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miR-499 protects cardiomyocytes from H_2O_2-induced apoptosis via its effects on *Pdcd4* and *Pacs2*

Jiaji Wang[1,§], Zhuqing Jia[1,§], Chenguang Zhang[1,‡], Min Sun[2], Weiping Wang[1], Ping Chen[1], Kangtao Ma[1], Youyi Zhang[3], Xianhui Li[1,†,*], and Chunyan Zhou[1,*]

[1]Department of Biochemistry and Molecular Biology; School of Basic Medical Sciences; Key Laboratory of Molecular Cardiovascular Sciences; Ministry of Education of China; Peking University, Beijing, P.R. China; [2]Department of Cardiology; Peking University Third Hospital; Beijing, P.R. China; [3]Institute of Vascular Medicine; Peking University Third Hospital; Key Laboratory of Molecular Cardiovascular Sciences; Ministry of Education of China; Peking University; Beijing, P.R. China

[†]Current affiliation: Department of Biochemistry and Molecular Biology; Logistics College of Chinese People's Armed Police Forces; Tianjin, P.R. China

[‡]Current affiliation: Department of Medical Genetics; Capital Medical University; Beijing, P.R. China

[§]Both authors contributed equally to this work and share first authorship.

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Abbreviations: *Myh7b*, myosin, heavy chain 7B, cardiac muscle, beta; β-MHC, β-myosin heavy chain; qRT-PCR, quantitative RT-PCR; PDCD4, programmed cell death 4; PACS2, phosphofurin acidic cluster sorting protein 2; DYRK2, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2; BID, BH3 interacting domain death agonist; EDAR, ectodysplasin A receptor; PHLDA2, pleckstrin homology-like domain, family A, member 2; PHF17, PHD finger protein 17; DCC, deleted in colorectal carcinoma; P53AIP1, tumor protein p53 regulated apoptosis inducing protein 1; AP-1, activator protein-1; I/R, ischemia/reperfusion; ChIP, chromatin immunoprecipitation

Background: microRNAs (miRNAs) are a class of small, non-coding endogenous RNAs that post-transcriptionally regulate some protein-coding genes. miRNAs play an important role in many cardiac pathophysiological processes, including myocardial infarction, cardiac hypertrophy, and heart failure. miR-499, specifically expressed in skeletal muscle and cardiac cells, is differentially regulated and functions in heart development. However, the function of miR-499 in mature heart is poorly understood.

Results: We report that cardiac-abundant miR-499 could protect neonatal rat cardiomyocytes against H_2O_2-induced apoptosis. Increased miR-499 level favored survival, while decreased miR-499 level favored apoptosis. We identified three proapoptotic protein-coding genes—*Pdcd4*, *Pacs2*, and *Dyrk2*—as targets of miR-499. miR-499 inhibited cardiomyocyte apoptosis through its suppressive effect on *Pdcd4* and *Pacs2* expression, thereby blocking *Bid* expression and BID mitochondrial translocation. We also found that H_2O_2-induced phosphorylation of c-Jun transcriptionally upregulated miR-499 expression via binding of phosphorylated c-Jun to the *Myh7b* promoter.

Conclusions: Our results revealed that miR-499 played an inhibiting role in the mitochondrial apoptosis pathway, and had protective effects against H_2O_2-induced injury in cardiomyocytes.

Introduction

miRNAs are endogenous small non-protein-coding RNAs that are about 22 nucleotides in length and act as negative regulators of gene expression by pairing with sites in the 3′ UTR regions of mRNAs of protein-coding genes. miRNAs are involved in cardiac events such as the conduction of electrical signals, the contraction, growth and morphogenesis of heart.[1] miR-499 was first reported functioning in coordination with miR-208a and miR-208b to control muscle-specific gene expression and performance by van Rooij et al.[2] Recently, miRNAs have been reported to participate in many pathophysiological processes in the heart, including myocardial infarction, cardiac hypertrophy, cardiac fibrosis, and heart failure.[3-5] It has been reported that in the myocardium of rats with acute myocardial infarction, the expression of some miRNAs was altered, including that of some cardiac-abundant miRNAs such as miR-1, -133, -208, and -499.[6-8] Thus, miRNAs might be attractive targets for the diagnosis and treatment of heart diseases.

*Correspondence to: Xianhui Li, Email: lixianhuiwj@163.com; Chunyan Zhou; Email: chunyanzhou@bjmu.edu.cn
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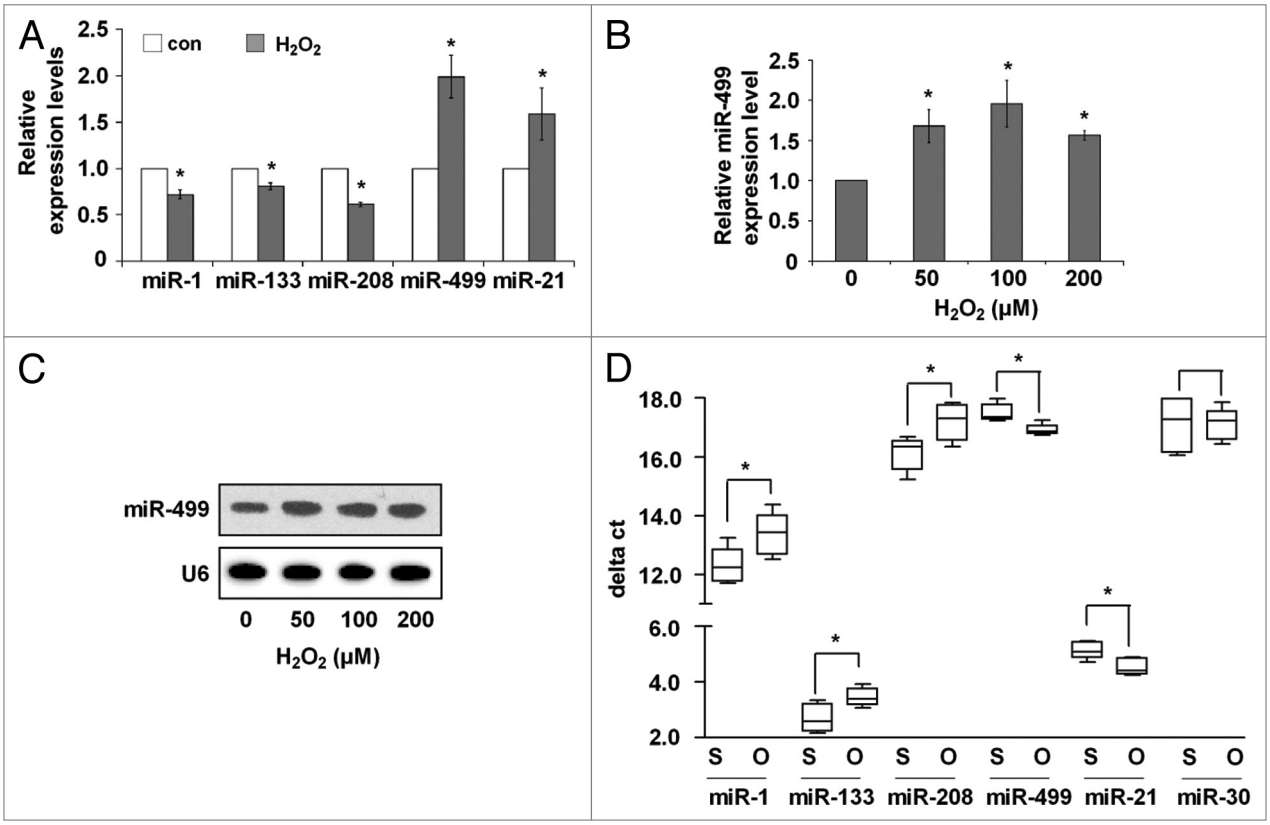

Figure 1. Cardiac-abundant miRNAs are aberrantly expressed in response to ROS stimulus. (**A**) Cardiac-abundant miRNAs levels in neonatal rat cardiomyocytes exposed to H_2O_2. Cardiomyocytes that were not treated with H_2O_2 were used as a negative control (con), while miR-21 was used as a positive control. (**B**) miR-499 level in neonatal rat cardiomyocytes exposed to different concentrations of H_2O_2. The cardiomyocytes not treated with H_2O_2 (0 μM) were used as a negative control. Data represent the results of three independent experiments. (**C**) A representative image shows the northern blot analysis of miR-499 level in neonatal rat cardiomyocytes exposed to different concentrations of H_2O_2. The cardiomyocytes not treated with H_2O_2 were used as a negative control. (**D**) The value of delta Ct for cardiac-abundant miRNAs levels in rat hearts exposed to I/R injury, while miR-21 was used as a positive control, and miR-30 was used as a negative control. O, rat hearts were exposed to I/R injury (n = 6); S, rat hearts were sham treated (n = 6). *P < 0.05 compared with the negative control.

miR-499 is specifically expressed in skeletal muscle and cardiac cells.[2] In the mouse, it is encoded by intron 19 of the myosin, heavy chain 7B, cardiac muscle, β gene (*Myh7b*), a little-studied myosin gene that shares extensive homology with β-myosin heavy chain (β-*MHC*).[9] *Myh7b* and miR-499 are expressed in the heart, but the expression of miR-499 and its host gene *Myh7b* is uncoupled because of an exon skipping mutation.[10] miR-499 has a high expression level in the heart although the expression of *Myh7b* is low, indicating that miR-499 might have some cardiac functions. Indeed, several studies have reported that miR-499 is differentially regulated and functions in heart development.[11-13] Shieh et al. reported that elevated miR-499 levels affect cardiac gene expression and predispose transgenic mice to cardiac stress-induced dysfunction.[14] miR-499 may modulate the cardiac response to stress in part by regulating the immediate early gene response. In addition, it was reported that the plasma level of miR-499 was significantly increased in rats with acute myocardial infarction. Interestingly, this induction was also verified in humans, indicating that the effect was not solely confined to the model animal.

Many heart diseases are associated with reactive oxygen species (ROS), including myocardial infarction, cardiac hypertrophy, and heart failure.[15,16] Hydrogen peroxide (H_2O_2), as an exogenous ROS, could activate caspase 3 either directly or through the truncation of BID, which subsequently activates the mitochondria-dependent pathway.[17] Recent studies have found that plasma miR-499 can be employed as a biomarker of acute myocardial infarction,[8,18] in which process ROS plays an important role. However, it is not clear whether H_2O_2 affects the expression of cardiac-abundant miRNAs and, therefore, whether miRNAs function in H_2O_2-mediated cardiac cell apoptosis and death. We therefore decided to investigate the relationship between H_2O_2 and miR-499 during the process of heart infarction.

To elucidate the molecular mechanisms by which miR-499 regulates apoptosis, we analyzed its potential targets according to the prediction by TargetScan.[19,20] Potential target genes were functionally classified using GO (Gene Ontology).[21,22] A panel of seven putative targets, *Pdcd4, Pacs2, Dyrk2, Edar, Phlda2, Phf17,* and *Dcc*, predicted to be related to apoptosis, was selected

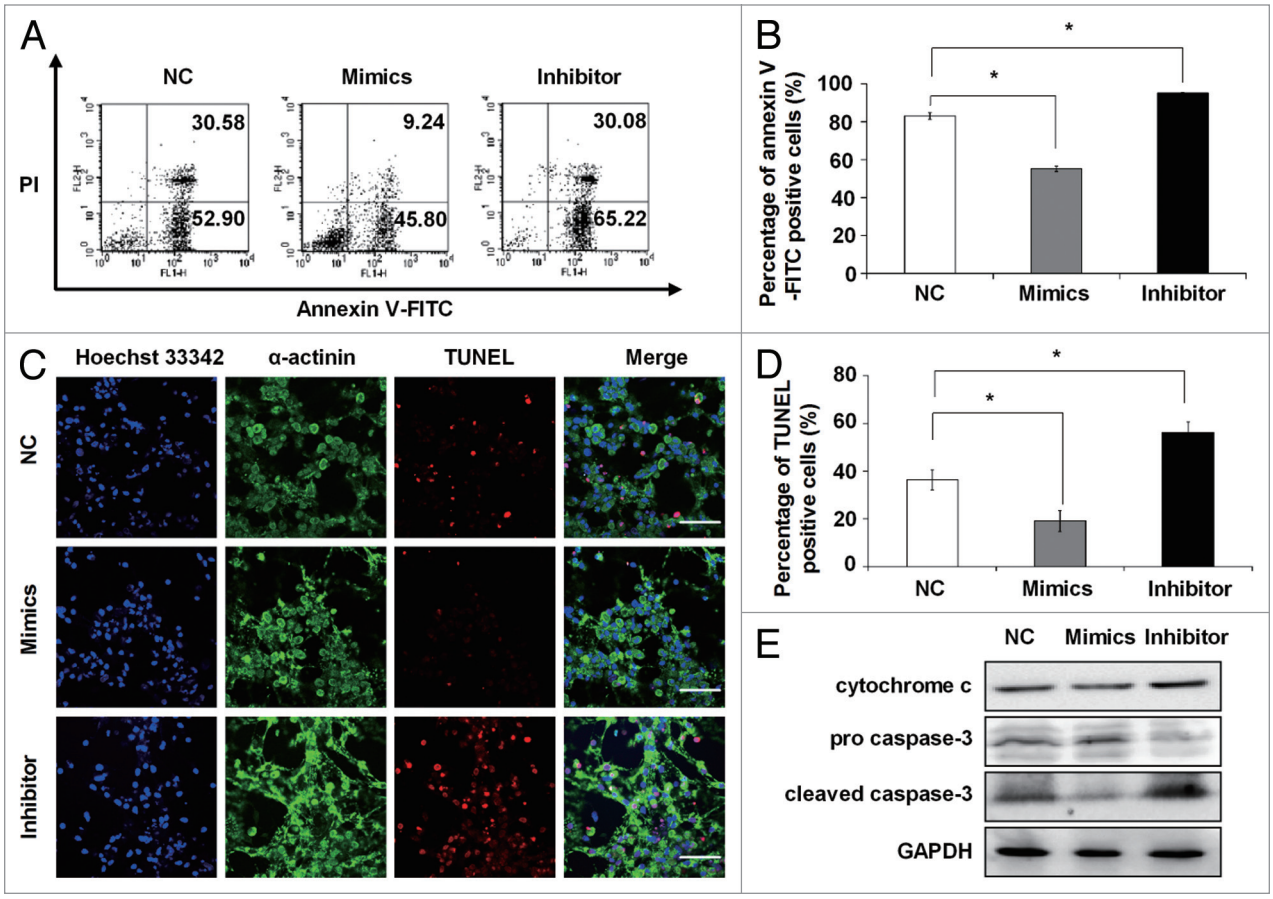

Figure 2. miR-499 could protect cardiomyocytes from H_2O_2-induced apoptosis. (**A**) Representative images for flow cytometry analysis of Annexin V/PI staining of cardiomyocytes treated with miR-499 negative control (NC), mimics or inhibitor and then exposed to H_2O_2. Quantitative results for Annexin V-positive cardiomyocytes from three independent experiments are shown in (**B**). (**C**) Representative images of TUNEL-stained cardiomyocytes treated with miR-499 NC, mimics, and inhibitor, and then exposed to H_2O_2. Red, TUNEL-positive myocyte nuclei; blue, Hoechst 33342-stained nuclei; scale bars, 50 μm. Quantitative results for apoptotic cells from three independent experiments are shown in (**D**). (**E**) Representative immunoblots of cytochrome c, procaspase-3, and cleaved caspase-3 in cardiomyocytes treated with NC, mimics, or inhibitor. The experiments were repeated at least three times. *P < 0.05 compared with the negative control. All the experiments were repeated at least three times.

for further validation. Interestingly, all of these seven proteins are pro-apoptosis factors. We found *Pdcd4, Pacs2,* and *Dyrk2* to be direct targets, so we used only these three proteins and BID (a known target of PACS2 and PDCD4) for the subsequent overexpression and knockdown experiments.

In addition to the involvement of these proteins, it has been reported that H_2O_2-induced apoptosis is preceded by rapid activation of all three classes of MAPKs (mitogen-activated protein kinases): ERK, JNK, and p38.[23] So, we also studied whether miR-499 was regulated by H_2O_2-induced activation of MAPKs.

We found that in response to H_2O_2 stimulation, the expression of miR-499 was significantly increased due to the binding of phosphorylated c-Jun to the *Myh7b* promoter, which in turn, protected cardiomyocytes against H_2O_2-induced injury by targeting two proteins, PDCD4 and PACS2, which are related to the mitochondrial apoptosis pathway through BID. Our results shed new light on the protective mechanism of cardiomyocytes in response to oxidative stress.

Results

Cardiac-abundant miRNAs are aberrantly expressed in response to ROS stimulus

miR-499 is one of cardiac-abundant miRNAs (**Fig. S1A**). To examine its roles together with other cardiac-abundant miRNAs in cellular response to H_2O_2 stimulus, we treated cardiomyocytes with 100 μM H_2O_2. Short exposure (6 h) of cardiomyocytes to H_2O_2 induced the activation of caspase 8 and caspase 3 (**Fig. S1B**), and resulted in decreased expression of miR-1, -133, and -208 but increased expression of miR-499. A known H_2O_2-responsive miRNA, miR-21 was used as a positive control (**Fig. 1A**).[24] RT-PCR and northern blot analysis showed that H_2O_2-induced expression of miR-499 in cardiomyocytes exhibited concentration-dependent changes: it increased from 0 μM (control) to 50 μM, peaked at 100 μM, and decreased thereafter at 200 μM (**Fig. 1B and C**). The average standardized values of grayscale scanning on the northern blots from three independent experiments are 1.45 (50 μM), 1.66 (100 μM), and 1.29 (200 μM), respectively, (the values for 0 μM are set as 1).

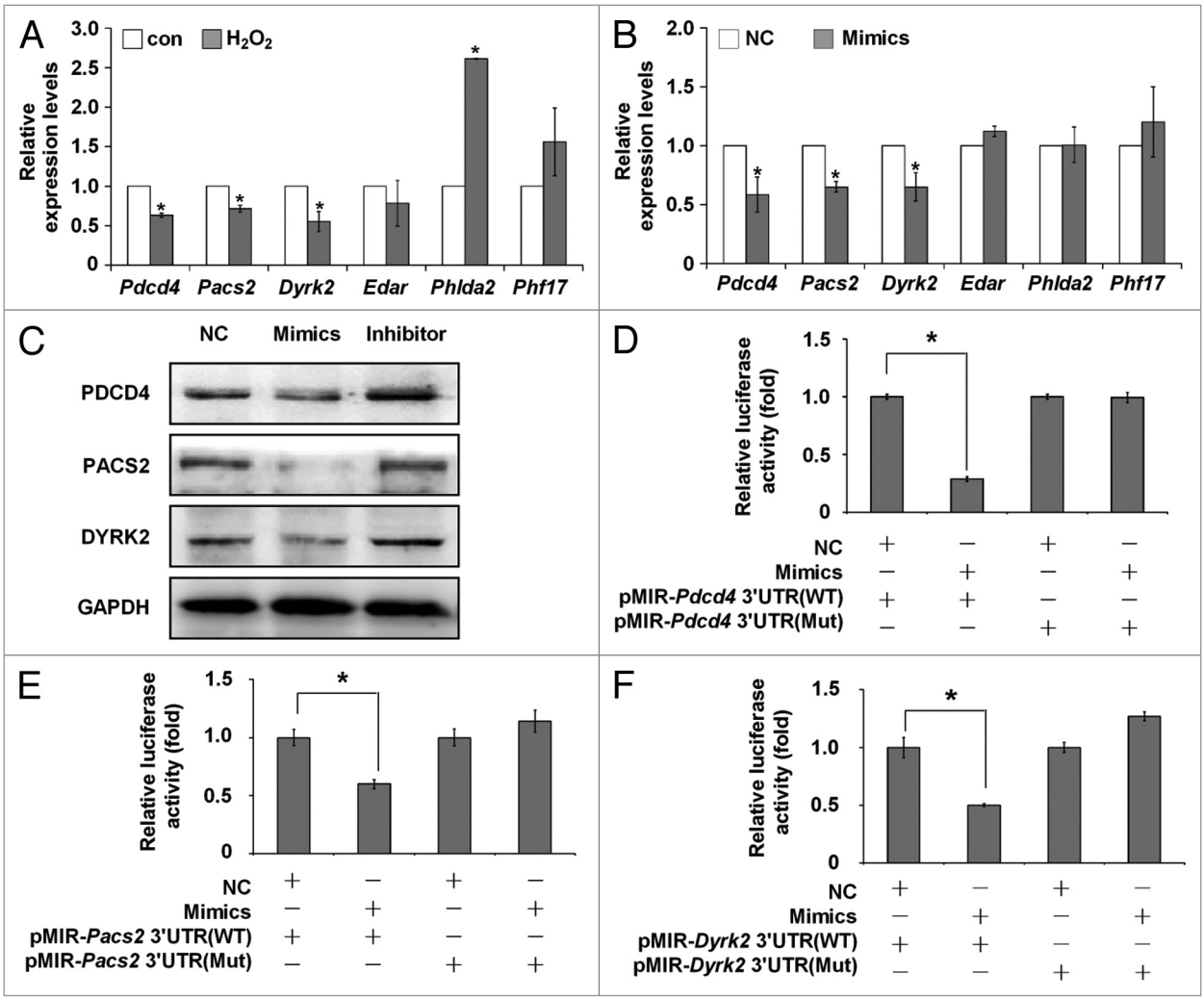

Figure 3. *Pdcd4*, *Pacs2,* and *Dyrk2* are targets of miR-499. (**A**) The mRNA level of predicted potential targets of miR-499 in cardiomyocytes exposed to H_2O_2 (100 μM). Cardiomyocytes that were not treated with H_2O_2 were used as a negative control (con). (**B**) The mRNA level of predicted targets of miR-499 in cardiomyocytes treated with miR-499 NC or mimics. (**C**) Representative images of three independent experiments show the protein level of PDCD4, PACS2, and DYRK2 in cardiomyocytes treated with miR-499 NC, mimics, and inhibitor. GAPDH was used as an internal control. (**D–F**) Luciferase analysis of the effect of miR-499 on its potential targets. Luciferase activity was measured in HeLa cells co-transfected with miR-499 and pMIR-*Pdcd4*-3′UTR(WT) or pMIR-*Pdcd4*-3′UTR(Mut) (**D**), pMIR-*Pacs2*-3′UTR(WT) or pMIR-*Pacs2*-3′UTR(Mut) (**E**), pMIR-*Dyrk2*-3′URT(WT), or pMIR-*Dyrk2*-3′UTR(Mut) (**F**). *$P < 0.05$ compared with the negative control. Data represent the results of three independent experiments.

Next, we detected the change of miR-499 expression in the in vivo rat model of myocardial ischemia/reperfusion (I/R) injury. Ischemia was successfully achieved after occlusion of the left anterior descending coronary artery for 1 h; this was confirmed by visual observation (cyanosis) and continuous electrocardiograph monitoring (**Fig. S1C**). After 1 h of myocardial ischemia, the suture was loosened for 6 h for reperfusion.

To determine the potential involvement of miRNAs in cardiac I/R injury, we employed qRT–PCR analysis to determine miRNA levels in rat hearts after I/R in vivo. Decreased expression of miR-1, -133, and -208 was observed, whereas the expression of miR-499 appeared to be increased, miR-21 was used as a positive control and miR-30 was used as a negative control (**Fig. 1D**). This is consistent with our ex vivo results, suggesting that the upregulation of miR-499 in response to ROS stimulus may exert some biological effect.

miR-499 could protect cardiomyocytes from H_2O_2-induced apoptosis

To study whether miR-499 indeed participates in H_2O_2-induced apoptosis, we performed flow cytometry analysis (**Fig. 2A and B**) and TUNEL assay (**Fig. 2C and D**) to verify apoptotic cell death. First, we tested whether ectopic expression of miR-499 could influence cell death. We transiently overexpressed miR-499 in cardiomyocytes by transfection with miR-499 mimics or negative control (NC) (**Fig. S2A**). The cardiomyocytes treated with mimics were less susceptible to H_2O_2, with lower apoptosis rates than cardiomyocytes treated with NC; in contrast, the knockdown of endogenous miR-499 by transfection with

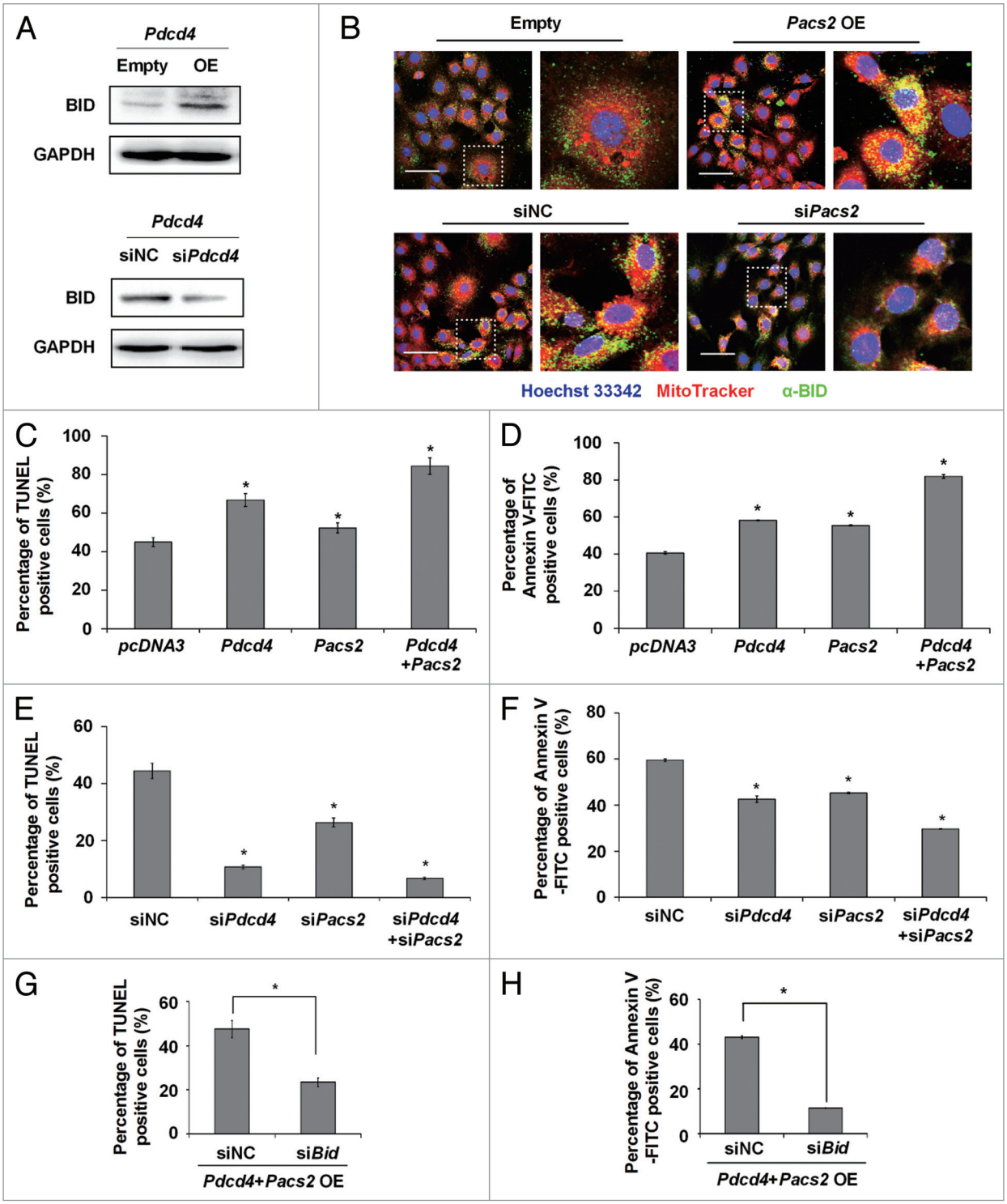

Figure 4. PDCD4 and PACS2 participate in the regulation of apoptosis in cardiomyocytes exposed to H_2O_2 via their effect on *Bid* expression. (**A**) BID protein level in H9c2 cells treated with *Pdcd4*-overexpression constructs (OE) or *Pdcd4* siRNA (si*Pdcd4*). In the OE system, cells transfected with empty plasmid (Empty) were used as a negative control. In the *Pdcd4* knockdown system, cells transfected with negative control siRNA (siNC) were used as the negative control. (**B**) Representative images showing subcellular localization of BID in response to H_2O_2 stimulus. Red, Mitotracker-stained mitochondria; blue, Hoechst 33342-stained nuclei; green, BID antibody staining. Left row, merged picture, scale bar, 50 μm; right row, magnified area, indicated with the white frame in the merged image. In the OE system, cells transfected with empty plasmid (Empty) were used as a negative control. In the *Pacs2*-knockdown system, cells transfected with negative control siRNA (siNC) were used as a negative control. (**C–F**) Quantitative analysis of the results of the TUNEL assay and FACS assay on H9c2 cells treated with *Pdcd4* OE, *Pacs2* OE, si*Pdcd4*, or si*Pacs2*, and then exposed to H_2O_2. pcDNA3 was used as a negative control plasmid for OE system (**C and D**). siNC was used as the negative control for knockdown system (**E and F**). (**G and H**) Quantitative analysis of the results of TUNEL assay and FACS assay on H9c2 cells treated with *Pdcd4* and *Pacs2* overexpression plasmids and siRNA against *Bid* (si*Bid*), and then exposed to H_2O_2. siNC was used as a negative control. **P* < 0.05 compared with the negative control. Data represent the results of three independent experiments.

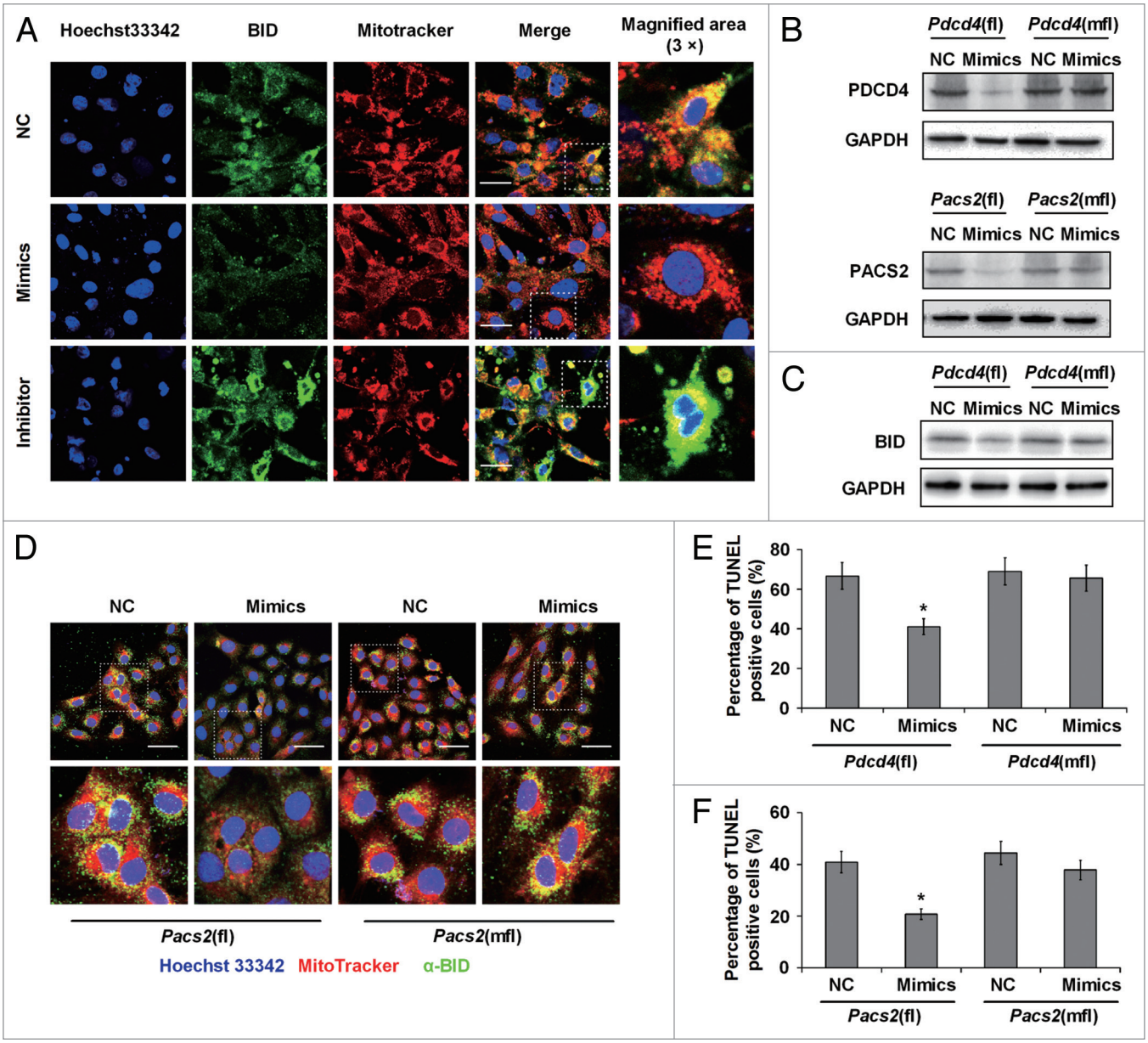

Figure 5. miR-499 inhibits H_2O_2-induced apoptosis through its effect on PDCD4 and PACS2. (**A**) Subcelluar localization of BID in response to H_2O_2 stimulus in cardiomyocytes transfected with miR-499 NC, mimics, and inhibitor. Blue, Hoechst 33342-stained nuclei; green, BID antibody staining; red, Mitotracker-stained mitochondria. Merge, merged from Hoechst 33342-stained nuclei, BID and mitochondria; scale bar, 50 μm; right row, magnified area, indicated with the white frame in the merged images. (**B**) Expression of *Pdcd4* (top) and *Pacs2* (bottom) in H9c2 cells co-transfected with miR-499 NC or mimics in combination with full-length *Pdcd4*(fl) or *Pacs2*(fl), or full-length plasmid with a 3′ UTR mutation of *Pdcd4*(mfl) or *Pacs2*(mfl). (**C**) *Bid* expression at the protein level in H9c2 cells co-transfected with *Pdcd4*(fl) or *Pdcd4*(mfl) and miR-499 NC or miR-499 mimics. (**D**) Representative images showing BID mitochondrial translocation in H9c2 cells co-transfected with miR-499 NC or mimics and *Pacs2*(fl) or *Pacs2*(mfl). Blue, Hoechst 33342-stained nuclei; green, BID staining, red, Mitotracker-stained mitochondria. Top row, merged images; scale bar, 50 μm; bottom row, magnified area, indicated with the white frame in the merged images. (**E and F**) Quantitative analysis of the results of the TUNEL assay on H9c2 cells exposed to H_2O_2 as well co-transfected with miR-499 NC or mimics and *Pdcd4*(fl) or *Pdcd4*(mfl) (**E**), *Pacs2*(fl) or *Pacs2*(mfl) (**F**). Data represent the results of three independent experiments. $^*P < 0.05$ compared with the negative control.

miR-499 inhibitor could induce cardiomyocyte apoptosis compared with the NC group (**Fig. 2A–D**).

As a consequence, compared with NC, the miR-499 inhibitor led to accumulation of cytochrome c in the cytosol and cleaved caspase-3, while miR-499 mimics attenuated the release of cytochrome c and caspase-3 activation (**Fig. 2E**).

Pdcd4, *Pacs2*, and *Dyrk2* are targets of miR-499

We next studied the effect of miR-499 on the pro-apoptosis factors PDCD4, PACS2, DYRK2, EDAR, PHLDA2, PHF17, and DCC. The mRNA expression of *Pdcd4, Pacs2,* and *Dyrk2* was downregulated in cardiomyocytes exposed to H_2O_2, whereas the mRNA expression *of Edar, Phlda2,* and *Phf17* showed an

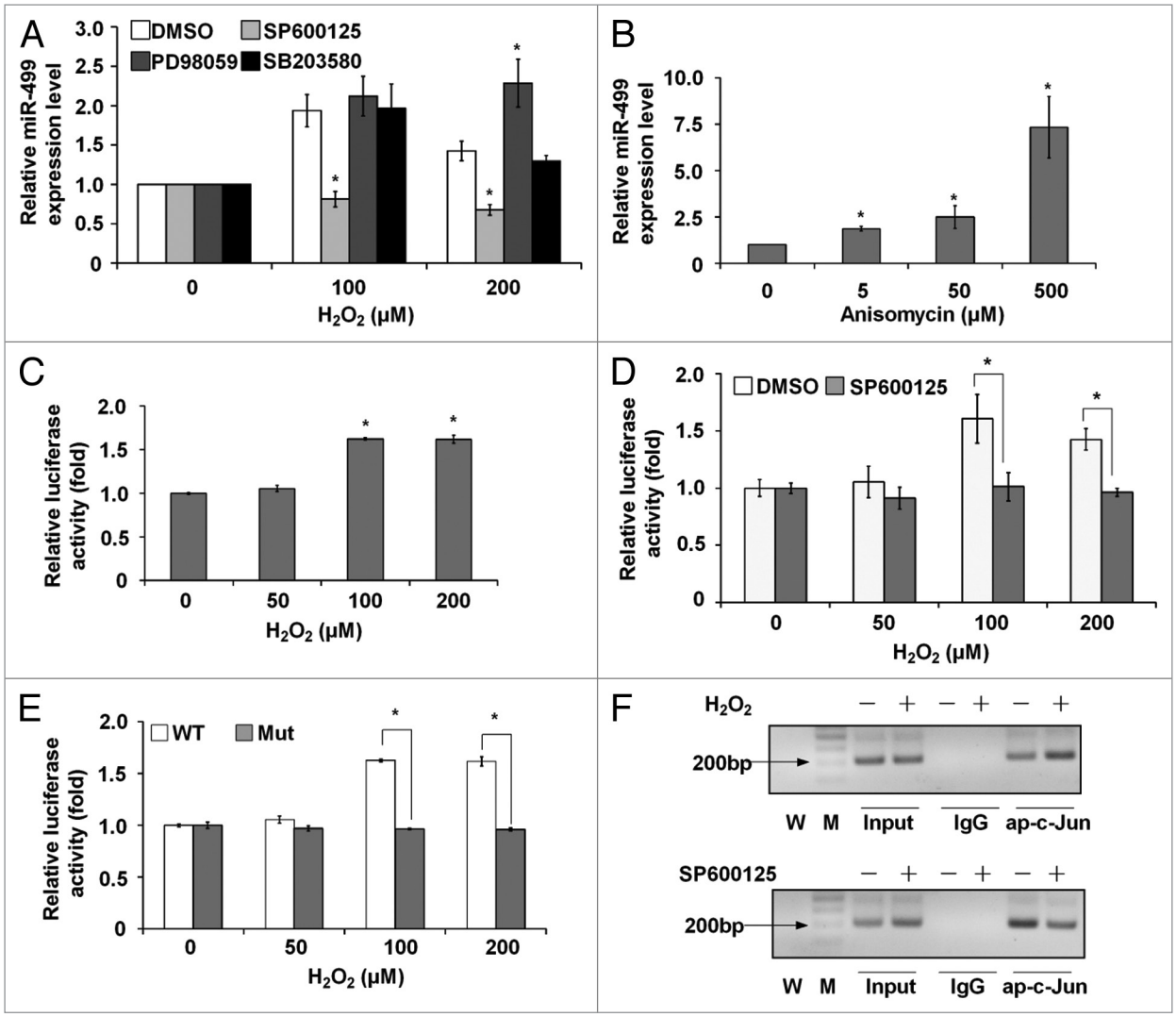

Figure 6. Phosphorylation of c-Jun regulates miR-499 expression in response to H_2O_2 treatment. (**A**) The miR-499 level in cardiomyocytes treated with antagonists of JNK (SP600125), ERK (PD98059), or p38 (SB203580). Cardiomyocytes treated with DMSO were used as a control. (**B**) The miR-499 level in cardiomyocytes treated with an agonist of JNK (anisomycin) at different concentrations for 6 h. Cardiomyocytes treated with DMSO (0 μM) were used as a control. (**C**) Luciferase activity measured from H9c2 cells transfected with constructs containing the *Myh7b* promoter, and then treated with H_2O_2 at different concentrations for 24 h. (**D**) Luciferase activity measured from H9c2 cells transfected with constructs containing the *Myh7b* promoter, and then treated with DMSO or SP600125 for 2 h before exposure to H_2O_2. (**E**) Luciferase activity measured from H9c2 cells transfected with constructs containing the wild-type (WT) or mutant (Mut) putative AP-1 binding sites in the *Myh7b* promoter, and then treated with H_2O_2. (**F**) ChIP analysis of p-c-Jun binding to the *Myh7b* promoter in cardiomyocytes exposed to H_2O_2. W, water; M, marker. ap-c-Jun, antibody against p-c-Jun. **P* < 0.05 compared with the control. Data represent the results of three independent experiments.

inconsistent pattern (**Fig. 3A**); *Dcc* mRNA was not detected. In addition, miR-499 mimic treatment also decreased mRNA expression of *Pdcd4, Pacs2,* and *Dyrk2* (**Fig. 3B**). Western blot analysis indicated that administration of miR-499 mimics attenuated *Pdcd4, Pacs2,* and *Dyrk2* expression, whereas miR-499 inhibitor treatment elevated the expression of *Pdcd4, Pacs2,* and *Dyrk2* (**Fig. 3C**).

To further confirm that *Pdcd4*, *Pacs2,* and *Dyrk2* are direct miR-499 targets, we tested whether miR-499 could suppress their expression by binding to the 3′ UTR of these genes. Sequence analysis indicated that *Pdcd4, Pacs2,* and *Dyrk2* had conserved binding sites for miR-499 in their 3′ UTR (**Fig. S3A**). We generated luciferase reporters containing the 3′ UTR of *Pdcd4, Pacs2,* and *Dyrk2*, respectively. Addition of miR-499 mimics into these systems resulted in a significant reduction in luciferase activity compared with miR-499 NC, whereas no effect was observed with a construct containing a mutated segment of *Pdcd4, Pacs2,* and *Dyrk2* (seed sequence AGUCUUA was mutated to AGUCUGC). Moreover, no change was seen in luciferase reporter activity when miR499 NC was co-transfected with each reporter construct (**Fig. 3D–F**).

PDCD4 and PACS2 participate in the regulation of apoptosis in cardiomyocytes exposed to H_2O_2 via their effect on *Bid* expression

Next, we explored how PDCD4, PACS2, and DYRK2 participate in the cell death program triggered by H_2O_2. We

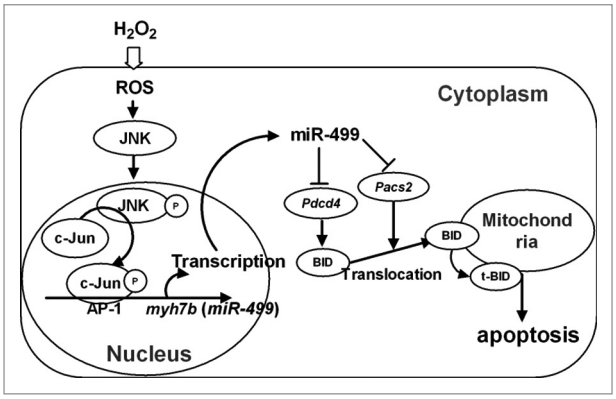

Figure 7. A schematic model of the role of miR-499 in regulating cardiomyocyte apoptosis.

chose the H9c2 cell line because it is not only a cardiac myoblast cell line, but also expresses a high level of miR-499, similar to primary cardiomyocytes.[14]

To investigate how PDCD4, PACS2, and DYRK2 regulate H_2O_2-induced apoptosis, we first prepared *Pdcd4, Pacs2,* and *Dyrk2* overexpression plasmids as well as siRNAs and used them to transfect H9c2 cells. Western blot analysis results showed the overexpression or silencing of all three proteins by transfecting their respective plasmids or siRNAs (**Fig. S4A**). Meanwhile, western blot analysis results also showed that the *Pdcd4*-overexpression plasmid induced upregulation of *Bid* expression, whereas the *Pdcd4* siRNA resulted in downregulation of *Bid* (**Fig. 4A**).

To determine whether PACS2 recruits BID to mitochondria, H9c2 cells were transiently transfected with the *Pacs2*-overexpression construct, and then treated with H_2O_2 to induce apoptosis. We found that overexpression of *Pacs2* in H_2O_2-treated H9c2 cells enhanced BID recruitment to the mitochondria, while knockdown of *Pacs2* by siRNA blocked BID recruitment to the mitochondria (**Fig. 4B**).

Taira et al. reported that DYRK2 could induce *P53aip1* expression in response to the apoptotic stimulus,[25] but we did not observe any significant changes in *P53aip1* expression in *Dyrk2*-overexpressing or *Dyrk2*-silenced cardiomyocytes that were treated with H_2O_2 (**Fig. S4B**), so the function of DYRK2 might not be very important in the cardiomyocyte apoptosis induced by H_2O_2. Therefore, we focused on PDCD4 and PACS2.

To understand how PDCD4 and PACS2 exert their function in H_2O_2-induced apoptosis, we conducted TUNEL assay (**Fig. 4C**; **Fig. S4C**) and flow cytometry analysis (**Fig. 4D**) on the cells. We found that in response to H_2O_2 stimulation, overexpression of *Pdcd4* or *Pacs2* alone could enhance apoptosis, and co-transfection with both *Pdcd4*- and *Pacs2*-overexpressing constructs had a synergistic effect and resulted in enhanced apoptosis (**Fig. 4C, 4D**). The knockdown of either *Pdcd4* or *Pacs2* could inhibit apoptosis, while knockdown of both *Pdcd4* or *Pacs2* had a synergistic effect and showed enhanced apoptosis inhibition (**Fig. 4E and F**).

To investigate the role of BID in the apoptosis system, siRNA specially targeting *Bid* was used to transfect cells (**Fig. S4D**). As demonstrated by TUNEL assay (**Fig. 4G**; **Fig. S4E**) and flow cytometry analysis (**Fig. 4H**), *Bid* knockdown could reverse the high apoptosis rate induced by the overexpression of both *Pdcd4* and *Pacs2* (**Fig. 4G and H**).

miR-499 inhibits H_2O_2-induced apoptosis through its effect on PDCD4 and PACS2

First, we studied the effect of miR-499 on BID. Rat cardiomyocytes were transfected with miR-499 NC, mimics, or inhibitor, and then treated with H_2O_2. The overexpression of miR-499 not only inhibited *Bid* expression, but also blocked BID translocation to mitochondria; in contrast, the miR-499 inhibitor upregulated the protein level of BID, as well as BID translocation to mitochondria (**Fig. 5A**).

Next, we co-transfected H9c2 cells with miR-499 NC or mimics and a series of constructs encoding *Pdcd4* and *Pacs2* with wild-type 3′UTR or mutated 3′UTR. Western blot analysis results showed that miR-499 mimics could inhibit *Pdcd4* and *Pacs2* expression at the protein level in the case of co-transfection with wild-type miR-499 3′UTR but not mutated 3′UTR (**Fig. 5B**). *Bid* expression (**Fig. 5C**) and mitochondrial translocation (**Fig. 5D**) were also inhibited in the presence of constructs encoding *Pdcd4* or *Pacs2* with wild-type miR-499 3′UTR but not mutated 3′UTR, indicating that miR-499 indeed suppressed BID through PDCD4 and PACS2. TUNEL assay showed that miR-499-mediated protective effects were only observed in the presence of constructs encoding *Pdcd4* or *Pacs2* with wild-type 3′UTR but not with mutated 3′UTR (**Fig. 5E and F**).

Phosphorylation of c-Jun regulates miR-499 expression in response to H_2O_2 treatment

In cardiomyocytes treated with inhibitors of JNK, ERK, and p38, that is, SP600125,[26] PD98059,[27] and SB203580,[28] respectively, we found that only SP600125 could reverse H_2O_2-induced miR-499 upregulation (**Fig. 6A**). H_2O_2 could induce JNK and c-Jun phosphorylation, and SP600125 could attenuate the phosphorylation of JNK and c-Jun (**Fig. S5A**), which is consistent with previous reports.[29,30] To determine the effect of the JNK pathway, we treated cardiomyocytes with the JNK agonist anisomycin.[31] The results showed that the activation of the JNK pathway was sufficient to upregulate the expression of miR-499 in cardiomyocytes (**Fig. 6B**).

Sequence analysis of the promoter region of *Myh7b* shows that it contains one binding site for the transcription factor AP-1, which is conserved among rats, mice, and humans (**Fig. S5B**). To determine how H_2O_2 stimulus upregulates the miR-499 level, a 1-kb region of the *Myh7b* regulatory genomic fragment upstream of the translational start site was cloned into the pGL3-luciferase reporter,[10] and then transfected into H9c2 cells. We found that H_2O_2 treatment could increase the *Myh7b* promoter activity approximately 1.6-fold (**Fig. 6C**). Also, SP600125 treatment attenuated the *Myh7b* promoter activity (**Fig. 6D**). When the AP-1-binding site was mutated from TGACTCA to TGCCTCG, the H_2O_2-induced luciferase activity was reduced (**Fig. 6E**).

Chromatin immunoprecipitation (ChIP) analysis revealed that p-c-Jun (phosphorylated c-Jun) bound to the AP-1-binding site region even without H_2O_2 stimulus, whereas H_2O_2 treatment increased p-c-Jun binding activity; while, when the JNK pathway was blocked by SP600125, the binding activity of p-c-Jun was decreased (**Fig. 6F**).

These data demonstrate that upregulation of miR-499 by H_2O_2 in cardiomyocytes was dependent on the phosphorylation of c-Jun.

Discussion

We have demonstrated through this study that miR-499 plays a role in ROS-induced apoptosis in cardiac cells in vivo and in vitro. In response to H_2O_2 treatment, the expression of miR-499 was upregulated depending on JNK activation and c-Jun phosphorylation. Two novel target proteins of miR-499, PDCD4, and PACS2 were identified. PDCD4 upregulates the expression of *Bid*, and PACS2 translocates BID to the mitochondria to induce apoptosis. This is in agreement with the findings of other reports.[32,33] PDCD4 is a novel tumor suppressor that brings its effects by interacting with translation initiation factors eIF4A and eIF4G and inhibiting translation.[34,35] In *Pdcd4*-deficient islet β cells, the mRNA level of proapoptotic genes (*Bad*, *Bax*, and *Bid*) was significantly decreased, whereas the mRNA level of the anti-apoptotic gene *Bcl-xl* was increased.[32] PACS2, a multifunctional sorting protein, integrates ER-mitochondria communication, ER homeostasis, and apoptosis. In response to apoptotic stimuli, PACS2 potentiates the translocation of BID to mitochondria, which initiates a sequence of events that lead to cell death.[33] BID belongs to the BH3-only subgroup of pro-apoptotic molecules of the Bcl-2 protein family and modulates diverse apoptotic signals.[36] Once full-length BID is activated, it forms truncated BID (tBID), which induces the release of apoptotic factors, including cytochrome c and Smac/Diablo after its translocation from the cytoplasm into mitochondria.[37,38] Thus, BID activation (tBID formation) is an important upstream event in the mitochondrial death pathway.[39]

Our results indicated that the proapoptotic protein DYRK2 may not participate in H_2O_2-induced apoptosis in cardiomyocytes, even though it is a direct target of miR-499. It has been shown that in cells exposed to genotoxic stress, DYRK2 is translocated to the nucleus where it phosphorylates p53, and subsequently, induces *P53aip1* expression, which leads to apoptosis in various cancer cells.[25] Therefore, DYRK2 may play little roles in cardiac cells.

Previous studies by Wang et al. showed that miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1.[40] Our data therefore revealed a novel aspect of the cellular functions of miR-499 in the regulation of apoptosis: miR-499 not only represses *Bid* expression by targeting *Pdcd4*, but also blocks the translocation of BID from the cytoplasm to mitochondria by targeting *Pacs2*. We found that *Pdcd4*, *Pacs2,* and *Dyrk2* are direct targets of miR-499, but whether this miRNA has other potential targets remains to be determined.

Our study has demonstrated that induction of miR-499 by H_2O_2 was dependent on JNK activation and also dependent on c-Jun phosphorylation. We found that the phosphorylated c-Jun bound to the AP-1-binding site of the *Myh7b* promoter and induced upregulation of miR-499. The transcription factor AP-1 has been reported to be involved in the induction of apoptosis in cells in response to stress factors and growth factor withdrawal.[41]

We have also noticed that the expression of pro-apoptosis genes *Pdcd4*, *Pacs2,* and *Dyrk2* was downregulated in response to H_2O_2 treatment. We repeated the experiments many times, and the results were all the same. After searching literature, we found that the similar phenomenon was reported previously. Sheedy et al.[42] reported that treatment of human peripheral blood mononuclear cells with lipopolysaccharide resulted in lower *Pdcd4* expression, which was due to induction of the miR-21 via the adaptor myD88 and NFκB. Zhang et al.[24] reported that the expression of miR-21 was upregulated, but the pro-apoptosis gene *Pdcd4*, as the target of miR-21, was downregulated. H_2O_2 treatment could result in the upregulation of endogenous miR-499, as the target genes of miR-499, *Pdcd4*, *Pacs2,* and *Dyrk2* was downregulated through post-transcriptional regulation.

Through this study, we establish a regulatory pathway that H_2O_2 induces JNK and c-Jun phosphorylation in cardiomyocytes, the activation of the JNK pathway upregulates the expression of miR-499 by binding p-c-Jun to the AP-1 site on the *Myh7b* promoter, miR-499 acts as a upstream mediator of the mitochondrial pathway through PDCD4 and PACS2 to inhibit *Bid* expression and mitochondrial translocation (**Fig.** 7). Therefore, control of the mitochondrial apoptosis pathway by miR-499 may be important for blocking the effect of pathological insults to the heart. Based on all these results, we propose that miR-499 may serve as an indicator of cardiac disease, and can be used as a target for cardiac disease treatment strategies.

Table 1. siRNAs used for transfection

Gene	Sequence (5′–3′)
Pdcd4	Sense: GUCUAAAGGU GGAAAGCGUd TdT
	Anti-sense: ACGCUUUCCA CCUUUAGACd TdT
Pacs2	Sense: CAACAGAACU UCAAGCAGAd TdT
	Anti-sense: UCUGCUUGAA GUUCUGUUGd TdT
Dyrk2	Sense: GCAUGAACCU CUAUGAGCUd TdT
	Anti-sense: AGCUCAUAGA GGUUCAUGCd TdT
Bid	Sense: CCGAAACAAU GACCGUGAUd TdT
	Anti-sense: AUCACGGUCA UUGUUUCGGd TdT

Materials and Methods

Cell culture

Neonatal rat ventricular myocytes were prepared from 1- or 2-d-old Sprague-Dawley rats as previously described.[43] The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 15% fetal bovine serum (FBS) for 36 h; then, the culture medium was changed to serum-free medium and the cells were cultured for another 12 h before further experiments. H9c2 cells (ATCC® CRL-1446™) and HeLa cells (ATCC® CCL-2™) were maintained in high-glucose DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml).

Plasmid constructs

The construct *Myh7b*-luc was a generous gift from Dr Leslie Leinwand (Department of Molecular, Cellular, and Developmental Biology, University of Colorado); Bid-GFP was

Table 2. Primers used in qRT-PCR

Primers	Primer sequences (5′–3′)	Product size (bp)
18S RNA	F: GTAACCCGTT GAACCCCATT	151
	R: CCATCCAATC GGTAGTAGCG	
Pdcd4	F: TGCCCGTGTT GGCAGTGTCC	190
	R: TGGCCCACCA ACTGTGGTGC	
Pacs2	F:TCTGGATCGT CTCCCTGTCC	187
	R: TTTCCCACAT CAAAGTCGTC C	
Dyrk2	F: GCTCACGTCC CCTGATGC	95
	R: GAGCAAATAA AAACAGGTCT GAGC	
Edar	F: CCAACTGTGG TGAGAACGAA T	118
	R: TCGTCGTCTT TAGTGCCGTAT	
Phlda2	F: CTCCGACGAG ATCCTTTGCG	194
	R: ACACGTACTT AGAGGTGTGC TC	
Phf17	F: CGAAAGCCTT CTGAGGTGTT T	96
	R: AGCCAGCACG TAGTAGTCAT C	
Dcc	F: CAAGCTGGCT TTTGTACTCT TCG	176
	R: GAACTCCTCG GTCGGACTCT	

from Dr Heesun Byun (Department of Pharmacology, College of Medicine, Chungnam National University); pCMV-SPORT-*Pdcd4* was from Dr Iwata Ozaki (Health Administration Center, Department of Internal Medical, Saga Medical School, Saga University); pcDNA3-*Pacs2* was from Dr Gary Thomas (Vollum Institute, Oregon Health and Science University); pEGFP-*Dyrk2* was from Dr Walter Becker (Institute of Pharmacology and Toxicology, Medical Faculty of the RWTH Aachen University); and pGL3-*Pdcd4*-3′UTR was from Dr Giridhar Mudduluru (Department of Experimental Surgery Mannheim/Molecular Oncology of Solid Tumors, Deutsches Krebsforschungszentrum and University Heidelberg). pMIR-REPORT-*Pdcd4*-3′UTR was constructed by cloning the *Pdcd4*-3′UTR from pGL3-*Pdcd4*-3′UTR into pMIR-REPORT. pMIR-REPORT-*Pacs2*-3′UTR and pMIR-REPORT-*Dyrk2*-3′UTR were commercially constructed by RiboBio (RiboBio); pcDNA3.1-*Pdcd4*+3′UTR [*Pdcd4*(fl)] and pcDNA3.1-*Pacs2*+3′UTR [*Pacs2*(fl)] were commercially constructed by GeneChem (GeneChem); mutation of the potential miR-499-binding sites on pMIR-REPORT-*Pdcd4*-3′UTR, pMIR-REPORT-*Pacs2*-3′UTR, pMIR-REPORT-*Dyrk2*-3′UTR, *Pdcd4*(fl) and *Pacs2*(fl) was performed by TransGen. siRNAs targeting *Pdcd4*, *Pacs2*, *Dyrk2,* and *Bid* (**Table 1**) were commercially synthesized by Sigma.

Expression of cardiac-abundant miRNAs in cardiac cells exposed to H_2O_2

Cultured rat cardiomyocytes were treated with H_2O_2 (0–200 μM) for 6 h, and miRNAs were then isolated from the cultured cells using the RNAvzol Reagent. For northern blot analysis, cultured rat cardiomyocytes were treated with H_2O_2 (0–200 μM) for 6 h. For western blot analysis, cultured rat cardiomyocytes were treated with H_2O_2 (0–200 μM) for 12 h. The MAPK pathway inhibitors, SP600125, SB203580, and PD98059, and the JNK pathway agonist anisomycin were purchased from Sigma, and the cardiomyocytes were pretreated with these substances at 50 μM for 2 h, and then stimulated with H_2O_2 for 6 h.

Oligonucleotide transfection in cultured cardiomyocytes

The miR-499 NC, mimics, and inhibitor were obtained from RiboBio. miR-499 mimics are chemically synthesized fragments that have the same sequence as miR-499 and enhance endogenous miR-499 function, whereas miR-499 inhibitors are chemically synthesized fragments designed as with reverse complementary sequence to miR-499, which could weaken endogenous miR-499 effects. The negative control was a scramble miRNA, which was 22 nucleotides long. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The medium was replaced with new culture medium 6–8 h after transfection.

Real-time RT-PCR

Briefly, RNAs from cardiomyocytes were isolated using an RNAvzol reagent (Vigorous Biotech) based on the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA with M-MLV reverse transcriptase (Promega) in a 25-μl reaction mixture, which contained 2 μg total RNA, 400 mM reverse transcriptional primers, 4 U/μl M-MLV, 1 U/μl RNAsin, and 0.4 mM dNTP mix. qRT-PCR was performed in the ABI 7300 real-time RT-PCR system with reagents from the SYBR® Green Real-time PCR Master Mix (TOYOBO) and the appropriate primers (**Table 2**). All annealing temperatures were 60 °C. Transcription levels were normalized to 18S rRNA level, and miRNA levels were normalized to U6 level. Each value presents the average of the results from at least three independent experiments. The random primer was purchased from AUGCT Company (AUGCT) and the primers for miR-1, -133, -208, -499, -21, and U6 were purchased from RiboBio.

Western blot analysis

Western blot analysis was performed as previously described.[44] Cells were seeded into a 60-mm plate at 4×10^5 cells per plate and harvested after 48 h of culture. Total protein was prepared and subjected to 12% SDS PAGE and subsequently transferred onto nitrocellulose membranes. The primary antibodies used were antibodies against PDCD4 (code: sc-27123), PACS2 (code: sc-160645), DYRK2 (code: sc-66867), p-JNK (code: 4668, Cell Signaling Technology), total JNK (code: 9252, Cell Signaling Technology), p-c-Jun (code: sc-822, Santa Cruz), total c-Jun (code: sc-166540), cytochrome c (code: sc-13156), pro-caspase-3 (code: sc-7148), cleaved-caspase-3 (code: ab13847, Abcam), BID (code: sc-6538), and GAPDH (code: TA-08, ZSGB-BIO); horseradish peroxidase-conjugated anti-goat, anti-mouse, and anti-rabbit antibodies were used as secondary antibodies.

Luciferase assays

HeLa cells were seeded at a density of 1×10^5 per well in a 24-well plate. When the cells reached 60% confluence, they were transfected with constructs of miR-499 mimics, NC, pMIR-REPORT-*Pdcd4*-3′UTR, pMIR-REPORT-*Pacs2*-3′UTR, pMIR-REPORT-*Pdcd4*-3′UTR(mut), pMIR-REPORT-*Pacs2*-3′UTR(mut), and *Myh7b*-luc, respectively, using VigoFect (Vigorous) according to the manufacturer's instructions. The total amount of DNA was kept constant using pcDNA3.1 or

pGL3-basic plasmid. Luciferase activity was measured and normalized to Renilla luciferase activity. All experiments were done in triplicate, and each experiment was repeated three times.

ChIP assay

Neonatal rat cardiomyocytes were treated with H_2O_2 or SP600125 before H_2O_2 treatment and cells were harvested 6 h later. ChIP experiments were performed according to a previously described method.[43] After crosslink reversal, precipitated DNA was analyzed by PCR for fragments of the *Myh7b* promoter by using the following primers: 5′-GCTCCCTTCC CTGGTCTTTC-3′ (forward) and 5′-GGACAAGGCA GCGAGGCACT-3′ (200 bp). The annealing temperature was 60 °C. The data obtained were normalized to that of corresponding DNA precipitated by IgG.

Role of miR-499 in H_2O_2-induced apoptosis

Briefly, rat cardiomyocytes cultured in serum-free medium were treated with H_2O_2 for 6 h. Cell death (apoptosis and necrosis) was then measured by flow cytometry analysis, and cell apoptosis was measured by terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL) staining. For flow cytometry analysis, briefly, cultured cells were harvested by trypsinization and washed with PBS. Cells (1×10^6) from each sample were processed for Annexin V/PI apoptosis detection (Biosea Biotech) according to the manufacturer's instructions. For TUNEL analysis, cardiomyocytes cultured on coverslips in 24-well plates were fixed in 4% paraformaldehyde. TUNEL staining was done using the in situ cell death detection kit (Roche) according to the manufacturer's protocol. The number of TUNEL-positive cells was counted under a fluorescence microscope. In the western blot analysis, the mitochondria were isolated with the Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific).

Immunoprecipitation and confocal imaging

For confocal microscopy, cells were cultured on confocal dishes and transfected with plasmids or oligonucleotides. One day later, cells were fixed with 4% formaldehyde, permeabilized with phosphate-buffered saline containing 0.1% Triton X-100, and incubated with primary antibodies against α-actinin (code: A7811, Sigma) and BID (code: sc-6538), which was followed by incubation with the anti-mouse or anti-goat secondary antibodies conjugated to Alexa Fluo 488. Nuclei were stained with Hoechst 33342. The confocal dishes were examined with an Olympus confocal microscope (Olympus Corporation). For staining of cells with Mitotracker (Invitrogen), the cultured cells were incubated with Mitotracker dye for 15 min in the incubator at 37 °C before washing and fixation for staining.

Northern blot analysis

Northern blot analysis was performed with the miRNA northern blot assay kit following the manufacturer's instructions. Total miRNA was isolated using the miRcute miRNA extraction kit (TIANGEN) following the manufacturer's instructions. The oligonucleotide probes used to detect miR-499 and U6 snRNA were purchased from Signosis (Signosis Inc.). U6 was used as an internal control.

Rat myocardial I/R model

Male SD rats were anesthetized by intraperitoneal injection using composite anesthetic (0.1 ml/30 g body weight). The trachea was intubated for artificial respiration with room air. Thoracotomy was performed between the sternum and left costa. The pericardium was opened to expose the heart. A 6–0 suture was passed around the left anterior descending coronary artery, and the coronary artery was occluded by pulling on the suture tightly.[45] After 1 h of myocardial ischemia, the suture was loosened for 6 h for reperfusion. In the sham-control group, rats were exposed to all surgical procedures except ligation of the anterior descending coronary artery. Each group had six rats. This study was approved by the Institutional Laboratory Animal Care and Use Committee of Peking University Health Science Center. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the China National Institute of Health.

Statistical analysis

The data are expressed as mean ± standard deviation (S.D.). Comparisons between groups were analyzed using Student's *t* test or ANOVA, and the Student–Newman-Kleuss method was used to estimate the level of significance. Differences were considered to be statistically significant at $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here:
www.landesbioscience.com/journals/rnabiology/article/28300/

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