the fixed transverse sections and longitudinal sections of the hearts were embedded in paraffin, cut into 5 μm cross sections, and stained with hematoxylin-eosin (HE). Other rats were executed by decapitation, and heart weight was determined. The left ventricle (LV) was then carefully trimmed away from the right ventricle and atria. Heart weight (HW) and left ventricle weight (LVW) were expressed relative to body weight (BW).

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from cultured cells or heart tissues was extracted with Trizol reagent (Invitrogen) according to manufacturer's instruction. One microgram of total RNA was reverse transcripted using One-step RT Kit (Takara Biotechnology) and the resulting cDNA was used as a PCR template. The mRNA expression levels were determined using SYBR-Green Quantitative PCR kit (Takara Biotechnology) by iCycler iQ system (Bio-Rad). Amplification conditions were 15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 1 min at 55 °C and 30 s at 72 °C. A dissociation curve was generated to verify that the majority of fluorescence detected could be attributed to the labeling of specific PCR products, and to verify the absence of primer–dimers and sample contamination. All PCR reactions were done in triplicate. Rat-specific primers (Supplementary Table 1) for PARP-1, PARP-2, SIRT1, atrial natriuretic factor (ANF), brain natriuretic polypeptide (BNP), and myosin heavy chain β (β-MHC) were synthesized by Invitrogen. GAPDH served as an endogenous control.

2.5. Western blot analysis

Goat anti-PARP-2 polyclonal antibody (Sigma, diluted 1:200) and rabbit anti-SIRT1 polyclonal antibody (Sigma, diluted 1:1000) were used as primary antibodies. Mouse anti-α-tubulin monoclonal antibody (Sigma, diluted 1:10,000) served as loading control. Briefly, protein was separated by SDS–PAGE gel electrophoresis, and then transferred to PVDF membranes (Millipore). After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies, followed by incubation with appropriate horseradish peroxidase (HRP)-labeled second antibodies. Immunoreactive bands were detected with the Super-Signal West Pico Chemiluminescent Substrate (Pierce). The intensity of protein bands was analyzed by LabWorks software (Bio-Rad).

2.6. Measurement of cell surface area

Rhodamine–phalloidin (Invitrogen) was employed to visualize actin fragment by fluorescence microscopy. Cardiomyocytes grown on coverslips were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and further incubated with 0.1% rhodamine–phalloidin and 0.1% saponin for 1 h. After washed with PBS, coverslips were mounted in prolong Gold anti-fade reagent with DAPI (Invitrogen) and inspected with a confocal microscope (Zeiss 710).

2.7. RNA interference

Three different duplex siRNAs (Supplementary Table 2) for PARP-2 (si001, si002, and si003), SIRT1 (siSIRT1), and negative control siRNA were purchased from Genepharma (Shanghai, PR China). Rat cardiomyocytes were transfected with siRNAs, respectively, by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 h after transfection, qRT-PCR and Western blot were employed to compare silencing efficiency of different duplex siRNAs.

2.8. Statistical analysis

Data were presented as mean ± SE. Statistical analyses between two groups were performed by unpaired Student's *t*-test. Differences among groups were tested by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. In all cases, differences were considered statistically significant with $P < 0.05$.

3. Results

3.1. Expression of PARP-2 in neonatal rat cardiomyocytes treated with AngII

AngII is an well-established neurohumoral factor that can stimulate cardiac hypertrophy [24]. As shown in Fig. 1A and B, the mRNA levels of PARP-2 in cultured neonatal rat cardiomyocytes were enhanced in a dose and time dependent manner responding to AngII stimulation. AngII treatment also resulted in significant upregulation of PARP-2 protein expression (Fig. 1C and D).

3.2. Expression of PARP-2 in heart tissues of AAC rats

Abdominal aortic constriction surgery was performed to produce pressure overload hypertrophy in rats. At 8 weeks after surgery, the hearts of rats submitted to AAC presented typical hypertrophic changes (Fig. 2A and B) and obvious cross-sectional area of cardiomyocytes as indicated by HE staining (Fig. 2C). The heart weight to body weight (HW/BW) ratio and left ventricle weight to body weight (LVW/BW) ratio were both increased in AAC rats comparing with sham-operated animals (Supplementary Table 3). The echocardiography revealed that IVSd, IVSs, LVPWd and LVPWs were significantly increased, whereas LVIDs was decreased after AAC (data not shown). And the expression of hypertrophy marker genes including ANF, BNP and β-MHC mRNA was significantly increased in AAC rats (Fig. 2D). These results convergently suggested the successful induction of cardiac hypertrophy by AAC surgery. In addition, as shown in Fig. 2E and F, both protein and mRNA expression of PARP-2 were significantly upregulated after 8 weeks of pressure overload induced by AAC.

3.3. PARP-2 knockdown protected cardiomyocytes from AngII-induced hypertrophy in neonatal rat cardiomyocytes

To investigate the potential effect of PARP-2 on cardiac hypertrophy, we took the approach of RNA interference to knock down the endogenous PARP-2 in neonatal rat cardiomyocytes. Quantitative RT-PCR and Western blot was performed to evaluate the efficacy of three independent siRNAs, marked si001, si002, and si003. As shown in Fig. 3A and B, si003 reduced the mRNA and protein expression of PARP-2 by 79% and 81%, respectively ($P < 0.01$, compared with control), and did not interfere with PARP-1, exhibiting potential efficacy and selectivity for PARP-2 silence. Therefore, it was used in the following experiments. The cell surface area and mRNA levels of ANF, BNP and β-MHC were measured after transfection (Fig. 3C and D). The results revealed that the downregulation of PARP-2 by si003 (siPARP-2) could inhibit the increase in cell surface area as well as high expression of hypertrophic biomarkers induced by AngII treatment (100 nM for 24 h).

3.4. SIRT1 participates in the anti-hypertrophic effect of PARP-2 knockdown

PARP-2 was knocked down in neonatal rat cardiomyocytes by using siRNA, and then SIRT1 expression was detected by qRT-PCR and Western blot, respectively. As shown in Fig. 4A and B, the mRNA and protein levels of SIRT1 were both significantly upregulated following PARP-2 knockdown. Although PARP-2 deletion attenuated AngII-induced increase in cell surface area and mRNA

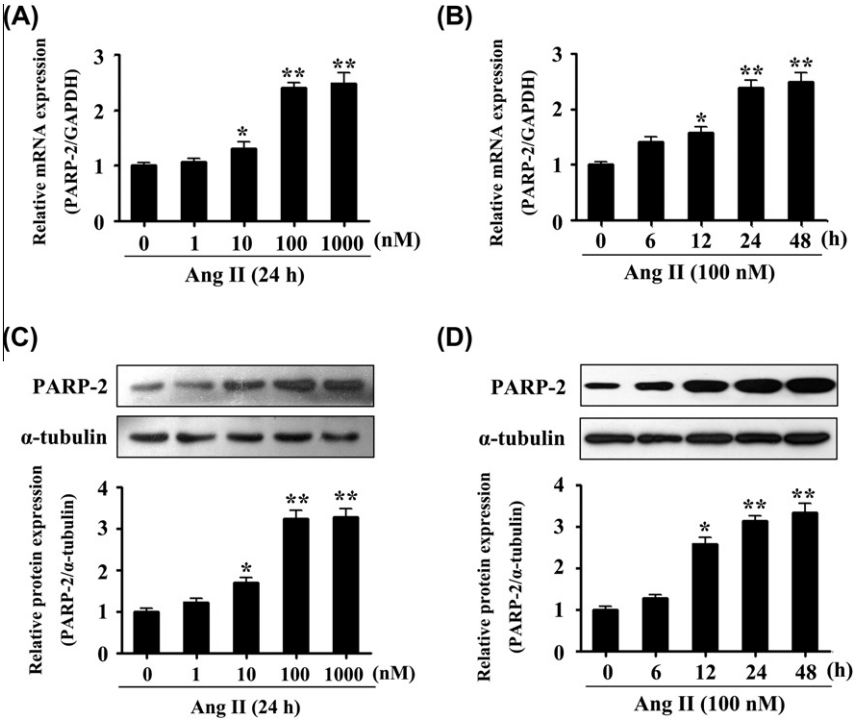

Fig. 1. PARP-2 expression was increased in neonatal rat cardiomyocytes following AngII treatment. Cultured neonatal rat cardiomyocytes were treated with various concentrations of AngII for 24 h or with 100 nM AngII for indicated time points. The mRNA and protein levels of PARP-2 were determined by qRT-PCR (A and B) and Western blot (C and D), respectively. The results were normalized (mRNA by GAPDH and protein by α-tubulin) and then presented as the fold of control levels. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control, $n = 6$.

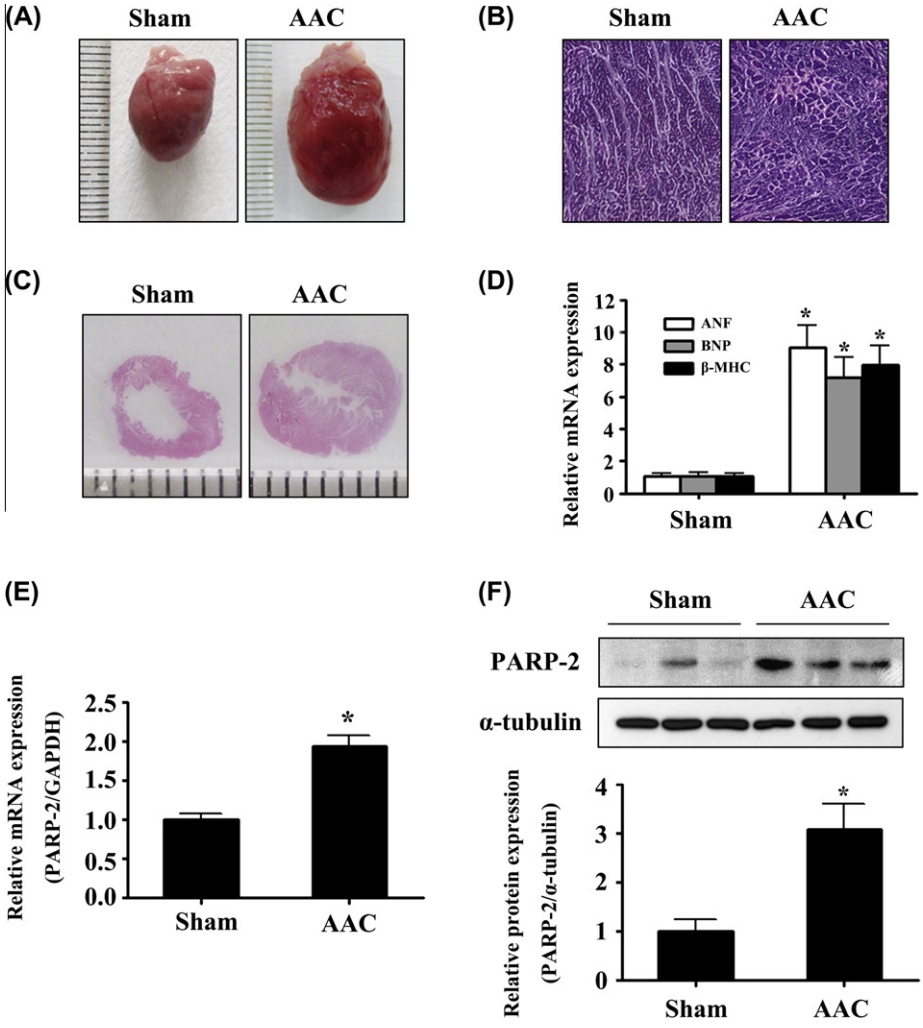

Fig. 2. PARP-2 mRNA and protein expression were upregulated in abdominal aortic constriction rats. Pressure overload was induced by AAC in rats. At 8 weeks after surgery, the hypertrophic changes and PARP-2 expression in heart tissues were determined. (A) Gross hearts from AAC and sham-operated rats (Sham). (B and C) HE staining for transverse section and longitudinal section of hearts. (D) The mRNA levels of ANF, BNP and β-MHC were determined by qRT-PCR. (E and F) The protein and mRNA levels of PARP-2 were detected by Western blot and qRT-PCR, respectively. $^{*}P < 0.05$ *vs.* sham, $n = 8$. Scale: 1 mm (A and B); magnification: ×40 (C).

expression of ANF, BNP and β-MHC, this anti-hypertrophic effect was nearly abolished when SIRT1 was simultaneously knocked down (Fig. 4C and D). The potential efficacy of siSIRT1 was shown in Supplementary Fig. 1. These results suggested the involvement of SIRT1 activation in the protective effect of PARP-2 knockdown against cardiac hypertrophy.

4. Discussion

PARP-2 participates in abundant cellular processes such as DNA repair and maintenance of genomic integrity [8,18]. It is essential for the transcriptional regulation of metabolism and oxidative stress responses [25,26]. However, the potential roles of PARP-2 in cardiovascular system have not yet been reported. In the present study, we found that PARP-2 mRNA and protein levels were significantly unregulated following AngII treatment in cardiomyocytes (Fig. 1). Elevated PARP-2 expression was also observed in heart tissues from SD rats submitted to AAC-induced pressure overload (Fig. 2E and F). Furthermore, our results showed that specific knockdown of PARP-2 by RNA interference could markedly block the hypertrophic responses stimulated by AngII (Fig. 3C and D). To our best knowledge, these findings provide the first evidence suggesting the involvement of PARP-2 in cardiac hypertrophy.

AngII is an established neurohumoral factor that induces cardiac hypertrophy [24]. During the last decades, multiple signaling pathways that regulate AngII-mediated cardiac hypertrophy response have been identified, including activation of PKC, MAPKs, and the production of reactive oxygen and nitrogen species. However, the downstream targets and nuclear integrators of AngII-mediated cardiac growth response are not yet fully understood [27]. PARP is known to be activated by cell oxidative stress. Because oxidative stress is also considered a main component of AngII-mediated cell signaling, it was postulated that PARP could be a downstream target of AngII-induced signaling leading to cardiac hypertrophy [15]. AngII can activate membrane-bound NADPH oxidases, leading to increased production of reactive oxygen and nitrogen species. In addition, AngII stimulation of cardiomyocytes causes DNA damage [28,29], which is another profound stimulus of PARP activation. PARP-1 is the founding PARP family member, which has been the most extensively studied [9]. Recently, Pillai et al. has demonstrated that PARP-1 is activated during AngII-induced cardiomyocyte hypertrophy, and mice deficient in the PARP-1 gene are protected from AngII-mediated hypertrophy [27]. Our data presented here extend these previously reported intracellular cascades of AngII pathway and document that PARP-2 is a downstream nuclear target of AngII-mediated signaling, leading to myocardial hypertrophy.

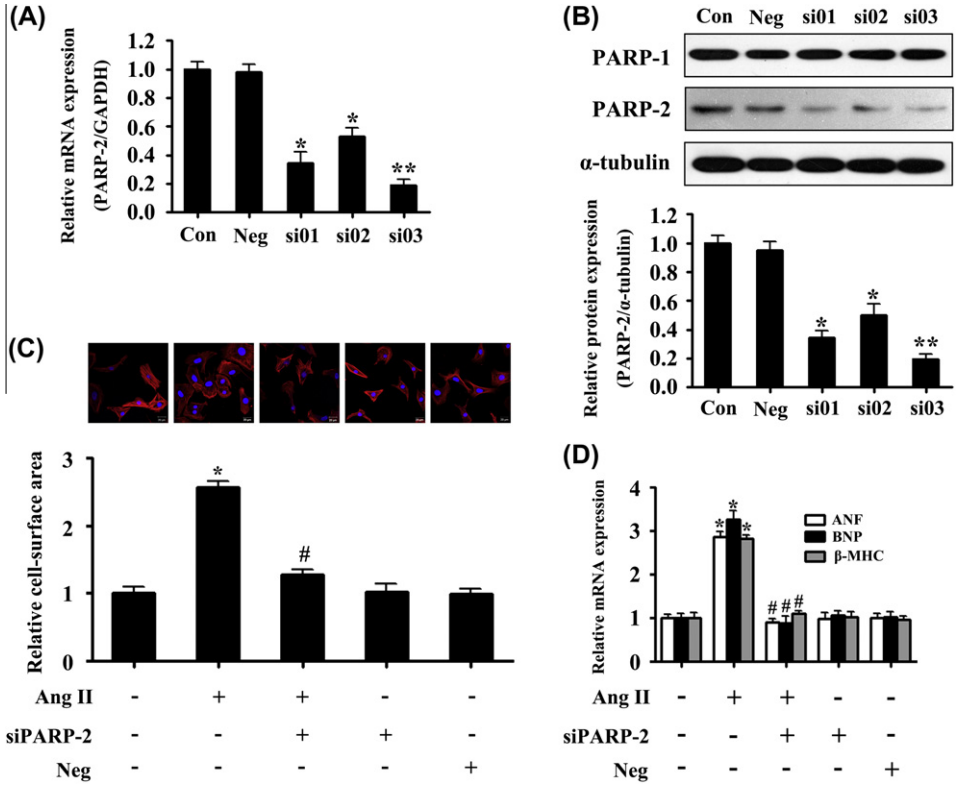

Fig. 3. PARP-2 knockdown blocked the hypertrophic responses induced by AngII. Neonatal rat cardiomyocytes were transfected with three independent siRNAs for PARP-2 (marked si001, si1002 and si003, respectively) and negative control (Neg). The efficacy for PARP-2 knockdown was determined by qRT-PCR (A) and Western blot (B). Cells were treated with or without 100 nM AngII for 24 following PARP-2 knockdown by si003 (siPARP-2). Subsequently, the cell surface area (C) and mRNA levels of ANF, BNP and β-MHC (D) were measured. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control; $^{\#}P < 0.05$ *vs.* AngII treatment, $n = 6$.

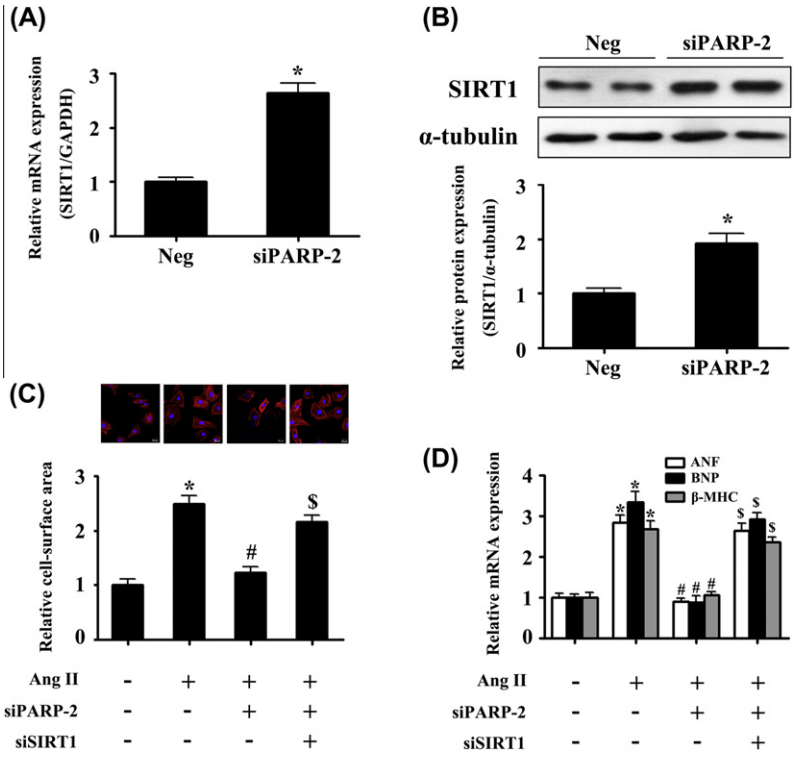

Fig. 4. SIRT1 activation was involved in the anti-hypertrophic effect of PARP-2 knockdown. Neonatal rat cardiomyocytes were transfected with siRNA for PARP-2 (siPARP-2) and negative control (Neg). The mRNA and protein expression of SIRT1 were detected by qRT-PCR (A) and Western blot (B), respectively. Cardiomyocytes transfected with siPARP-2 or siRNA for SIRT1 (siSIRT1) were treated with AngII for 24 h. The cell surface area (C) and mRNA levels of hypertrophic biomarkers (D) were measured, respectively. $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* AngII treatment, $^{\$}P < 0.05$ *vs.* siPARP-2 group $n = 6$.

PARP-2 was discovered as a result of the presence of residual DNA-dependent PARP activity in embryonic fibroblasts derived from PARP-1-deficient mice. Its catalytic domain has the strongest resemblance to that of PARP-1 with 69% similarity. Most of the published data on PARP-1 and PARP-2 integrate the unique property of these two DNA-dependent enzymes to detect DNA interruptions acting as potent enzymatic coactivators during various physiological or pathological processes [18]. They clearly participate in the first line of defense of the genome as efficient DNA-break sensors and signaling molecules as a part of a survival program [30]. However, PARP-2 deficient mice display specific phenotypes, indicating that PARP-1 and PARP-2 functions are complementary but do not fully overlap [17,31]. PARP-2 preferentially heteromodifies histone H2B, whereas PARP-1 preferentially modifies histone H1 in isolated nuclei. The DNA-binding domain of PARP-2 differs from that of PARP-1 and it targets DNA gaps but not nicks. Furthermore, specific partners of PARP-2 are beginning to be discovered, such as the telomeric protein TRF2 [32]. These evidences convergently suggest that a better understanding of the specific molecular pathways involving PARP-2 will be essential in the identification of new target molecules and the design of new therapeutic approaches.

Poly(ADP-ribosyl)ation by PARP requires NAD as substrate for generating ADP-ribose monomers. The overactivation of PARP may deplete the stores of cellular NAD and induce a progressive ATP depletion, thereby altering the functions and activities of NAD-dependent enzymes. The sirtuin family, which belongs to class III of histone deacetylases (HDACs), is implicated in the control of critical cellular processes such as differentiation, proliferation, apoptosis, metabolism and aging [33]. Since the activity of sirtuins is NAD-dependent and susceptible to the availability of NAD content, reduced cellular levels of NAD will attenuate the SIRT deacetylase activity. The best-characterized sirtuins, SIRT1, is indicated to protect the heart from hypertrophy, metabolic dysregulation, and inflammation [34,35]. Previous studies have demonstrated that the modulation of intracellular NAD levels by PARP-1 critically influences SIRT1 activity [36]. However, PARP-2 is not a major PARP enzyme in the cell and, therefore, is not likely to significantly influence NAD homeostasis. Rather, PARP-2 impacts on SIRT1 by directly interacting with the SIRT1 promoter, where it acts as a negative regulator for SIRT1 expression [25]. Consistently, we found that the mRNA and protein levels of SIRT1 were both significantly upregulated following PARP-2 knockdown in cultured neonatal rat cardiomyocytes (Fig. 4A and B). Moreover, PARP-2 deletion attenuated AngII-induced hypertrophic responses as indicated by decrease in cell surface area and fetal gene expression, but its effect was abolished when SIRT1 was simultaneously knocked down by RNA interference (Fig. 4C and D). These findings suggest that the protective effect of PARP-2 knockdown against cardiac hypertrophy may be at least partially attributed to SIRT1 activation.

In summary, our present study shows that PARP-2 expression is unregulated in response to hypertrophic stimuli in vitro and in vivo. PARP-2 knockdown protects cardiomyocytes from hypertrophy, which may be mediated via activation of SIRT1. Understanding this novel role of PARP-2 might provide clues to the rational development and exploitation of specific PARP-2 inhibitors in a clinical setting and the design of new therapeutic approaches in different pathophysiological conditions including cardiac hypertrophy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.132.

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