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Original Article

β-catenin regulates myocardial ischemia/reperfusion injury following heterotopic heart transplantation in mice by modulating PTEN pathways

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Abstract: Ischemia reperfusion (I/R) injury, an inevitable event accompanying heart transplantation, is the primary factor leading to organ failure and graft rejection. In order to prevent I/R injury, we established murine heart transplantation model with I/R and cell culture system to determine whether β-catenin is a mediate factor in preventing I/R injury in heart transplantation. After successfully established heterotopic heart transplantation mice model, the I/R injury was induced, and two dynamic temporal were studied during different I/R phases. With the increase of ischemia and reperfusion time, heart damage was more severe. In the initial study, we observed that β-catenin was significantly decreased, while ROCK1 and PTEN increased during the perfusion phase from day 0 to day 1, and remain the same level until 3 days later. The similar pattern that β-catenin was down-regulated while ROCK1 and PTEN were up-regulated was also observed in the dynamic temporal ischemia study. To further investigate the role of β-catenin signaling in I/R injury *in vitro*, β-catenin over-expressing plasmid was transfected into HL-1 cells, a cardiac cell line. We noted that β-catenin over-expressing cardiomyocytes showed decreased ROCK1/PTEN expression both at mRNA and protein levels. In addition, cobalt dichloride ($CoCl_2$) -induced oxidative stress model was further established to mimic cardiac I/R injury. We observed that $CoCl_2$-induced activation of ROCK1/PTEN signaling pathway were attenuated by transient transfection of a β-catenin over-expressing plasmid. Taken together, our results suggest that cardiac transplant induced IR injury is closely associated with the down-regulation of β-catenin and up-regulation of ROCK1 and PTEN expression.

Keywords: Heart transplantation, Ischemia reperfusion (I/R) injury, β-catenin, ROCK/PTEN

Introduction

Cardiac transplantation is the preferred treatment for patients with end-stage heart disease, i.e, advanced heart failure [1-3]. It offers a significant improvement of life quality and the most beneficial survival of heart failure patients. However, in cardiac transplantation, the big challenge we have to encounter with is Ischemia/reperfusion (IR) injury [4, 5]. As after transplantation, cardiac grafts inevitably suffer from ischemia when separated from the donor. After revascularization, blood flow in post-ischemic hearts could activate a sequence of events that aggravates cardiac injury, i,e., ischemia-reperfusion injury (IRI), and contributes to a high rate of morbidity. Hence, it is critical important to minimize IR injury.

Recently, a robust of studies mainly on molecular level, regarding the important genes, gene networks, key signaling cascades, participating in I/R injury have been reported, and modulation of these pathway by endogenous factors or

pharmacological agents has been postulated as a relevant way to prevent I/R injury [6-9]. However, the molecular mechanism of heart transplantation induced I/R injury, involving activation of multiple and interlocked signaling pathways remains exclusive. Given the limited efficacy of these current therapeutic strategies, it is of critical importance to gather information about the status of key cardiac signaling pathways at early stages after I/R, to identify potential new targets for interventional therapies.

β-catenin pathway is an important regulator of cell development, regeneration, and carcinogenesis [10]. It has been shown to possess multiple regulatory functions during the inflammatory response [11, 12]. There are a lot of literature suggests that β-catenin and its related pathways PTEN/ROCK participates in the regulatory mechanism of IR injury events, such as: liver, lung, cerebral, etc. Kamo et al. (2013) found that β-catenin directly inhibits NF-κB activation, resulting in suppression of pro-inflammatory gene programs that otherwise drive the hepatocellular damage in IR-stressed liver forward [13]. Ke et al. (2013) demonstrated that β-catenin inhibits PTEN and promotes PI3K/Akt pathway, which acts as a novel regulator of innate and adaptive immune responses in the mechanism of liver IRI [14]. Zhou et al. (2019) found that PTEN/β-catenin signaling regulates the immune response and thus alleviates the inflammation of lung injury [15]. Li et al. (2019) found that Wnt/β-catenin pathway may play an important role in the protection against cerebral ischemia/reperfusion injury in rats [16]. However, currently, few reports have been published on the molecular mechanism of β-catenin events in cardiac I/R injury. The cross talk between β-catenin and other signal pathways, pivotal in the mechanism of cardiac IR injury, remains to be elucidated.

The heterotopic heart transplantation in rodents, like mice, rat, is a widely accepted animal model as which is mainly used to investigate transplantation biology [17-23]. Heterotopic abdominal mice heart transplantation has been extensively used to investigate ischemic-reperfusion injury, immunological consequences during heart transplantations [22].

Due to our initial experience on the model establishment of heterotopic heart transplantation, herein, we carried out studies to investigate the molecular mechanisms of β-catenin regulation involved in cardiac I/R injury. In the present work, firstly, using an *in vivo* mice model, we found that the β-catenin participates in myocardial ischemia/reperfusion injury following heterotopic heart transplantation in mice by modulating PTEN regulation.

Materials and methods

Experimental animals

The male C57BL/6 Mice, 8-10 weeks of age, with mean weight of 24-27 g, were maintained in a specific pathogen-free facility of Xiamen University. The mice were kept in individual stainless steel cages in a room with controlled temperature (23 ± 3°C) and humidity (55 ± 15%) on a 12-hour light/dark cycle, and provided with standard rodent food and free access to water prior to the experiments. The animals were fasted overnight before the experiments, but were given free access to water. All procedures involving mice were conducted in accordance with the guidelines of the Animal Care and Committees of Xiamen University and complied with the National Research Council's Guide for the Humane Care and Use of Laboratory Animals.

Cell line and cell culture

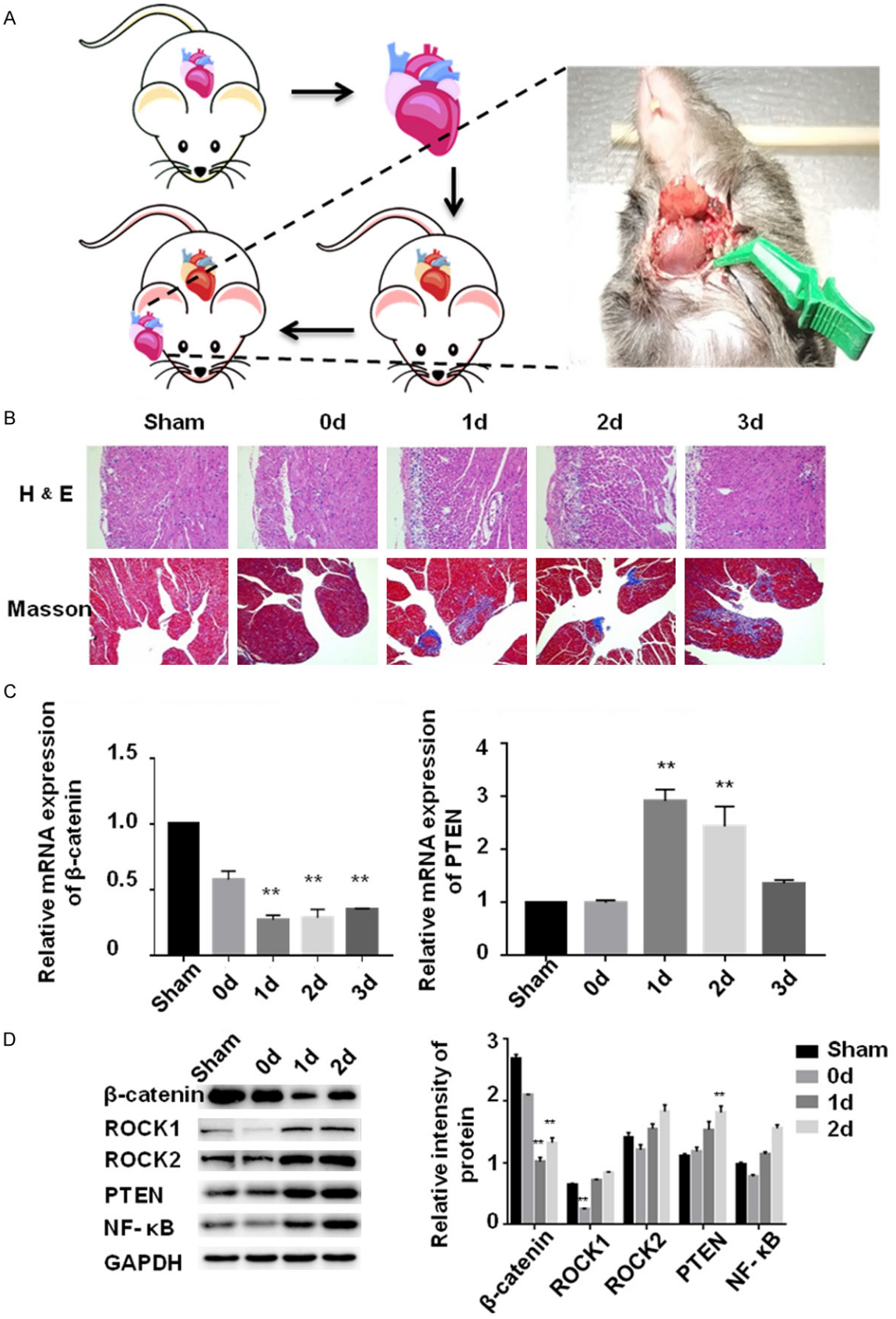
The murine cardiac HL-1 cell lines were originally purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured and maintained in 2 mL DMEM (50:50), supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin mixture (100 U/ml) (Gibco, Grand Island, NY). Cell lines were incubated at 37°C with 100% humidity in 5% CO_2.

Experimental design

The experimental design is schematically illustrated in **Figure 1A**.

Heterotopic heart transplantation procedure

Heterotopic heart transplantation procedure was demonstrated as previously described [24]. The rats were placed on the white corkboard and their limbs were fixed with tape. The rats were anaesthetized using 1% pentobarbital. Finding the right external jugular vein


A
B
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C
Relative mRNA expression of β-catenin
1.5
1.0
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0d
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Relative mRNA expression of PTEN
4
3
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β-catenin
ROCK1
ROCK2
PTEN
NF-κB
GAPDH
Relative intensity of protein
Sham
0d
1d
2d

Figure 1. Ischemia/reperfusion injury following heterotopic heart transplantation in mice. A. Schematic illustration of mice heterotopic heart transplantation in the study. Donor hearts were harvested from C57BL/6 mice (n=3, each) and preserved at 4°C for 24 h, followed by a heterotopic syngeneic transplantation. B. Histology analysis. The upper panel is HE staining. After transplantation, heart grafts were harvested for H&E staining, at different perfusion time points. Endocardium infiltrated with neutrophils. (day 0) Cardiomyocyte damage (day 1). Epicardium thickening (day 2). Trichrome staining for fibrosis (day 3). The lower panel is Mason staining. Representative images from 3 independent grafts per group. Images were taken at ×200 magnification. C. Real-time PCR was used to detect the mRNA expression of β-catenin (Left) and PTEN (Right) in the mice underwent different period of perfusion varying from day 0 to days 3: sham-operated mice, I/R mice with perfusion at day 0 (samples were obtained immediately when transplantation was done), I/R mice with perfusion at day 1, I/R mice with perfusion at day 2, and I/R mice with perfusion at day 3, (n=3/group). (Left) Compared with shame group, mRNA expression of β-catenin was decreased during the perfusion phase from day 0 to day 1, and remain the same level until 3 days after perfusion. (Right) Compared with shame group, mRNA expression of PTEN was significantly increased during the perfusion phase from day 0 to day 3 ($P<0.01$ both on day 1 and day 2). The PTEN level was significantly increased at 1 day of reperfusion ($P<0.01$), but then decreased at the IR 2 days compared with IR 1 day of reperfusion time point, however, when compared with controls, it was still significantly up-regulated ($P<0.01$). Columns, mean of three independent experiments; bars, SD (n=3). *, $P<0.05$, **, $P<0.01$. D. Western blot analysis was used to detect the expression of β-catenin, ROCK1, ROCK2, PTEN and NF-KB (Left) in the mice underwent different period of perfusion at 0, 1 and 2 days time point. (Right Pannel) Band intensity was expressed compared with total GAPDH. β-catenin protein accumulation was decreased at IR 1 day and 2 days reperfusion time points. ROCK1, ROCK2, and NF-KB protein levels were decreased at 0 day of reperfusion, but increased at 1 day and 2 days reperfusion compared with sham control.

(REJV), the tributaries of the REJV were cut off. The right common carotid artery (RCA) was exposed and external carotid arteries. The RCA was occluded by vessel clip and tied, then incised between the clip and the tie. The RCA was then overturn and ligature of 9-0 silk. The REJV was prepared in the same way. The donor was intravenously injected with 1 mL of 4°C heparinized saline (100 U/mL). The thorax was opened and the anterior chest wall was pulled in a cranial direction. After removal of thymus and pericardium, we performed a second reverse perfusion through the thoracic aorta to completely drain the remaining blood from the donor heart. Then we cut around the ascending aorta (AO) and the pulmonary artery (PA), just above the PA bifurcation. All blood vessels, except AO and PA, are connected to the posterior surface of the heart. The donor heart was placed in the right side of the neck of the recipient immediately. The aorta of the donor heart is connected to the common carotid artery of the recipient heart; Connect the pulmonary artery of the donor heart to the external jugular vein of the recipient heart. The heart rapidly reperfusion and gradually developed a sinus rhythm.

Myocardial I/R model

Myocardial I/R model mice were subjected to myocardial I/R as previously described [15]. Briefly, to induce ischemia, mice were first intraperitoneally anesthetized with sodium 1% pentobarbital (50 mg/kg, purchased from Sigma, St. Louis, USA). Surgical sutures were cut to expose the heart and right common carotid artery (RCA). The right common carotid artery (RCA) was sutured with 7-0 silk for 10 min before release for reperfusion. At the 0, 1, 2, 3 day of reperfusion, the hearts were obtained for further investigations.

For ischemia study, the same procedures were administered to mice exposed to ischemia for 5 min, 10 min, and 15 min, followed by 1 day of reperfusion. At the end of the procedures, samples were immediately harvested. Blood samples were collected by cardiac puncture and centrifuged at 3000 g for 10 minutes to abstract serum, which was stored at -80°C and to determine biochemical parameters. Tissues were snap frozen in liquid nitrogen for gene expression detection or fixed in 4% buffered formalin for histopathological evaluation.

In vitro ischemia reperfusion model

An in vitro $CoCl_2$-induced apoptosis model was established to mimic cardiac IRI. In the present study, the $CoCl_2$ induced cell model was established to mimic the cardiac IRI in vitro. HL-1 cells were plated in a 6 well plate (80,000 cells/well) and cultured in DMEM medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin overnight. DMEM culture medium was replaced by deoxygenized PBS and HL-1 cells were treated with $CoCl_2$ (500-1000 μM) for 12 h, 24 h and 36 h, respectively. OD was detected in HL-1 cells treated with different concentrations (500,

600, 700, 800, 900, and 1000 μM). The optimal concentration was 600 μM at 24 h was selected for the further study, Prior to cell collection, some cells were treated with $CoCl_2$ (600 μM) for 24 h. The cells were then lysed and processed for protein analysis or Real-time PCR detection. Cells were seeded into 6-well plates at 80% confluency and transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen, United States) for 72 h. The cells were then lysed and processed for protein analysis or Real-time PCR detection.

Real-time PCR

Total RNA was extracted from cells and heart tissues using Trizol (TransGen BiotechChina) according to the manufacturer's instructions. One μg of total RNA was used to synthesize cDNA using reverse transcriptase kit (YEASEN, China). Gene expression of β-catenin, ROCK1, ROCK2, PTEN, NF-κB, and GAPDH were detected by qPCR using the SybGreen method. Primer sequences used for PCR were listed: β-catenin F: 5'-ACTGGAGCTCTCCACATCC-3' and R: 5'-GTGGCTCCCTCAGCTTCAAT-3', ROCK1 F: 5'-AACGCTCCGAGACACTGTAG-3' and R: 5'-ACTTTCCTGCAAGCTTTTATCCAC-3,' ROCK2 F: 5'-CATCAACGTGGAGAGTCTGCTG-3' and R: 5'-TCTGTGATGCCTTATGACGAAC-3', PTEN F: 5'-CTGCAGAAAGACTTGAAGGTGT-3' and R: 5'-CTAGCTGTGGTGGGTTATGGT-3', NF-κB F: 5'-GCTATGTGTGTGAAGGCCCA-3' and R: 5'-TTGCAAATTTTGACCTGTGGGT-3', GAPDH F: 5'-CAGGAGAGTGTTTCCTCGTCC-3' and R: 5'-TTCCCATTCTCGGCCTTGAC-3'. qPCR was conducted in a 10 μL PCR with 1× SybrGreen mixture (Bio-Rad), 2.5 μM primers, and 1 μL of cDNA, with the following thermal profiling: an initial activation step was carried with at 95°C for 5 mins, followed by 40 cycles of: 95°C for 10 s, 60°C for 30 s, and 72°C for 20 s. Data were quantified automatically using the Light Cycler 480 (Roche, Switzerland) and the relative expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Western blotting analysis

Total cell protein was abstracted from cardiac tissue, or HL-1 cells after transfection. The concentration of the protein was measured by BCA protein assay kit (Solarbio, Beijing, China), following manufacturer's instruction. Samples were electrophoresed by using 10% SDS-PAGE. The protein was then transferred onto a PVDF (polyvinylidene fluoride) membrane (Bio-Rad, USA). After blocking in 5% dry milk for 4 hours, the membranes were incubated with incubated with primary antibodies: GAPDH, (Product# 60004-1-Ig, Proteintech), β-catenin (Product# 66379-1-Ig, Proteintech), PTEN (Product# 60300-1-Ig, Proteintech), ROCK1 (Product# 66782-1-Ig, Proteintech), ROCK2 (Product# 66633-1-Ig, Proteintech), P65 (Product# 10745-1-AP, Proteintech) The membranes were then incubated with HRP-conjugated secondary antibodies: HRP-conjugated Affinpure Goat Anti-Rabbit IgG (H+L) (SA00001-2, Proteintech), HRP-conjugated Affinpure Goat Anti-Mouse IgG (H+L) (SA00001-1, Proteintech). Specific protein bands were visualized using an enhanced chemiluminescence (ECL) kit and then processed using Image J for quantification. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad). GAPDH was used as internal reference.

Histology

After reperfusion, tissues from the donor mice hearts were fixed in 4% paraformaldehyde (Solarbio, Beijing) for 14 h at 4°C, embedded in paraffin. Formalin-fixed paraffin-embedded heart tissues were then cut in 5 μm sections. Serial slides were used for hematoxylin and eosin (H&E) staining and Masson staining according to standard procedures.

The pathological features were carefully examined under 200× magnification. Images were analyzed by an independent investigator in blind manner. H&E stained slides were evaluated for the area of necrosis and inflammatory cells infiltration. The area of necrosis and inflammatory cells infiltration was determined as previously described and expressed as a percentage of the whole section.

Cardiac fibrosis was assessed using Masson Trichrome staining as previously described by us (Chu et al. 2011). 4 μm thick sections of cardiac tissue were sectioned and for each section and measurement of fibrosis, 10 fields were chosen at random and imaged under light microscopy using the Olympus BH2 microscope at ×40 magnification. Fibrosis was then quantified using ImagePro Plus software (Adept Electronic Solutions Pty Ltd, Moorabin, Australia).

Cell viability assay

Cell viability was analyzed by using Cell Counting Kit-8 (CCK-8) (TransGene, Beijing, China), followed by manufacturer's instructions. Briefly, the cells were seeded into the 96-well plate. 10 μL of Cell Counting Kit-8 solution with 100 μL MES were then added into each well. Followed by incubation at 37°C for 1 h, cells were subjected to detect the OD450 values by using the SpectraMicroplate Reader (BIOTEC). Results were showed as percentage of the vehicle control levels set at 100%. Each treatment was performed in triplicate.

Serum analysis

Blood samples were obtained from mice with different ischemia time. Serum was collected and stored at -80°C until analysis was performed. Serum levels of cytokines, including proinflammatory cytokines (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-10) and TGF-β, were measured using enzyme-linked immunosorbent assays (ELISAs) performed according to the manufacturer's instructions.

Statistical analysis

The data was presented as mean ± SD. The students test and one-way ANOVA were used to conduct the comparison of the different protein, mRNA, luciferase reporter and miRNA expression levels. Statistical analyses were performed by SPSS 16.0 software. A *P*-value less than 0.05 was considered a statistically significant difference.

Results

Ischemia/reperfusion injury following heterotopic heart transplantation in mice

We first examined the cardiac IR injury in the heart tissue underwent different reperfusion time point by histology analysis. We found that in the sham group, the cardiac cells were arranged neatly, and no bleeding or inflammatory cell infiltration was observed, however, 0d group showed obvious bleeding. Either on 1 d, 2 d and 3 d after reperfusion, biopsies revealed that significant infiltrations of inflammatory (mononuclear cells) and edema of the intercellular substance in myocardial tissue, in comparison with biopsies from sham groups (HE staining, **Figure 1B**). Masson Trichrome staining demonstrated that cardiac perivascular fibrosis in biopsies of mice with 1 d, 2 d and 3 d after reperfusion was significantly greater than in sham controls and mice with 0 day of ischemia reperfusion (**Figure 1B**).

Since PTEN-mediated β-catenin signaling regulates innate immune responses in mouse liver ischemia/reperfusion injury [13], we were keen to determine the effect of PTEN-mediated β-catenin signaling in heart ischemia/reperfusion injury.

Cardiac ischemia/reperfusion induces rapid and time-dependent degradation of β-catenin

Real-time PCR was used to detect the mRNA expression of β-catenin in mice underwent different period of perfusion varying from day 0 to days 3: sham-operated mice, I/R mice with perfusion at day 0 (samples were obtained immediately when transplantation was done), I/R mice with perfusion at day 1, I/R mice with perfusion at day 2, and I/R mice with perfusion at day 3 (n=3/group). Compared with shame group, β-catenin mRNA expression was decreased during the perfusion phase from day 0 to day 1, and remained the same level until 3 days after perfusion (**Figure 1C**). We investigated whether such β-catenin reduction was related to changes in other key cardiac signaling pathways.

Compared with shame group, mRNA expression of PTEN was significantly increased during the perfusion phase from day 0 to day 3 ($P<0.01$ both on day 1 and day 2). The PTEN level was significantly increased at 1 day of reperfusion ($P<0.01$), but then decreased at the IR 2 days compared with IR 1 day of reperfusion time point, however, when compared with controls, it was still significantly up-regulated ($P<0.01$) (**Figure 1C**). We further investigated whether the decreased β-catenin or increased PTEN transcript levels detected by real-time PCR correlated to decreased or increased levels of the corresponding protein, as well as what is the event of its upstream (ROCK1/ROCK2) or downstream of PTEN pathways during IR injury. By using Western Blot analysis, β-catenin, ROCK1, ROCK2, PTEN, and NF-KB protein levels were detected. Results showed that β-catenin protein accumulation was decreased at IR 1 day and 2 days reperfusion time points.

ROCK1, ROCK2, and NF-κB protein levels were decreased at 0 day of reperfusion, but increased at 1 day and 2 days reperfusion compared with sham control (**Figures 1D** and S1).

Cardiac transplant induced heart IR injury is closely associated with the down-regulation of β-catenin and up-regulation of ROCK1 and PTEN expression

However, whether β-catenin levels are altered at early time points of cardiac transplantation induced myocardial ischemia, the molecular mechanisms involved and the impact of such changes in β-catenin related signaling networks has not been fully investigated. In our study, when heart was removed from the donor, it was subjected to 5 min, 10 min, and 15 min of cold ischemia followed by 1 day of subsequent reperfusion. To our knowledge, the pattern of β-catenin levels at such initial times of I/R has not been previously described.

In order to evaluate the injury of cardiac myocytes during reischemia reperfusion, HE and Masson staining were performed. According to the results of HE staining, myocardial cell structure was intact in the sham operation group, and no bleeding or inflammatory cell infiltration was observed. However, with the prolonged period of ischemia, myocardial tissue begun to be surrounded by thick layer of inflammation, and the cells were disordered and degraded. When at 15 min time point, myocardial tissue showed obvious bleeding and necrosis. The results of Masson staining also showed that the degree of myocardial fibrosis increased greatly when ischemia time was prolonged (**Figure 2A**).

Real-time PCR was used to detect the mRNA expression of β-catenin, ROCK1, ROCK2, PTEN, and NF-κB in mice subjected to different ischemic time point (sham, 5 min, 10 min and 15 min after ischemia). Compared with shame group, mRNA expression of β-catenin underwent a decreased pattern with the ischemic time point increased. However, there were no significant changes observed for ROCK1 mRNA expression during the whole period. For ROCK2, in general, there was a decline trend although it underwent an up-regulation at 10 min. It is obvious that mRNA expression of β-catenin was significantly increased. A similar pattern of concomitant up-modulation of NF-κB levels in early I/R was observed in mice hearts, although a little difference at 15 min time point (**Figure 2B**). Western blot analysis showed that except for a decreased β-catenin protein levels were observed, others all increased with the ischemic time increased (**Figures 2C**, **2D** and S2).

The pattern of inflammatory factors including proinflammatory cytokines (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-10) and TGF-β were evaluated in the blood serum of mice with different time point of ischemia (Sham, 5 min, 10 min, and 15 min). A decrease pattern of IL-6 was observed during the whole time course. We found that serum levels of IL-1β and TNF-α proinflammatory cytokines began to increase at 5 min time point, although decrease and remain the base level at 15 min. These cytokines are crucial in tissue injury and inflammation [25-27]. However, no significant difference was observed between each group. For IL-10, at 5 min time point, the expression of IL-10 increased significantly, and peaked at 10 min, compared to other time points. However, the whole time course witnessed a decreased pattern of TGF-β levels (**Figure 2E**).

The over-expression of β-catenin inhibited the expression of PTEN and ROCK

Since it is well known that EGFR mutations usually lead to the abnormal expression of itself and promote cellular proliferation in lung cancer, we investigated the potential fact whether β-catenin over-expression on ROCK/PTEN signal transduction exists in cardiomyocyte. HL-1 cell lines were used in the present study, β-catenin plasmid DNA with different dosage (1, 2, 3, 4, 5 ug), and plasmid with blank were transfected into cells. 4 μg were selected as the optimal dosage for β-catenin plasmid, as at such a dosage (**Figure 3A**).

We found a significant increase in the expression of β-catenin mRNA and protein was observed after transfection with β-catenin plasmid DNA in HL-1 cells, when compared with PB-IP and control group. However, we also noted that β-catenin over-expressing cardiomyocytes showed decreased ROCK1, ROCK2, PTEN, and NF-KB mRNA expression. Significant down-regulation of PTEN both at mRNA and protein levels were observed in β-catenin over-expressing HL-1 cells ($P<0.01$), when compared with others (**Figures 3B**, **3C** and S3). Our *in vitro*

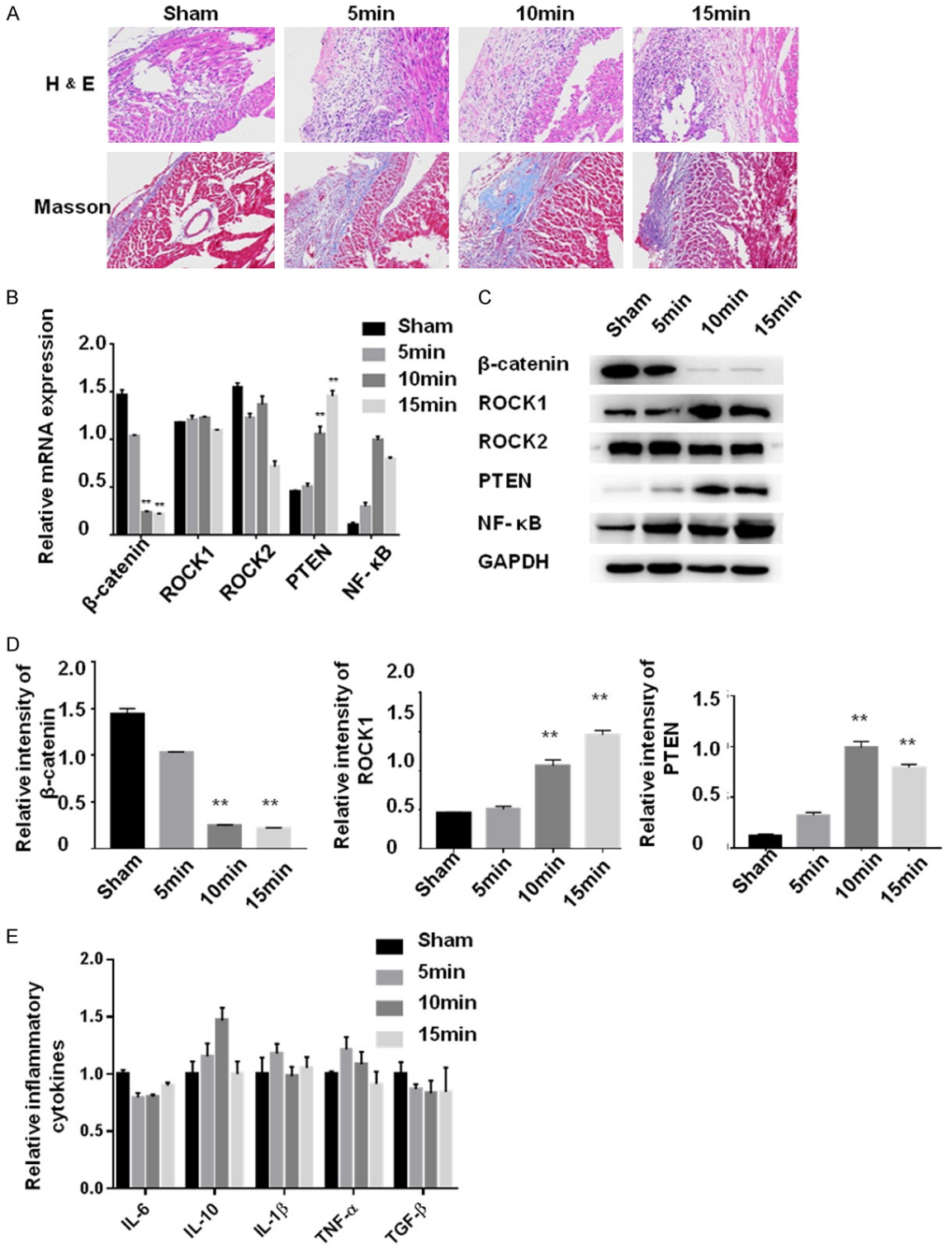

Figure 2. Cardiac ischemia/reperfusion induces rapid and time-dependent degration of β-catenin. A. Histology analysis. The upper panel is HE staining. After transplantation, heart grafts were harvested for H&E staining, at different ischemia time points, 5 min, 10 min and 15 min. The lower panel is Mason staining. Representative images from 3 independent grafts per group. Images were taken at ×200 magnification. B. Real-time PCR was used to detect the mRNA expression of β-catenin, ROCK1, ROCK2, PTEN, and NF-κB in the mice exposed to different period of ischemic at 5 min, 10 min, and 15 min: sham-operated mice, I/R mice with ischemic for 5 min, I/R mice with ischemic for 10 min, and I/R mice with ischemic for 15 min (n=3/group). Compared with shame group, mRNA expres-

sion of β-catenin underwent a decreased pattern with the ischemic time point increased. However, there were no significant changes observed for ROCK1 mRNA expression during the whole period. For ROCK2, in general, it was a decline trend although underwent an up-regulation at 10 min. It is obvious that mRNA expression of β-catenin was significantly increased. Similar trend was observed at NF-KB, although a little difference at 15 min time point. Columns, mean of three independent experiments; bars, SD (n=3). *, $P<0.05$, **, $P<0.01$. C. Western blot analysis was used to detect the expression of β-catenin, ROCK1, ROCK2, PTEN and NF-κB in the mice with cold ischemia at 5 min, 10 min and 15 min time point. GAPDH is used as a loading control. D. β-catenin protein accumulation was decreased at 5 min, 10 min and 15 min time points. PTEN and NF-KB protein levels were decreased at 0 day of reperfusion, but increased at 1 day and 2 days reperfusion compared with sham control. (Right Panel) Band intensity was expressed compared with total GAPDH. E. ELISA was used to detect the serum pattern of inflammatory factors including proinflammatory cytokines (IL-1β, IL-6, TNF-α), anti-inflammatory cytokines (IL-10) and TGF-β mice with different time point of ischemia (Sham, 5 min, 10 min, and 15 min). Columns, mean of three independent experiments; bars, SD (n=3). *, $P<0.05$, **, $P<0.01$.

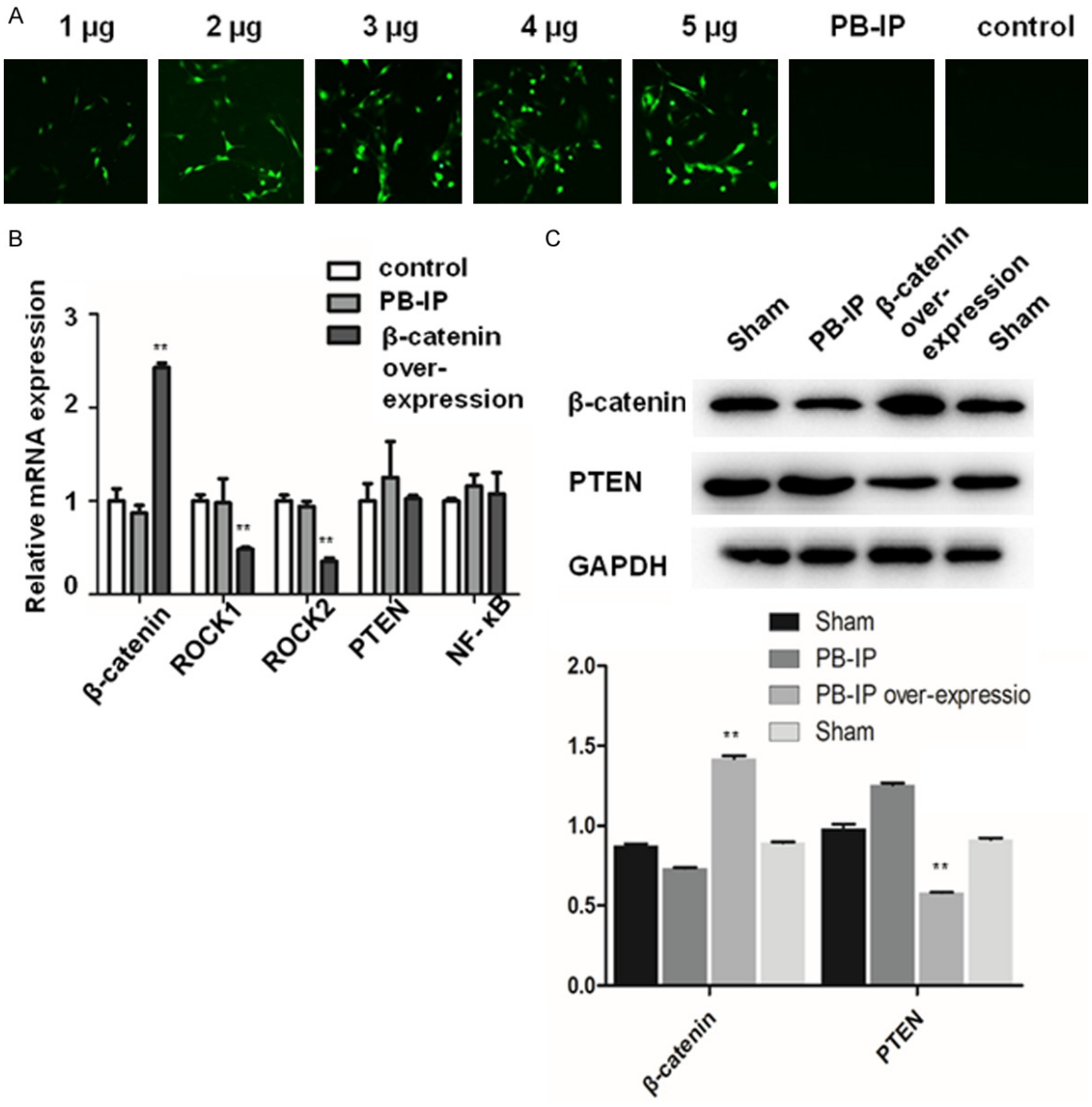

Figure 3. Over-expression of β-catenin inhibited the expression of PTEN and ROCK in HL-1 cells. A. β-catenin plasmid DNA with different dosage (1, 2, 3, 4, 5 ug), and black plasmid were transfected into HL-1 cells. 4 ug were chose and cells were divided into three groups: Control, PB-IP and β-catenin over-expression group. Cell density was monitored after treatment. Typical pictures are shown. Original magnification, ×100. B. The results of the real-time PCR. Real-time PCR was used to detect the mRNA expression of β-catenin, ROCK1, ROCK2, PTEN, and NF-κB in the three

groups. Columns, mean of three independent experiments; bars, SD (n=3). *, $P<0.05$, **, $P<0.01$. C. Western blot analysis. Western blot was used to determine the protein expression of β-catenin, and PTEN in the three groups, which were detected significantly altered for the mRNA level. GAPDH is used as a loading control.

study suggests that β-catenin could inhibit ROCK, and PTEN pathways.

$CoCl_2$-induced activation of ROCK1/PTEN signaling pathway was attenuated by transient transfection of a β-catenin over-expressing plasmid

To further investigate the role of β-catenin and its related signaling in injury in *vitro*, we utilized $CoCl_2$-mediated oxidant injury as an *in vitro* model of heart ischemic reperfusion injury, since $CoCl_2$ was often used as hypoxia mimic reagent and is able to establish hepatic I/R injury *in vitro* [28]. Here, HL-1 cells were treated with $CoCl_2$ (500-1000 μM) for 12 h, 24 h and 36 h, respectively. OD was detected in HL-1 cells treated with different concentrations (500, 600, 700, 800, 900, and 1000 μM). We revealed that the OD value in cells treated with 600 μM at 24 h time point was significantly lower than that in other groups (**Figure 4A**), the optimal concentration was 600 μM at 24 h and it was therefore selected for the further study. Cells were selected into the four groups: Control group, $CoCl_2$ group (HL-1 cells treated with $CoCl_2$), $CoCl_2$+PB-IP group (HL-1 cells with blank plasmid transfection and treated with $CoCl_2$), and β-catenin+$CoCl_2$ group (Cells transfected with β-catenin over-expressing plasmid and treated with $CoCl_2$). Real-time PCR and Western Blot analysis were used to detect ROCK1, ROCK2, PTEN, and NF-kB levels. Compared with controls, $CoCl_2$ treated cells showed significantly increased ROCK1, ROCK2, PTEN, and NF-κB levels (**Figures 4B**, **4C** and S4). However, in β-catenin+$CoCl_2$ group, we demonstrated that $CoCl_2$-induced activation of ROCK1/PTEN signaling pathway was attenuated by transfection of a β-catenin over-expressing plasmid. **Figure 4D** displayed schematic illustration of the role of β-catenin and its related PTEN/ROCK pathways during myocardial Ischemia/Reperfusion injury following heterotopic heart transplantation in mice.

Discussion

Ischemia and reperfusion injury (IRI) is a major pathogenesis to cause heart dysfunction and failure following heart transplantation, which is a key cause of death and disability. Prompt restoration of blood flow to the ischemic area is crucial for reducing infarct size and mortality, but it can itself trigger additional myocardial damage, termed reperfusion injury. Despite advances in the protocols allowing rapid and effective reperfusion in myocardial ischemia patients, therapies adequately targeting reperfusion injury remain elusive. Therefore, it is crucial to gather information about the status of key cardiac signaling pathways at early stages after I/R to identify potential new targets for intervention. Experiments on myocardial ischemia/reperfusion model demonstrated that β-catenin regulates IR injury, but the underlying mechanism in heart IR remains unknown. Even less is known about the effects of ischemia/reperfusion in the heart transplantation models.

In the present study, we performed systematic analysis of β-catenin and ROCK/PTEN signal pathways during ischemia and reperfusion (with different time points) using heterotopic heart transplantation mice model. We have investigated (a) β-catenin and the activations of ROCK/PTEN signal pathways during reperfusion, as during heart transplantation, difference may exist between the gene responses evident in initial, intermediate, and prolonged reperfusion times when following an acute ischemic insult. (b) β-catenin and its related signal pathways during early ischemia time, since early ischemia may decrease the ischemia-reperfusion (IR) injury than prolonged ischemia, which is related to the survival rate after transplantation (c) the relationship between β-catenin and ROCK/PTEN signal pathways *in vitro* (d) the role of β-catenin playing in the process of I/R *in vitro* cell model.

β-catenin is a very relevant signaling hub in cardiac physiopathology [29]. In our study, firstly, we found that during transplantation, cardiac ischemia/reperfusion induces a rapid and time-dependent degradation of β-catenin either in reperfusion time point. Ke et al. (2013) reported that β-catenin could regulate immune responses in hepatic IR injury and is a novel

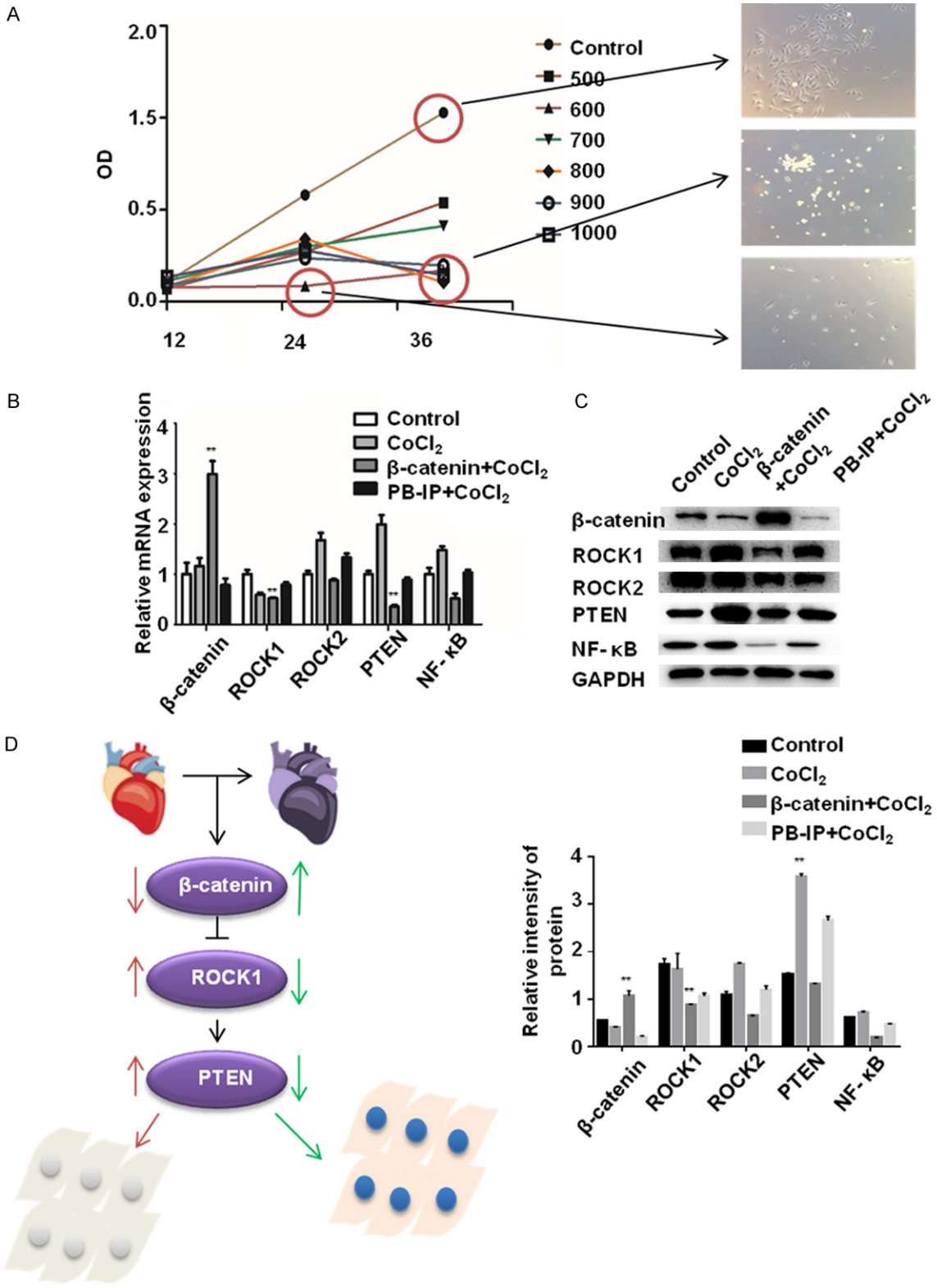

Figure 4. $CoCl_2$-induced activation of ROCK1/PTEN signaling pathway were attenuated by β-catenin over-expression. A. HL-1 cells were treated with $CoCl_2$ (500-1000 μM) for 12 h, 24 h and 36 h, respectively. OD was detected in HL-1 cells treated with different concentrations (500, 600, 700, 800, 900, and 1000 μM). Data are expressed as the OD value. Data shown are means ± SD (n=3). B. The results of the real-time PCR. Real-time PCR was used to detect the mRNA expression of β-catenin, ROCK1, ROCK2, PTEN, and NF-κB in the four groups: Control group, $CoCl_2$ group (HL-1 cells treated with $CoCl_2$), $CoCl_2$ +PB-IP group (HL-1 cells with black plasmid transfection and treated with

$CoCl_2$), and β-catenin+$CoCl_2$ group (Cells transfected with β-catenin over-expressing plasmid and treated with $CoCl_2$). C. Western blot analysis. Western blot was used to determine the protein expression of β-catenin, ROCK1, ROCK2, PTEN, and NF-KB in the four groups (Upper panel). Band intensity was expressed compared with total GAPDH (Down Panel). D. Schematic illustration of the role of β-catenin and its related PTEN/ROCK pathways during myocardial Ischemia/Reperfusion injury following heterotopic heart transplantation in mice.

regulator of innate and adaptive immune responses in the mechanism of liver IRI [14]. Others found that β-catenin is up-regulated in the long-term of patients and experimental models of heart failure. However, in our study, β-catenin mRNA expression was decreased during the perfusion phase from day 0 to day 1, and it remains the same level until 3 days after perfusion, which suggests a time dependent expression of β-catenin in heart I/R injury.

PTEN, a tumor suppressor gene, is an essential regulator of cell proliferation, apoptosis, differentiation, migration, etc. Recently, studies have found that PTEN plays an important role in myocardial remodeling, cardiac hypertrophy, myocardial fibrosis and myocardial ischemia reperfusion injury [30, 31]. Ruan et al. (2009) found that PTEN may play a role in ischemia/reperfusion injury by inhibiting antiapoptotic survival signals [32]. Inhibiting PTEN may serve as a potential approach to exert cardiac protection against ischemia reperfusion injury. In our study, we found that PTEN was significantly up-regulated with the prolonged reperfusion time, suggested that activation of PTEN plays an important role in the IR injury during heart transplantation, and its activation is positively associated with reperfusion time.

ROCK belongs to the family of serine/threonine kinases and includes two isoforms: ROCK1 and ROCK2. ROCK plays important roles in the regulation of platelet activation. ROCK is a major regulator of cell contractility, motility, and proliferation. Many of these ROCK-mediated processes in endothelial cells, vascular smooth muscle cells, pericytes, astrocytes, glia, neurons, leukocytes, and platelets are important in stroke pathophysiology, and the inhibition of such processes could improve stroke outcome. It has been demonstrated that ROCK participate in the event of heart I/R injury [33, 34]. ROCK1 promotes migration and invasion of non-small-cell lung cancer cells through the PTEN/PI3K/FAK pathway [35].

In our initial study designed to detect the molecular mechanism of β-catenin and its related signal pathways in mice underwent different period of reperfusion (0, 1, 2, and 3 days), we found that with the reperfusion time increased, an inhibition of β-catenin pattern occurred in transplanted induced IR injury, while ROCK1 and PTEN increased during the reperfusion phase from day 0 to day 1, and remain the same level until 3 days after reperfusion.

Since early ischemia time may decrease the ischemia and reperfusion injury than prolonged ischemia time [36, 37], the role of β-catenin in I/R dynamic injury during heart transplantation was conducted. When hearts were removed from the donor, a robust mouse was subjected to 5, 10, and 15 min of cold ischemia followed by 1 day of subsequent reperfusion. The molecular mechanism on β-catenin and ROCK/PTEN pathway in I/R injury following heart transplantation were also investigated. The time point selected in our study is different from previous studies which investigated IR injury in murine heart transplantation utilized ischemia time varying from 18 to 24 hours [38]. We found that mRNA expression of β-catenin underwent a decreased pattern with the ischemic time point increased. However, PTEN mRNA expression was significantly activated during the whole period. For ROCK2, in general, it has a decline trend although underwent an up-regulation at 10 min. It is obvious that mRNA expression of β-catenin was significantly increased. The above data suggest that cardiac transplant induced heart IR injury is closely associated with the down-regulation of β-catenin and up-regulation of ROCK1 and PTEN expression.

Subsequently, we poured our attention to analyze the relationship of β-catenin and ROCK/PTEN pathways. We found that β-catenin over-expressing cardiomyocytes showed decreased ROCK1/PTEN expression both at mRNA and protein levels. In addition, cobalt dichloride ($CoCl_2$)-induced oxidative stress model was further established to mimic cardiac I/R injury. We found that $CoCl_2$-induced activation of ROCK1/PTEN signaling pathway were attenuated by

transient transfection of a β-catenin over-expressing plasmid. It is, therefore, concluded that over-expression of β-catenin, might play a protective role in the process I/R injury, or at least participate in the process of transplantation induced heart I/R injury, by inhibit ROCK/PTEN pathways.

In summary, to our knowledge, our study is the first time to investigate the protective role of β-catenin and its related pathways in transplanted induced I/R injury. It suggests that over-expression of β-catenin seems to ameliorate transplanted induced I/R injury by inhibiting ROCK1/PTEN signaling pathway. However, more studies, like *in vivo* studies on over-expression of β-catenin to detect improved cardiac function, modulated oxidative injury, repressed inflammatory damage, should be carried out to verify this hypothesis.

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Disclosure of conflict of interest

None.

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References

[1] Ayesta A, Urrutia G, Madrid E, Vernooij RWM, Vicent L and Martinez-Selles M. Sex-mismatch influence on survival after heart transplantation. A systematic review and meta-analysis of observational studies. Clin Transplant 2019; 33: e13737.

[2] Kim IC, Youn JC and Kobashigawa JA. The past, present and future of heart transplantation. Korean Circ J 2018; 48: 565-590.

[3] Zheng SG, Meng L, Wang JH, Watanabe M, Barr ML, Cramer DV, Gray JD and Horwitz DA. Transfer of regulatory T cells generated ex vivo modifies graft rejection through induction of tolerogenic CD4+CD25+ cells in the recipient. Int Immunol 2006; 18: 279-289.

[4] Yellon DM and Hausenloy DJ. Myocardial reperfusion injury. N Engl J Med 2007; 357: 1121-1135.

[5] Chen C, Yao W, Wu S, Zhou S, Ge M, Gu Y, Li X, Chen G, Bellanti JA, Zheng SG, Yuan D and Hei Z. Crosstalk between Connexin32 and mitochondrial apoptotic signaling pathway plays a pivotal role in renal ischemia reperfusion-induced acute kidney injury. Antioxid Redox Signal 2019; 30: 1521-1538.

[6] Zhang Y, Moszczynski LA, Liu Q, Jiang J, Zhao D, Quan D, Mele T, McAlister V, Jevnikar A, Baek SJ, Liu K and Zheng X. Over-expression of growth differentiation factor 15 (GDF15) preventing cold ischemia reperfusion (I/R) injury in heart transplantation through Foxo3a signaling. Oncotarget 2017; 8: 36531-36544.

[7] Al-Awar A, Almasi N, Szabo R, Takacs I, Murlasits Z, Szucs G, Torok S, Posa A, Varga C and Kupai K. Novel potentials of the DPP-4 inhibitor sitagliptin against ischemia-reperfusion (I/R) injury in rat ex-vivo heart model. Int J Mol Sci 2018; 19: 3226.

[8] Zhang W, Ren H, Xu C, Zhu C, Wu H, Liu D, Wang J, Liu L, Li W, Ma Q, Du L, Zheng M, Zhang C, Liu J and Chen Q. Hypoxic mitophagy regulates mitochondrial quality and platelet activation and determines severity of I/R heart injury. Elife 2016; 5: e21407.

[9] Tan Z, Jiang R, Wang X, Wang Y, Lu L, Liu Q, Zheng SG, Sun B and Ryffel B. RORgammat+IL-17+ neutrophils play a critical role in hepatic ischemia-reperfusion injury. J Mol Cell Biol 2013; 5: 143-146.

[10] Manigandan K, Manimaran D, Jayaraj RL, Elangovan N, Dhivya V and Kaphle A. Taxifolin curbs NF-kappaB-mediated Wnt/beta-catenin signaling via up-regulating Nrf2 pathway in experimental colon carcinogenesis. Biochimie 2015; 119: 103-112.

[11] Jang J, Jung Y, Chae S, Bae T, Kim SM, Shim YJ, Chung SI and Yoon Y. XAV939, a Wnt/beta-catenin pathway modulator, has inhibitory effects on LPS-induced inflammatory response. Immunopharmacol Immunotoxicol 2019; 41: 394-402.

[12] Mohamed HT, Gadalla R, El-Husseiny N, Hassan H, Wang Z, Ibrahim SA, El-Shinawi M, Sherr DH and Mohamed MM. Inflammatory breast

cancer: activation of the aryl hydrocarbon receptor and its target CYP1B1 correlates closely with Wnt5a/b-beta-catenin signalling, the stem cell phenotype and disease progression. J Adv Res 2019; 16: 75-86.
[13] Kamo N, Ke B, Busuttil RW and Kupiec-Weglinski JW. PTEN-mediated Akt/beta-catenin/Foxo1 signaling regulates innate immune responses in mouse liver ischemia/reperfusion injury. Hepatology 2013; 57: 289-298.
[14] Ke B, Shen XD, Kamo N, Ji H, Yue S, Gao F, Busuttil RW and Kupiec-Weglinski JW. beta-catenin regulates innate and adaptive immunity in mouse liver ischemia-reperfusion injury. Hepatology 2013; 57: 1203-1214.
[15] Zhou M, Fang H, Du M, Li C, Tang R, Liu H, Gao Z, Ji Z, Ke B and Chen XL. The modulation of regulatory T cells via HMGB1/PTEN/beta-Catenin axis in LPS induced acute lung injury. Front Immunol 2019; 10: 1612.
[16] Li P, Zhang Y and Liu H. The role of Wnt/beta-catenin pathway in the protection process by dexmedetomidine against cerebral ischemia/reperfusion injury in rats. Life Sci 2019; 236: 116921.
[17] Steinbruchel DA, Nielsen B, Salomon S and Kemp E. A new model for heterotopic heart transplantation in rodents: graft atrial septectomy. Transplant Proc 1994; 26: 1298-1299.
[18] Fukunaga N, Bissoondath V and Rao V. Custom-made Clamp facilitating stabilization of cuffs in murine heterotopic cervical heart transplantation. Transplantation 2019; 103: e62-e63.
[19] Hikawa H, Lotze MT and Rosenblum WD. Induction of peripheral tolerance by local delivery of dendritic cell progenitors to cardiac allografts in a murine heterotopic heart transplantation model. Gen Thorac Cardiovasc Surg 2007; 55: 307-314.
[20] Szabo G, Batkai S, Bahrle S, Dengler TJ, Vahl CF, Zimmermann R and Hagl S. Effects of nitric oxide synthesis on reperfusion injury and catecholamine responsiveness in a heterotopic rat heart-transplantation model. J Cardiovasc Pharmacol 1998; 31: 221-230.
[21] Fiorillo C, Pace S, Ponziani V, Nediani C, Perna AM, Liguori P, Cecchi C, Nassi N, Donzelli GP, Formigli L and Nassi P. Poly(ADP-ribose) polymerase activation and cell injury in the course of rat heart heterotopic transplantation. Free Radic Res 2002; 36: 79-87.
[22] Westhofen S, Jelinek M, Dreher L, Biermann D, Martin J, Vitzhum H, Reichenspurner H, Ehmke H and Schwoerer AP. The heterotopic heart transplantation in mice as a small animal model to study mechanical unloading - Establishment of the procedure, perioperative management and postoperative scoring. PLoS One 2019; 14: e0214513.
[23] Benke K, Sayour AA, Matyas C, Agg B, Nemeth BT, Olah A, Ruppert M, Hartyanszky I, Szabolcs Z, Radovits T, Merkely B and Szabo G. Heterotopic abdominal rat heart transplantation as a model to investigate volume dependency of myocardial remodeling. Transplantation 2017; 101: 498-505.
[24] Li Y, Xie B, Zhu M, Li X, Du F, Li Y, Li M, Yuan Q, Hua J, Xue M, Wang Z, Xia J and Qi Z. A highly reproducible cervical cuff technique for rat-to-mouse heterotopic heart xenotransplantation. Xenotransplantation 2017; 24.
[25] Luo Y and Zheng SG. Hall of fame among pro-inflammatory cytokines: interleukin-6 gene and its transcriptional regulation mechanisms. Front Immunol 2016; 7: 604.
[26] Yang S, Wang J, Brand DD and Zheng SG. Role of TNF-TNF receptor 2 signal in regulatory t cells and its therapeutic implications. Front Immunol 2018; 9: 784.
[27] Kida Y, Xia Z, Zheng S, Mordwinkin NM, Louie SG, Zheng SG, Feng M, Shi H, Duan Z and Han YP. Interleukin-1 as an injury signal mobilizes retinyl esters in hepatic stellate cells through down regulation of lecithin retinol acyltransferase. PLoS One 2011; 6: e26644.
[28] Tan R, Tian H, Yang B, Zhang B, Dai C, Han Z, Wang M, Li Y, Wei L, Chen D, Wang G, Yang H, He F and Chen Z. Autophagy and Akt in the protective effect of erythropoietin helix B surface peptide against hepatic ischaemia/reperfusion injury in mice. Sci Rep 2018; 8: 14703.
[29] Ivanyuk D, Budash G, Zheng Y, Gaspar JA, Chaudhari U, Fatima A, Bahmanpour S, Grin VK, Popandopulo AG, Sachinidis A, Hescheler J and Saric T. Ascorbic acid-induced cardiac differentiation of murine pluripotent stem cells: transcriptional profiling and effect of a small molecule synergist of wnt/beta-catenin signaling pathway. Cell Physiol Biochem 2015; 36: 810-830.
[30] Yan B, Singla RD, Abdelli LS, Singal PK and Singla DK. Regulation of PTEN/Akt pathway enhances cardiomyogenesis and attenuates adverse left ventricular remodeling following thymosin beta4 Overexpressing embryonic stem cell transplantation in the infarcted heart. PLoS One 2013; 8: e75580.
[31] Agbo E, Liu D, Li M, Saahene RO, Chen L, Zhao L, Wang Y and Tian G. Modulation of PTEN by hexarelin attenuates coronary artery ligation-induced heart failure in rats. Turk J Med Sci 2019; 49: 945-958.
[32] Ruan H, Li J, Ren S, Gao J, Li G, Kim R, Wu H and Wang Y. Inducible and cardiac specific PTEN inactivation protects ischemia/reperfusion injury. J Mol Cell Cardiol 2009; 46: 193-200.
[33] Duan JS, Chen S, Sun XQ, Du J and Chen ZW. Urotensin-#receptor antagonist SB-706375

protected isolated rat heart from ischaemia-reperfusion injury by attenuating myocardial necrosis via RhoA/ROCK/RIP3 signalling pathway. Inflammopharmacology 2019; 27: 1309-1318.

[34] Ming D, Songyan L, Yawen C, Na Z, Jing M, Zhaowen X, Ye L, Wa D and Jie L. trans-Polydatin protects the mouse heart against ischemia/reperfusion injury via inhibition of the renin-angiotensin system (RAS) and Rho kinase (ROCK) activity. Food Funct 2017; 8: 2309-2321.

[35] Hu C, Zhou H, Liu Y, Huang J, Liu W, Zhang Q, Tang Q, Sheng F, Li G and Zhang R. ROCK1 promotes migration and invasion of nonsmallcell lung cancer cells through the PTEN/PI3K/FAK pathway. Int J Oncol 2019; 55: 833-844.

[36] Cai W, Liu S, Hu M, Huang F, Zhu Q, Qiu W, Hu X, Colello J, Zheng SG and Lu Z. Functional dynamics of neutrophils after ischemic stroke. Transl Stroke Res 2020; 11: 108-121.

[37] Cai W, Wang J, Hu M, Chen X, Lu Z, Bellanti JA and Zheng SG. All trans-retinoic acid protects against acute ischemic stroke by modulating neutrophil functions through STAT1 signaling. J Neuroinflammation 2019; 16: 175.

[38] Andreeva K, Zhang M, Fan W, Li X, Chen Y, Rebolledo-Mendez JD and Cooper NG. Time-dependent gene profiling indicates the presence of different phases for ischemia/reperfusion injury in retina. Ophthalmol Eye Dis 2014; 6: 43-54.

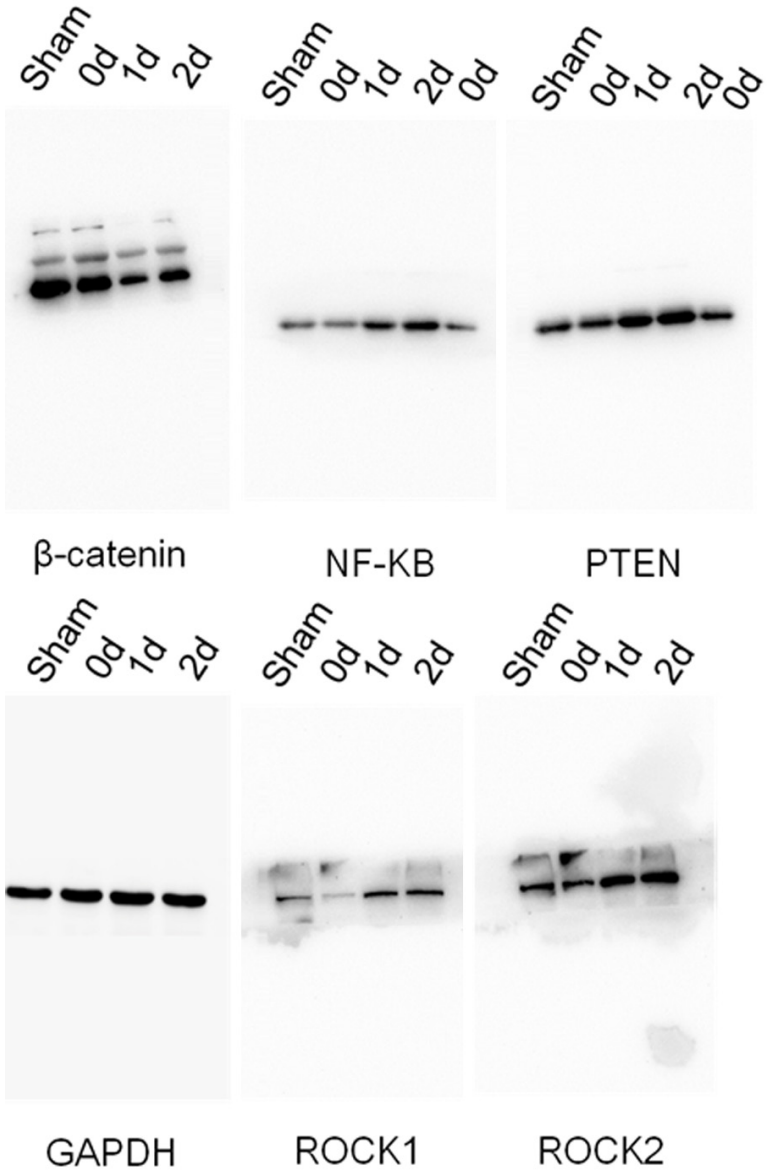
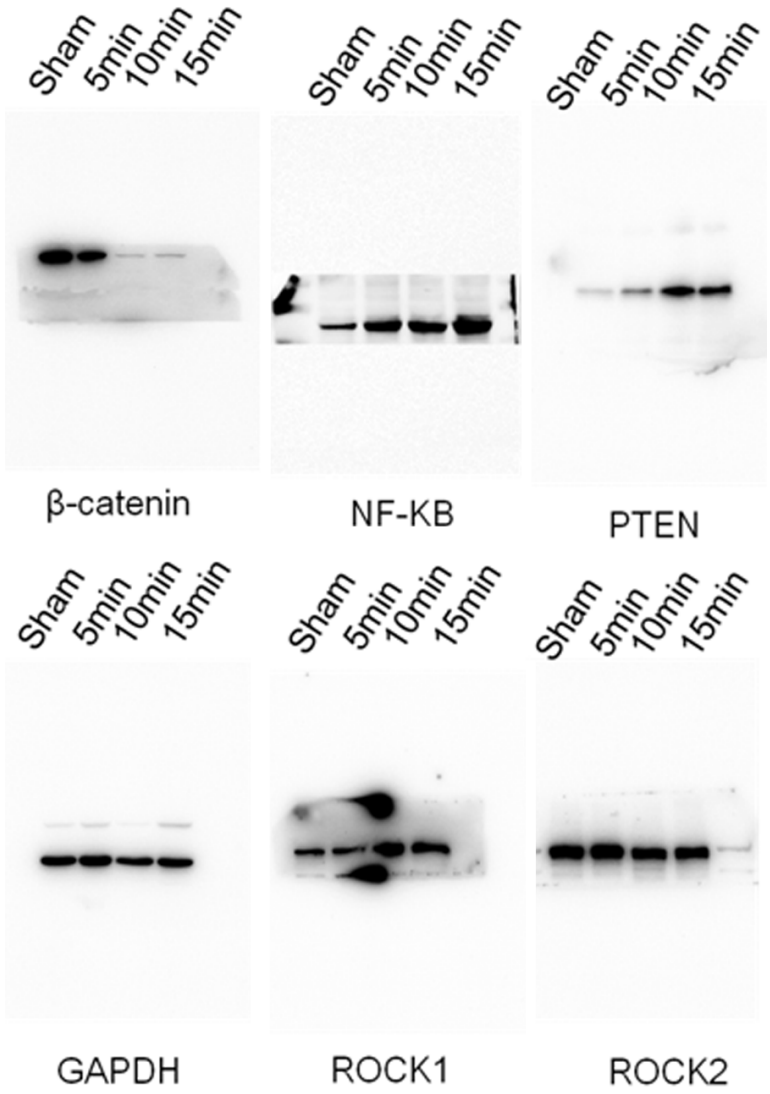


Figure S1. Original western images for relevant western blots of **Figure 1D**.



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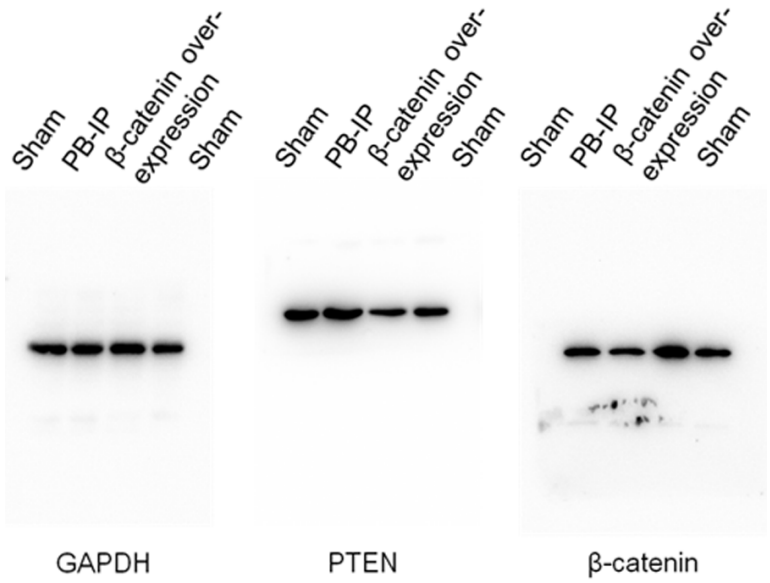

Figure S3. Original western images for relevant western blots of **Figure 3C**.

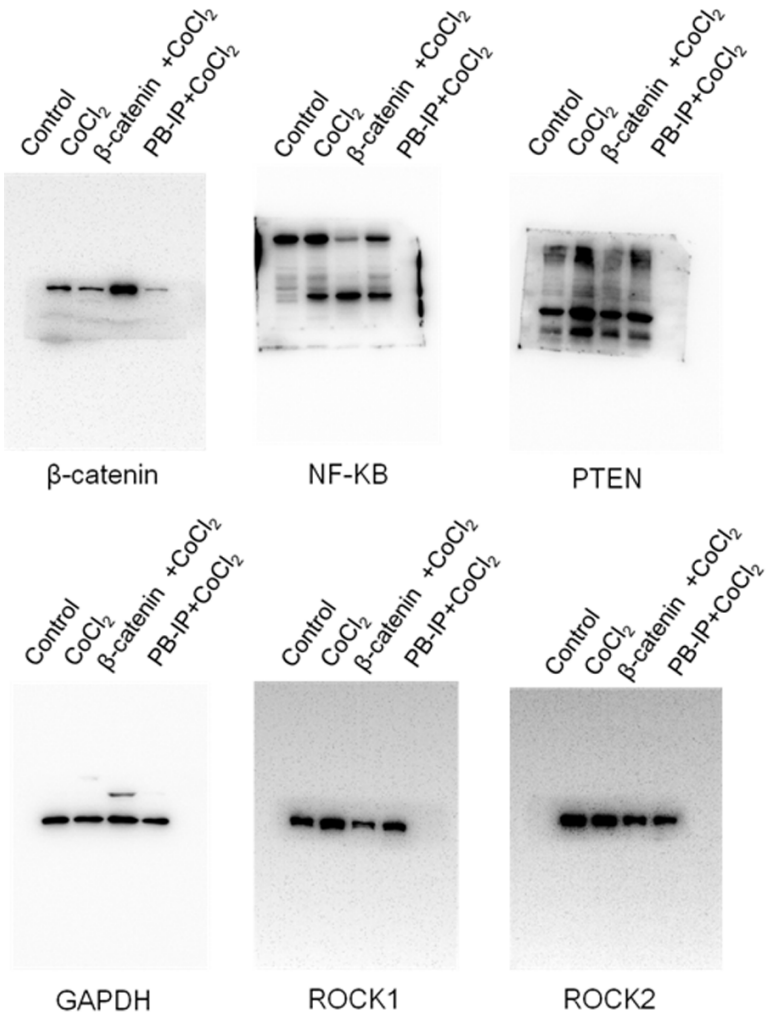

Figure S4. Original western images for relevant western blots of **Figure 4C**.