# Downregulation of cardiac PIASy inhibits Cx43 SUMOylation and ameliorates ventricular arrhythmias in a rat model of myocardial ischemia/reperfusion injury

**Tingting Wang[1], Jinmin Liu[1,2], Chenchen Hu[1], Xin Wei[1], Linlin Han[1], Afang Zhu[1], Rong Wang[1], Zhijun Chen[2], Zhengyuan Xia[3], Shanglong Yao[1], Weike Mao[1]**

[1]Department of Anesthesiology, Institute of Anesthesiology and Critical Care Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022, China;
[2]Department of Anesthesiology, Wuhan No. 1 Hospital, Wuhan, Hubei 430022, China;
[3]State Key Laboratory of Pharmaceutical Biotechnology, Department of Medicine, The University of Hong Kong, Hong Kong, China.

**Abstract**

**Background:** Dysfunction of the gap junction channel protein connexin 43 (Cx43) contributes to myocardial ischemia/reperfusion (I/R)-induced ventricular arrhythmias. Cx43 can be regulated by small ubiquitin-like modifier (SUMO) modification. Protein inhibitor of activated STAT Y (PIASy) is an E3 SUMO ligase for its target proteins. However, whether Cx43 is a target protein of PIASy and whether Cx43 SUMOylation plays a role in I/R-induced arrhythmias are largely unknown.

**Methods:** Male Sprague–Dawley rats were infected with PIASy short hairpin ribonucleic acid (shRNA) using recombinant adeno-associated virus subtype 9 (rAAV9). Two weeks later, the rats were subjected to 45 min of left coronary artery occlusion followed by 2 h reperfusion. Electrocardiogram was recorded to assess arrhythmias. Rat ventricular tissues were collected for molecular biological measurements.

**Results:** Following 45 min of ischemia, QRS duration and QTc intervals statistically significantly increased, but these values decreased after transfecting PIASy shRNA. PIASy downregulation ameliorated ventricular arrhythmias induced by myocardial I/R, as evidenced by the decreased incidence of ventricular tachycardia and ventricular fibrillation, and reduced arrythmia score. In addition, myocardial I/R statistically significantly induced PIASy expression and Cx43 SUMOylation, accompanied by reduced Cx43 phosphorylation and plakophilin 2 (PKP2) expression. Moreover, PIASy downregulation remarkably reduced Cx43 SUMOylation, accompanied by increased Cx43 phosphorylation and PKP2 expression after I/R.

**Conclusion:** PIASy downregulation inhibited Cx43 SUMOylation and increased PKP2 expression, thereby improving ventricular arrhythmias in ischemic/reperfused rats heart.

**Keywords:** Connexin 43; Myocardial reperfusion injury; PIASy; PKP2; SUMOylation; Arrhythmias

## Introduction

The incidence of coronary heart disease is increasing year by year worldwide. For patients with myocardial infarction, timely myocardial reperfusion therapy has become the main strategy for myocardial salvage.[1] However, reperfusion can further exacerbate myocardial ischemia/reperfusion (I/R) injury, which can cause reperfusion arrhythmia.[2] Therefore, intensive research has focused on the pathophysiological mechanisms related to reperfusion arrhythmia and the development of potential therapeutic strategies.

Connexin 43 (Cx43) is a major component of gap junctions and is present in the cardiac intercalated disc.[3] Cx43 plays a fundamental role in facilitating electrical transmission between adjacent cardiac myocytes in excitable tissues. In the heart, Cx43 is predominantly expressed in ventricular myocytes.[4,5] Without the expression of Cx43, normal propagation is disrupted, leading to arrhythmias.[6] Prolonged ischemia/hypoxia (>15 min) induces the cellular redistribution of Cx43 from the intercalated disc to the periphery of cardiomyocytes.[7] All these investigations indicate that Cx43 plays

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**Correspondence to:** Prof. Weike Mao, Department of Anesthesiology, Institute of Anesthesiology and Critical Care Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022, China
E-Mail: 1984xH0556@hust.edu.cn



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central roles in arrhythmias caused by myocardial I/R. However, the underlying mechanism remains unclear.

Small ubiquitin-like modifier (SUMO)ylation is a ubiquitin-like post-translational modification catalyzed by the activating (E1), conjugating (E2), and ligating (E3) enzymes.[8] Protein inhibitor of activated STAT Y (PIASy), a member of the PIAS family of SUMO E3 ligases, is highly expressed in neonatal myocardial tissues.[9] Kjenseth *et al*[10] proved that Cx43 can be regulated by the SUMO system. PIASy is an E3 ligase specifically for SUMO modification of some target proteins, such as p53 and caveolin-3, which play roles in cardiac pathogenesis, including I/R injury.[11] However, whether PIASy mediates SUMOylation of Cx43 as a target protein and what role SUMO-modified Cx43 plays are largely unknown.

Therefore, we investigated SUMO modification of Cx43 by PIASy and its subsequent role in abnormal Cx43 expression in gap junctions after cardiac I/R using a rat model with a heart transfected with PIASy short hairpin ribonucleic acid (shRNA).

## Methods

### Animal care

Male Sprague–Dawley rats weighing 120 to 160 g were purchased from Beijing Huafukang Biotechnology Co., Ltd. (Beijing, China). They were housed in comfortable conditions for 12 h and had free access to food and water. The animal protocol was approved by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology (No. SCXK [E] 2016-0057). The procedure was conducted in strict accordance with the National Institutes of Health standards stated in the Guide for the Care and Use of Laboratory Animals.

### Construction of adeno-associated virus vectors

The recombinant adeno-associated virus serotype 9 (rAAV9) vector carrying ZsGreen-shRNA-Scramble (rAAV9-ZsGreen-shRNA-Scramble) or shRNA-PIASy (rAAV9-ZsGreen-shRNA-PIASy) was packaged and synthesized by Biowit Technologies (Shenzhen, China).

### Vector administration

Rats were anesthetized with sodium pentobarbital (Darmstadt, Germany) at a dose of 65 mg/kg intraperitoneally. With the rats in the supine position, an apical approach for puncture was selected. The area around the apex beat was dehaired and disinfected. As the electrocardiogram (ECG) was continuously observed, an insulin syringe fitted with a 29-G needle was inserted near the apex of the heart and advanced through the chest wall to the anterior left ventricle. After slowly injecting 100 μL of rAAV9 vector ($1.0 \times 10^{12}$ vg/mL) diluted with saline, the rats were placed into the specific pathogen free (SPF) animal center for feeding. After 14 days, cardiac tissue was collected for Western blotting analysis to verify whether the protein expression of PIASy had successfully interfered with adenovirus rAAV9-ZsGreen-shRNA-PIASy administration. Rats were subjected to I/R 14 days after intraventricular injection of rAAV9 vectors.

### In vivo model of myocardial ischemia/reperfusion

An *in vivo* myocardial I/R model was established by ligation of the left anterior descending coronary artery (LAD) for 45 min, followed by 2 h of reperfusion, as we described previously.[12] At the end of the treatment, rats were anesthetized by sodium pentobarbital intraperitoneally. The trachea of each rat was cannulated, and artificial ventilation was performed with a tidal volume of 4 mL. Following the thoracotomy, the LAD artery was encircled with an 8–0 silk suture. Baseline parameters were recorded after a 15 min equilibration period. Myocardial ischemia was induced by tightening the suture, which was confirmed by blanching of the distal cardiac tissue and ST-elevation on the ECG. The snare was released after 45 min to allow reperfusion. Sham-operated rats underwent the same surgical procedure but without LAD ligation. ECG and arterial pressure were recorded during the entire procedure. Fresh ventricular tissue samples in the peri-infarct region were obtained, snap frozen in liquid nitrogen, and stored at −80°C.

### Experimental grouping

Rats were randomly divided into four groups ($n = 8$/group): (1) scramble group, in which rats were injected with rAAV9-ZsGreen-shRNA-Scramble; (2) scramble + I/R group, in which the scramble group was subjected to 45 min ischemia followed by 2 h reperfusion; (3) shRNA group, in which rats were injected with rAAV9-ZsGreen-shRNA-PIASy; and (4) shRNA + I/R group, in which the shRNA group was subjected to myocardial I/R as described.

### ECG recording and analysis

A standard limb lead II was used for ECG monitoring throughout the study as previously described.[12] The characteristics of ECG were analyzed using the LabChart software package from ADInstruments Pty, Ltd. (Sydney, NSW, Australia). The P duration, PR interval, QRS duration, and QTc were recorded at different time points. According to Wei *et al*[12] and Xue *et al*,[13] ventricular arrhythmias were found to be the main types of arrhythmias induced by myocardial I/R, including ventricular premature beats (VPBs), ventricular tachycardia (VT), and ventricular fibrillation (VF). A run of three or more consecutive VPBs was defined as VT, and a run lasting ≥10 s was considered sustained VT. The durations and episodes of ventricular arrhythmias within every consecutive 10 min block after ligation were determined.

### Immunofluorescence staining

Immunofluorescence staining of frozen heart sections was carried out as described previously.[12] In brief, the sections were incubated overnight with an anti-Cx43 primary antibody (1:200, #3512S, Cell Signaling Technology, Beverly, MA, USA) at 4°C. After washing, the

sections were incubated with Alexa Fluor DyLight 549-conjugated goat anti-rabbit antibody (1:200, A23320, Abbkine Scientific Co., Ltd., Wuhan, China) at room temperature for 1 h. The nuclei of the cells were stained with 4′,6-diamidino-2-phenylindole. Fluorescence images were observed using a fluorescence microscope (BX51; Olympus, Tokyo, Japan) and photographed using a multispectral imaging system (Nuance FX, Cri, Hopkinton, MA, USA).

### Co-immunoprecipitation (co-IP) of Cx43 and SUMO2/3

Rat ventricular tissues in the peri-infarct region were lysed with radio immunoprecipitation assay lysis buffer and incubated with Protein A Plus-Agarose (Santa Cruz Biotechnology, CA, USA) or anti-Cx43 antibody (1:1000, #3512S, Cell Signaling Technology) following the manufacturer's protocols. The collected mixture was centrifuged, and the supernatant was collected for Western blotting analysis as below.

### Western blotting analysis

After 2 h of reperfusion, rat ventricular tissues in the peri-infarct region were harvested, and the procedure for Western blotting was performed as described previously.[14] The primary antibodies used in this study were as follows: anti-Cx43 (1:1000, #3512S, Cell Signaling Technology), anti-phospho-Cx43-S368 (1:1000, #3511S, Cell Signaling Technology), anti-SUMO2/3 (1:500, ab3754, abcam, USA), anti-PIASy (1:1000, sc-166706, Santa Cruz Technology, Beverly, MA, USA), anti-voltage-gated Sodium Channel 1.5 ($Na_V1.5$) (1:200, GTX54798, Gentex, San Antonio, TX, USA), and anti-plakophilin 2 (PKP2) (1:1000, GTX54579, Gentex). The secondary antibody (1:5000) was purchased from Cell Signaling Technology.

### Statistical analysis

All the results are presented as the mean ± standard deviation. GraphPad Prism software (San Diego, CA, USA) was used for statistical analysis of the data. Independent *t* test was used for comparisons between two different groups. Differences between means were compared using oneway analysis of variance (ANOVA) for repeated measures or one-way ANOVA as appropriate, using Fisher's *post hoc* test. For non-normally distributed parameters, such as episodes of ventricular arrhythmias and duration of VT and VF, the Kruskal–Wallis rank sum test was employed. The incidence of ventricular arrhythmia was analyzed using Fisher's exact test. Statistical significance was defined as $P < 0.05$.

## Results

### Transfection of shRNA-PIASy decreased the protein expression of PIASy

As shown in Figure 1, the cardiac protein expression of PIASy was statistically significantly increased after myocardial I/R (scramble group *vs*. scramble + I/R group, $P < 0.01$; shRNA group *vs*. shRNA + I/R group, $P < 0.01$). Transfection of shRNA-PIASy markedly decreased the protein expression of PIASy (scramble group *vs*. shRNA group, $P < 0.01$).

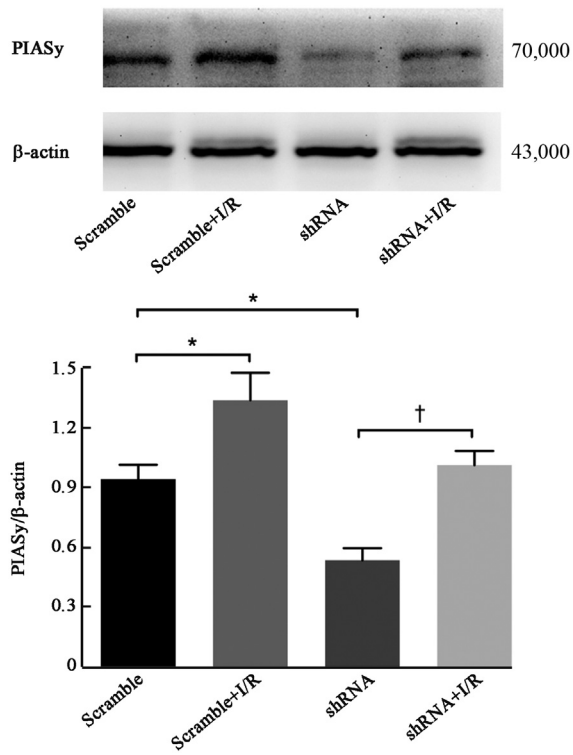


**Figure 1:** Transfection of shRNA-PIASy decreased PIASy protein expression of rats before and after myocardial I/R. (A) Representative Western blotting. (B) Myocardial protein expression levels of PIASy after I/R in the scramble and shRNA groups. The mean band density was normalized relative to β-actin. All values are presented as the mean ± standard deviation ($n = 8$/group). $^{*}P < 0.01$ compared with scramble group; $^{\dagger}P < 0.01$ compared with shRNA + I/R group. I/R: Ischemia/reperfusion; PIASy: Protein inhibitor of activated STAT Y; shRNA: Short hairpin ribonucleic acid.

### Downregulation of PIASy decreased the QRS duration and QTc interval

As shown in Figure 2, ECG was recorded before and after myocardial I/R. No statistically significant difference was found in P duration, QRS duration, QTc interval and PR interval at baseline between the scramble and shRNA groups ($P > 0.05$; Figure 2A). As shown in Figure 2B–2D, when compared with baseline in the scramble + I/R group, the QRS duration was statistically significantly increased at 45 min after ischemia ($P < 0.01$), 30 min after reperfusion ($P < 0.01$) and 2 h after reperfusion ($P < 0.05$), while the QTc interval was statistically significantly increased at 10 min and 45 min after ischemia ($P < 0.05$). When compared with the scramble + I/R group, the P duration of the shRNA + I/R group was significantly decreased at 45 min after ischemia ($P < 0.05$; Figure 2B); the QRS duration of the shRNA + I/R group was statistically significantly shortened at 45 min after ischemia ($P < 0.01$), and 30 min and 2 h after reperfusion ($P < 0.01$) compared with that of the scramble + I/R group [Figure 2C]; the QTc interval was

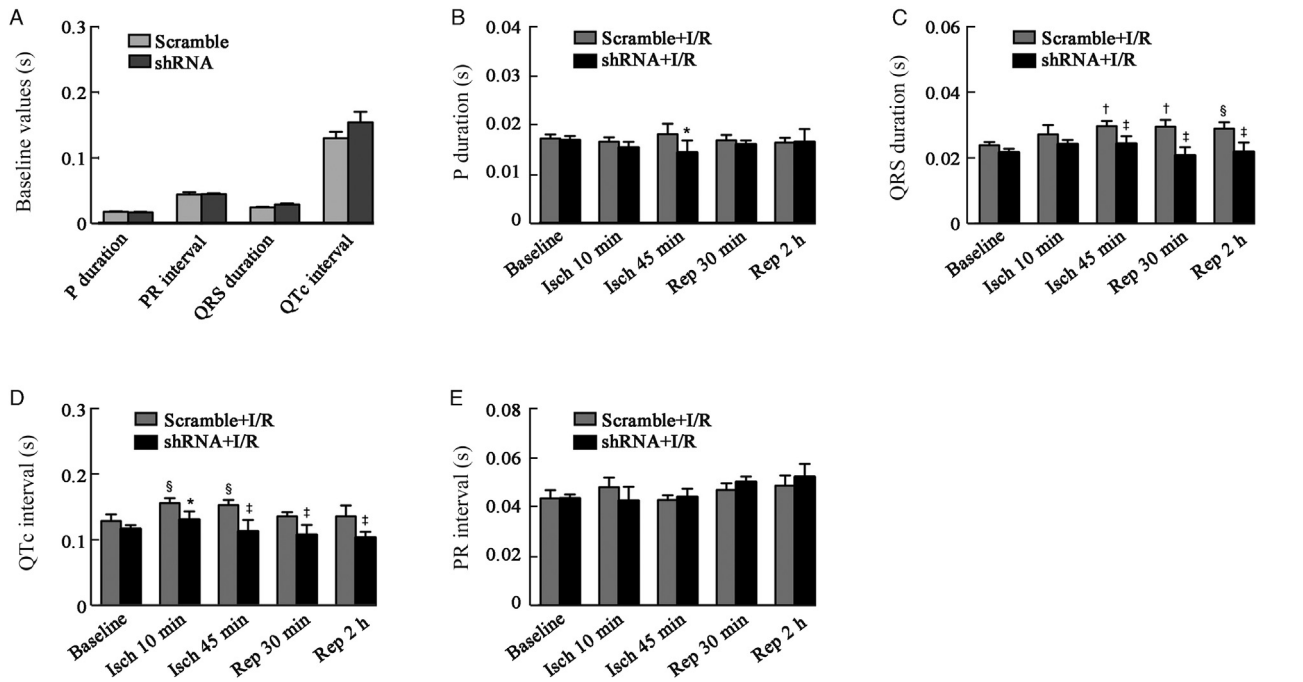


**Figure 2:** Downregulation of PIASy decreased the QRS duration and QTc intervals of rats during myocardial I/R. (A) The baselines of P duration, QRS duration, QTc interval and PR interval before I/R in the scramble and shRNA groups. (B–E) Time-course changes in P duration, QRS duration, QTc interval and PR interval during I/R after PIASy shRNA transfection. All values are presented as the mean ± standard deviation ($n = 8$/group). $^{*}P < 0.05$ and $^{\ddagger}P < 0.01$ compared with the corresponding scramble + I/R group; $^{\dagger}P < 0.01$, $^{\S}P < 0.05$ compared with the baseline of the scramble + I/R group. I/R: Ischemia/reperfusion; PIASy: Protein inhibitor of activated STAT Y; Isch: Ischemia; Rep: Reperfusion; shRNA: Short hairpin ribonucleic acid.

**Table 1: Occurrence of arrhythmia in rats during 45 min of ischemia, *n*.**

| Items | Scramble + I/R ($n = 8$) | shRNA + I/R ($n = 8$) |
|---|---|---|
| Incidence of VPBs | 8 | 8 |
| Incidence of VT | 8 | 8 |
| Incidence of sustained VT | 6 | 3* |
| Incidence of VF | 8 | 4* |

The incidence of arrhythmia was analyzed by Fisher's exact test. * $P < 0.05$ compared with scramble + I/R. I/R: Ischemia/reperfusion; shRNA: Short hairpin ribonucleic acid; VF: Ventricular fibrillation; VPBs: Ventricular premature beats; VT: Ventricular tachycardia.

**Table 2: The occurrence of arrhythmia in rats during 2 h of reperfusion, *n*.**

| Items | Scramble + I/R ($n = 8$) | shRNA + I/R ($n = 8$) |
|---|---|---|
| Incidence of VPBs | 8 | 8 |
| Incidence of VT | 4 | 2* |
| Incidence of sustained VT | 3 | 0* |
| Incidence of VF | 3 | 0* |

The incidence of arrhythmia was analyzed by Fisher's exact test. * $P < 0.05$ compared with scramble + I/R. I/R: Ischemia/reperfusion; shRNA: Short hairpin ribonucleic acid; VF: Ventricular fibrillation; VPBs: Ventricular premature beats; VT: Ventricular tachycardia.

markedly decreased at 10 min of ischemia ($P < 0.05$), 45 min of ischemia ($P < 0.01$), 30 min and 2 h after reperfusion ($P < 0.01$) in the shRNA + I/R group compared with the scramble + I/R group [Figure 2D]. However, the PR interval did not change statistically during I/R after downregulation of PIASy [Figure 2E].

### *Downregulation of PIASy ameliorated I/R-induced ventricular arrhythmias*

As indicated in Tables 1 and 2, ventricular arrhythmia, characterized by VPBs, VT, and sustained VT and VF, were observed in all rats after 45 min of myocardial ischemia and 2 h of reperfusion. The episodes of VT and VF were accompanied by an obvious decrease in arterial pressure [Supplementary Figure 1, http://links.lww.com/CM9/B455]. During 45 min of ischemia, downregulation of PIASy statistically significantly reduced the incidence of sustained VT and VF (both $P < 0.05$) [Table 1]. During 2 h of reperfusion followed by 45 min of ischemia, downregulation of PIASy reduced the incidence of VT and the incidence of sustained VT and VF [Table 2]. The incidence of VT was reduced from 4/8 to 2/8 after PIASy shRNA transfection ($P < 0.05$). No sustained VT or VF was observed in rats with the downregulation of PIASy ($P < 0.05$).

In addition, according to the recorded ECG, the arrhythmia severity was quantitatively analyzed using the Curtis and Walker[15] arrhythmia score. As shown in Figure 3A, the arrhythmia score was statistically significantly increased after I/R when compared with the corresponding control group (scramble group *vs.* scramble + I/R group, $P < 0.01$; shRNA group *vs.* shRNA + I/R group, $P < 0.01$). Downregulation of PIASy significantly reduced the arrhythmia score induced by I/R (scramble + I/R group

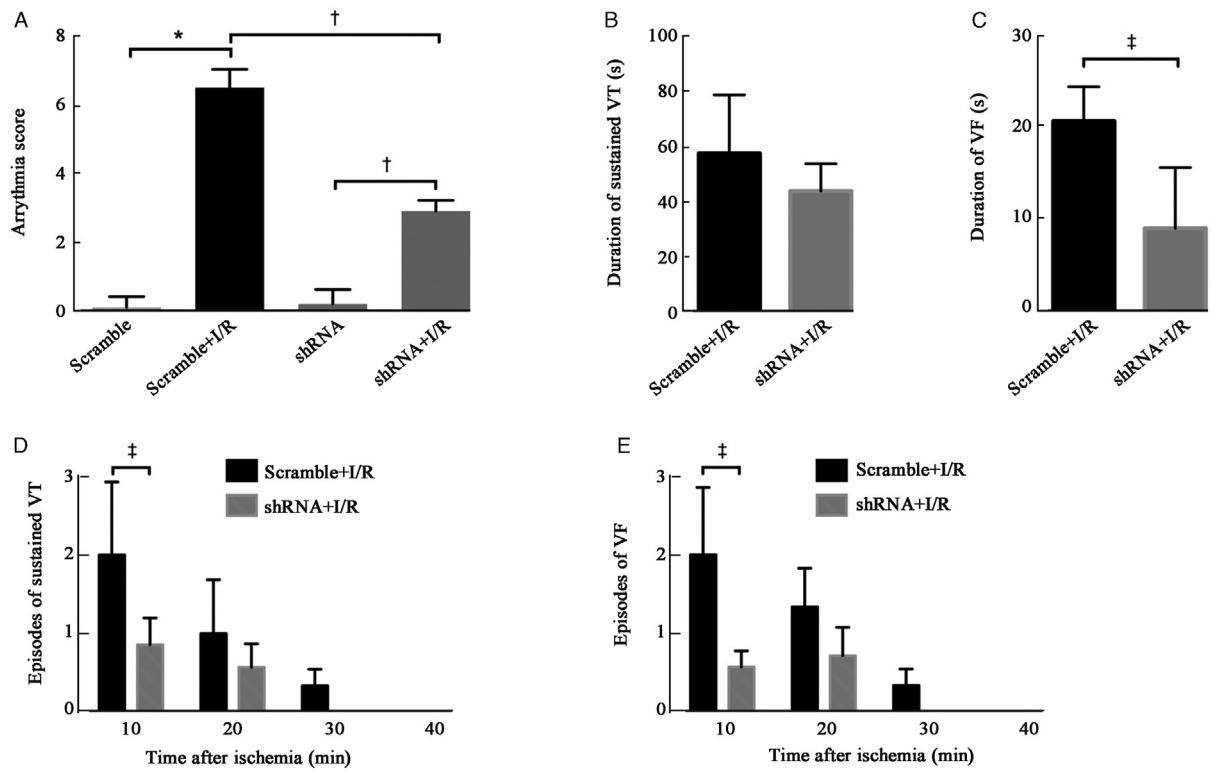


**Figure 3:** Downregulation of PIASy reduced ventricular arrythmia of rats induced by myocardial I/R. (A) Arrhythmia score of rats treated with PIASy shRNA or scramble shRNA after I/R. (B, C) Duration of sustained VT and VF after I/R. (D, E) Episodes of VF and sustained VT within every consecutive 10 min block after ligation of the LAD artery. All values are presented as the mean ± standard deviation ($n = 8$/group). $^{*}P < 0.01$ and $^{‡}P < 0.05$ compared with scramble + I/R group; $^{†}P < 0.01$ compared with shRNA + I/R group. I/R: Ischemia/reperfusion; LAD: Left anterior descending; PIASy: Protein inhibitor of activated STAT Y; shRNA: Short hairpin ribonucleic acid; VF: Ventricular fibrillation; VT: Ventricular tachycardia.

*vs*. shRNA + I/R group, $P < 0.01$). As shown in [Figure 3B and 3C], the duration of VF was significantly decreased in the shRNA + I/R group ($P < 0.05$) when compared with the scramble + I/R group, while the duration of sustained VT was decreased in the shRNA + I/R group but failed to reach a significant difference ($P > 0.05$). In addition, the episodes of VF and sustained VT were significantly decreased within 10 min of the onset of ischemia in the shRNA + I/R group when compared with the scramble + I/R group ($P < 0.05$; Figure 3D and 3E).

### Downregulation of PIASy increased the protein expression and phosphorylation of Cx43 after myocardial I/R

The expression and distribution of cardiac Cx43 after myocardial I/R were determined using Western blotting and immunofluorescence staining to investigate whether Cx43 protein plays roles in the PIASy downregulation that ameliorated I/R-induced ventricular arrhythmias. As shown in Figure 4A–4D, the total protein expression and phosphorylation of Cx43 were statistically significantly decreased after myocardial I/R in the scramble group ($P < 0.01$). Downregulation of PIASy markedly increased the total protein expression and the phosphorylation level of Cx43 after myocardial I/R when compared with the scramble + I/R group ($P < 0.01$). As shown in Figure 4E, myocardial Cx43, which was labeled with green fluorescence, was mainly expressed in the intercalated discs of the myocardium, as noted in the scramble group. After myocardial I/R, Cx43 expression in the intercalated discs decreased in the scramble + I/R group. In addition, downregulation of PIASy increased Cx43 protein expression in the intercalated discs after myocardial I/R compared with those in the scramble + I/R group.

### Downregulation of PIASy increased PKP2 protein expression after myocardial I/R

To investigate whether PKP2 and $Na_V1.5$ proteins play roles in the PIASy downregulation that ameliorated I/R-induced ventricular arrhythmias, the expression of PKP2 and $Na_V1.5$ in the left ventricle tissues was determined after I/R using Western blotting. As shown in Figure 5A–5C, the protein expression levels of both PKP2 ($P < 0.01$) and $Na_V1.5$ ($P < 0.05$) were statistically significantly decreased after myocardial I/R in the scramble group. Downregulation of PIASy markedly increased PKP2 protein expression after myocardial I/R when compared with the scramble + I/R group ($P < 0.01$). However, the protein expression of $Na_V1.5$ did not change statistically in the shRNA + I/R group when compared with the scramble + I/R group ($P > 0.05$).

### Downregulation of PIASy decreased the SUMOylation of Cx43 after myocardial I/R

To investigate the SUMOylation of Cx43 after myocardial I/R, co-IP of Cx43 and SUMO2/3 was performed in the left ventricle tissues. As shown in Figure 5D, the SUMOylation of Cx43 was significantly increased after myocardial I/R in the scramble + I/R group compared with the scramble group. However, the SUMOylation of Cx43

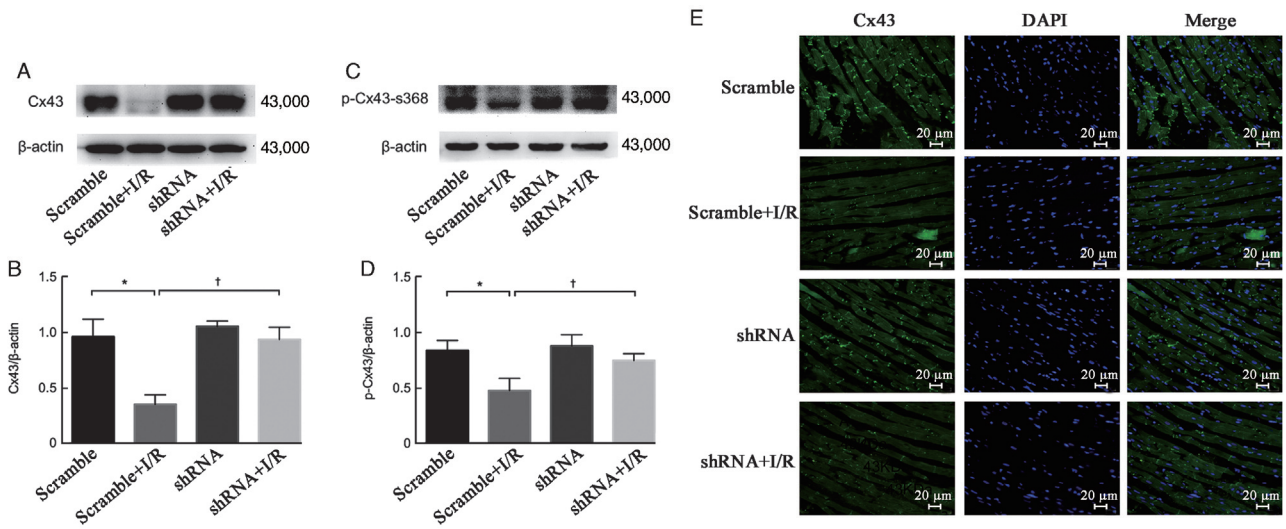


**Figure 4:** Downregulation of PIASy increased the protein expression and phosphorylation of Cx43 in rats after myocardial I/R. (A, B) Representative Western blotting and myocardial protein expression levels of Cx43 after I/R in the scramble and shRNA groups. The mean band density was normalized relative to β-actin. (C, D) Representative Western blots and myocardial phosphorylation of Cx43 after I/R in the scramble and shRNA groups. The mean band density was normalized relative to β-actin. (E) The distribution of Cx43 in cardiomyocytes assessed by immunofluorescence staining. The longitudinal cryostat sections of the left ventricles were immunostained with anti-Cx43 antibody (green) followed by 4′,6-diamidino-2-phenylindole staining (DAPI; blue: cell nuclei). All values are presented as the mean ± standard deviation ($n = 8$/group). $^{*}P < 0.01$ compared with scramble group; $^{\dagger}P < 0.01$ compared with scramble + I/R group. Cx43: Connexin 43; I/R: Ischemia/reperfusion; p-Cx43-s368: Phospho-Cx43-S368; PIASy: Protein inhibitor of activated STAT Y; shRNA: Short hairpin ribonucleic acid.

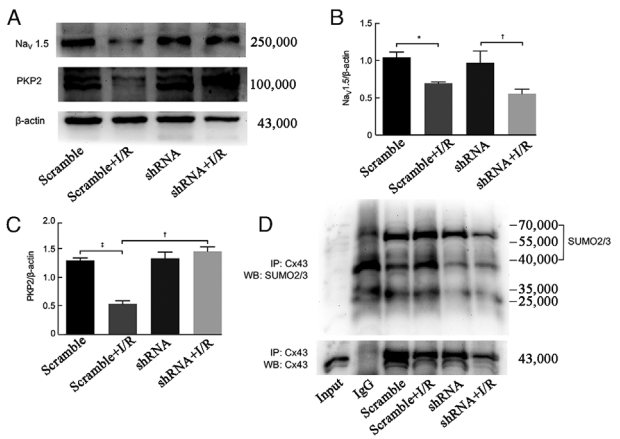


**Figure 5:** Downregulation of PIASy increased PKP2 protein expression and decreased Cx43 SUMOylation of rats after myocardial I/R. (A) Representative Western blotting. (B) Myocardial protein expression levels of $Na_v1.5$ after I/R in the scramble and shRNA groups. The mean band density was normalized relative to β-actin. (C) Myocardial protein expression levels of PKP2 after I/R in the scramble and shRNA groups. The mean band density was normalized relative to β-actin. All values are presented as the mean ± standard deviation ($n = 8$/group). (D) SUMO-modified Cx43 in the rat heart. Rat myocardial lysates were collected and subjected to IP with anti-Cx43 antibody, followed by immunoblotting with anti-SUMO2/3 antibody after I/R injury. $^{*}P < 0.05$ and $^{\ddagger}P < 0.01$ compared with scramble group; $^{\dagger}P < 0.01$ compared with shRNA + I/R group. Cx43: Connexin 43; IP: Immunoprecipitation; I/R: Ischemia/reperfusion; $Na_v1.5$: Voltage-gated Sodium Channel 1.5; PIASy: Protein inhibitor of activated STAT Y; PKP2: Plakophilin 2; shRNA: Short hairpin ribonucleic acid; SUMO: Small ubiquitin-like modifier; WB: Western blotting.

was markedly decreased after myocardial I/R in the shRNA + I/R group compared with the scramble + I/R group.

## Discussion

There are several novel findings in our study. First, using *in vivo* rat models of myocardial I/R, we demonstrated that myocardial I/R stimulation led to a significant increase in PIASy levels, which was associated with enhanced Cx43 SUMO modification by SUMO2/3, reduced expression of PKP2, and subsequent worsening of ventricular arrhythmia. Second, downregulation of cardiac PIASy by shRNA inhibited Cx43 SUMOylation and increased PKP2 expression, leading to increased Cx43 phosphorylation and subsequent amelioration of ventricular arrhythmias in myocardial I/R rat hearts.

Cx43, the most abundant member of the connexin family, forms gap junctions and is enriched in ventricular cardiomyocytes for electrical impulse transmission in the heart.[16] Notably, Cx43 is crucial in myocardial protection during the I/R process.[4,17] However, the underlying mechanisms remain unclear. Studies have shown that Cx43 is modified by SUMOylation, which regulates plasma membrane Cx43 protein levels and functional Cx43 gap junction levels.[10] In the rat heart model used in this study, we demonstrated that myocardial I/R significantly induced the SUMOylation of Cx43 by SUMO2/3 and reduced the phosphorylation of Cx43, in addition to decreasing Cx43 expression in intercalated discs. Given that Cx43 is cleared from the cytoplasm and degraded by autophagy,[18,19] these results may suggest that abnormalities in Cx43 localization between the plasma membrane and intercalated disc during I/R may be attributed to enhanced Cx43 SUMOylation, which subsequently downregulates Cx43 expression in gap junctions and promotes Cx43 degradation through the autophagic pathway.

PIASy, as an important SUMO E3 ligase, mediates the SUMOylation of various proteins, further regulating the activity, translocation, and autophagy of conjugated proteins.[20] In our study, we demonstrated that I/R stimulation led to a significant increase in PIASy levels, which was associated with enhanced SUMO modification of Cx43 by SUMO2/3, as evidenced by the co-IP results

and redistribution of Cx43 protein from the intercalated disc to the lateral membrane. When the cardiac PIASy level was downregulated by transfection with PIASy shRNA, SUMO2/3-modified Cx43 was decreased in both normal and I/R-treated hearts, contributing to resumed Cx43 expression in gap junctions. This report demonstrated that PIASy may be implicated in the Cx43 SUMOylation process and influence subsequent Cx43 functional consequences. However, the physical binding between Cx43 and PIASy protein should be studied by the mutual co-IP in both normal and I/R-treated rat cardiomyocytes. Since another post-transcriptional modification, phosphorylation, also plays a vital role in Cx43 expression and intracellular localization,[21,22] we sought to explore whether SUMO itself may consequently alter the phosphorylation of Cx43. Interestingly, we found that I/R caused a dramatic decrease in the S368 phosphorylation of Cx43 in ventricular tissue, while silencing PIASy by AAV9-mediated PIASy RNA interference partially recovered phos-368-Cx43 levels compared with those of the I/R group. These data may collectively demonstrate that interactions may exist between the SUMOylation and phosphorylation sites in the Cx43 protein.

It is well known that the mechanisms by which myocardial I/R causes arrhythmias include early or delayed afterdepolarization and re-entry of excitation, leading to life-threatening VT and VF.[23] The mechanism of IR-induced arrhythmia involves calcium overload, increases in extracellular potassium accumulation, and decreases in membrane $Na_V1.5$ density.[12,24] Studies have shown that loss of Cx43 is associated with abnormal sodium and potassium current conduction and facilitates arrhythmias,[25,26] which suggests that Cx43 plays a dominant role in the occurrence and development of I/R-induced arrhythmias. Our results have shown that I/R-induced increased Cx43-SUMO modification was associated with increased mortality in severe VT and VF, along with an extended duration of QRS and QTc. This may be attributed to abnormal redistribution of Cx43 from the intercalated disc to the lateral membrane fraction and cell-to-cell conductance between adjacent cardiomyocytes. In our study, shRNA-PIASy prevented Cx43 SUMO2/3 modification during I/R, which partially ameliorated the cardiac sensitivity to lethal ventricle arrhythmias, as evidenced by the decreased incidence, frequency, and duration of VT as well as sustained VT and VF during I/R. Although no significant upregulation of Cx43 after PIASy shRNA interference was found, we observed a partial recovery in Cx43 in PIASy shRNA + I/R hearts in comparison with scramble + I/R hearts. This may suggest that modulation of Cx43 SUMO by silencing PIASy ameliorates I/R-induced Cx43 dysregulation and subsequent susceptibility of the heart to severe arrhythmias, which may be a new target for cardiac protection against I/R. In addition, we observed that PIASy silencing increased the total levels of Cx43 protein and its functional phosphorylated forms in I/R, which may lead to attenuation of abnormal electrical conductance. Studies have shown that autophagy may reduce myocardial I/R injury and malignant arrhythmia by enhancing myocardial Cx43 phosphorylation and improving the acute remodeling of Cx43.[19] Thus, the elevated phosphorylation of Cx43 may be partially due to the increased PKCε isoform caused by PIASy interference. These results suggest that downregulation of PIASy inhibited Cx43 SUMOylation, leading to increased Cx43 phosphorylation and subsequent amelioration of ventricular arrhythmias in ischemic/reperfused rat hearts.

PKP2 is the main component of desmosomes and is known for its role in cell–cell adhesion.[27] PKP2 is codeposited with Cx43, and the loss of PKP2 leads to gap junction remodeling[28,29] and causes life-threatening arrhythmias.[30] Interestingly, Cx43 was found to coprecipitate with $Na_V1.5$ at the intercalated disc, while $Na_V1.5$ and PKP2 coexist in the same molecular complex.[27] In our study, we found that myocardial I/R decreased the protein expression of PKP2 and $Na_V1.5$, while downregulation of PIASy markedly increased PKP2 protein expression but not $Na_V1.5$ after myocardial I/R. These results may suggest that PKP2 and Cx43 are not independent but rather are protein complexes that may regulate the function of gap junctions, with significant consequences for ventricular arrhythmias.

Finally, several limitations to the present study should be considered. First, considering that we observed downregulation of cardiac PIASy to increase Cx43 phosphorylation, while Cx43 phosphorylation has been shown to decrease postischemic infarction,[31] we speculated that PIASy inhibition may reduce myocardial postischemic infarction by increasing Cx43 phosphorylation.

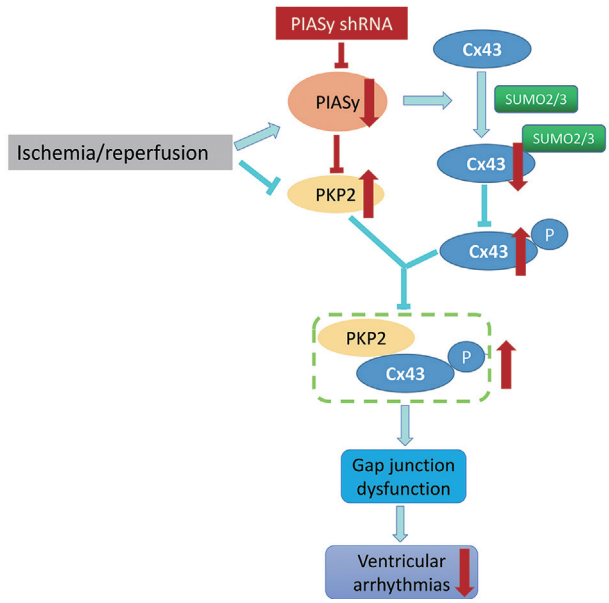


**Figure 6:** Schematic diagram of the proposed mechanism involving modulation of Cx43 SUMOylation by silencing PIASy, which ameliorated I/R-induced ventricular arrhythmias. Myocardial I/R stimulation led to a significant increase in the PIASy level, which was associated with enhanced Cx43 SUMO modification by SUMO2/3, reduced expression of PKP2, and subsequent worsening of ventricular arrhythmia. Downregulation of cardiac PIASy by shRNA inhibited Cx43 SUMOylation and increased PKP2 expression, leading to increased Cx43 phosphorylation and subsequent amelioration of ventricular arrhythmias in myocardial I/R rat hearts. "→" indicates promoted effects, and "⊣" indicates inhibited effects. Red indicates the changes under PIASy shRNA conditions. Cx43: Connexin 43; I/R: Ischemia/reperfusion; $Na_v1.5$: Voltage-gated Sodium Channel 1.5; PIASy: Protein inhibitor of activated STAT Y; PKP2: Plakophilin 2; shRNA: Short hairpin ribonucleic acid; SUMO: Small ubiquitin-like modifier.

However, this needs to be directly evaluated by measuring myocardial infarct sizes, which we are currently planning. Second, the physical binding between Cx43 and PKP2 during myocardial I/R merits further study.

In conclusion, our study demonstrates that Cx43 can either be SUMOylated or phosphorylated during cardiac I/R. Downregulation of cardiac PIASy inhibited Cx43 SUMOylation and increased PKP2 expression, which might lead to increased Cx43 phosphorylation and subsequent amelioration of ventricular arrhythmias in myocardial I/R rat hearts [Figure 6]. Our results provide insights into the effects and mechanisms of the modulation of Cx43 SUMOylation by silencing PIASy, which may contribute to the development of effective therapeutic regimens to combat myocardial I/R-induced ventricular arrhythmias.

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### Conflicts of interest

None.

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