Cardiovascular Research (2010) **87**, 431–439
doi:10.1093/cvr/cvq082

Ischaemic preconditioning-regulated miR-21 protects heart against ischaemia/reperfusion injury via anti-apoptosis through its target PDCD4

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Received 26 December 2009; revised 14 February 2010; accepted 5 March 2010; online publish-ahead-of-print 10 March 2010

Time for primary review: 32 days

Aims The aims of the present study are to determine the miRNA expression signature in rat hearts after ischaemic preconditioning (IP) and to identify an IP-regulated miRNA, miR-21, in IP-mediated cardiac protection, and the potential cellular and molecular mechanisms involved.

Methods and results The miRNA expression signature was investigated in rat hearts. Among the 341 arrayed miRNAs, 40 miRNAs were differentially expressed (21 up and 19 down) in rat hearts with IP, compared with their controls. Some of these differentially expressed miRNAs were further verified by quantitative reverse transcriptase–polymerase chain reaction. Remarkably, miR-21 was one of most upregulated miRNAs in hearts after IP. *In vivo*, IP-mediated cardiac protection against ischaemia/reperfusion injury was inhibited by knockdown of cardiac miR-21. In cultured cardiac myocytes, we identified that miR-21 also had a protective effect on hypoxia/reoxygenation-induced cell apoptosis that was associated with its target gene, programmed cell death 4. The protective effect of miR-21 on cardiac cell apoptosis was further confirmed in rat hearts after ischaemia/reperfusion injury *in vivo*.

Conclusion The results suggest that miRNAs are involved in IP-mediated cardiac protection. Identifying the roles of IP-regulated miRNAs in cardiac protection may provide novel therapeutic and preventive targets for ischaemic heart disease.

Keywords Ischaemic preconditioning • MicroRNA • Gene expression • Ischaemia/reperfusion injury • Ischaemic heart disease

1. Introduction

Despite current optimal treatment, ischaemic heart disease is still the number one killer in the USA.[1] Fortunately, mammals are able to evoke endogenous defensive mechanisms that protect the heart against ischaemic damages as demonstrated in a phenomenon known as ischaemic preconditioning (IP).[2,3] IP is a short period of ischaemia followed by reperfusion that arouses the endogenous mechanism of protection against a sustained ischaemic insult. IP itself is not clinically practical due to its innate character of injury, although it is a short ischaemia. However, elucidating the endogenous defensive molecular mechanisms in IP will definitely provide novel therapeutic approaches for ischaemic heart disease.

It is well established that, although the damage stimuli are diverse, the fate of a cell under any kind of damage conditions are decided by the gene changes inside the cells.[2,3] Some of these responsive genes are pro-injury genes leading the cells to injury and death, whereas some of them are intrinsic defensive genes that protect the cells against damages.[2,3] Currently, IP is one of the most powerful ways for cardiac protection by increasing multiple defensive gene expression and/or decreasing multiple pro-injury gene expression.[2] However, how are these genes regulated under IP and which are the key regulators for the cardiac protection are currently unclear.[3]

miRNAs have emerged as a novel class of endogenous, small, non-coding RNAs that negatively regulate gene expression via degradation

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or translational inhibition of their target mRNAs.[4–7] Currently, the expression profiles of miRNAs in cardiac IP are not identified. However, recent two reports have suggested that some miRNAs may be involved in cardiac IP. In an *in vitro* Langendorff IP mouse model, Yin *et al.*[8] demonstrated that cardiac miR-1, miR-21, and miR-24 were significantly increased by IP. In addition, injection of IP-miRNAs protected the hearts against ischaemia/reperfusion injury (I/R) in the *in vitro* model.[8] Another excellent study in this research area is reported by Rane *et al.*[9] These investigators demonstrated that both in cultured rat cardiac cells and in porcine hearts, miR-199a was downregulated by IP. In the current study, the expression signature of miRNAs in rat hearts after IP *in vivo* was determined by microarray analysis. Moreover, the role of miR-21, an IP-upregulated miRNA, in cardiac I/R, and the potential mechanisms involved were investigated.

2. Methods

2.1 Cardiac IP and I/R animal models

IP was induced in 10-week-old male Sprague-Dawley rat hearts as described in our previous study.[10] In brief, the rats were anaesthetized with ketamine (80 mg/kg ip) and xylazine (5 mg/kg ip). Under sterile conditions, the heart was exposed through a left thoracotomy in the fourth intercostal space. IP was achieved via four cycles of 5-min occlusion/5-min reperfusion cycles of the left anterior descending coronary artery (LAD). I/R was induced by 60-min occlusion, followed by 3-h reperfusion of LAD. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey, New Jersey Medical School, and were consistent with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, revised 1985).

2.2 Measurement of myocardial infarct size

At 3 h after reperfusion, rats were anaesthetized and 6 mL of 10% Evans blue dye was injected into the vena cava to define the area that was not supplied by LAD. The myocardial ischaemic area at risk (IAR) was identified as the region lacking blue staining. The ventricles of the hearts were sliced transversely into 2-mm thick slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) at 37°C for 10 min to identify the non-infarcted and infarcted areas. TTC staining was displayed as red colour. The infarcted area was defined as TTC unstained area (write colour). Infarct size was expressed as a percentage of the IAR (% IAR).[10]

2.3 miRNA expression signature array

At 6 h after IP, the LAD supplied left ventricles were isolated for miRNA microarray analysis using the chip contained 341 mature miRNAs (Chip ID miRRat 12.0 version; LC Sciences).[11,12] Each group had six rats, and miRNA expression profiling was done by miRNA microarray analysis using a chip containing 341 mature miRNAs (Chip ID miRRat 12.0 version; LC Sciences). Data were analysed by subtracting the background and then normalizing the signals using a LOWESS filter (locally weighted regression). The miRNA expression was demonstrated by the mean of the six biological replicates. For the two-colour experiments, the ratio of the two sets of detected signals (log 2 transformed, balanced) and *P*-values of the *t*-test were calculated; differentially detected signals were those with *P*-values < 0.01. Proprietary 'spike-in' controls were used at each step of the process.

2.4 Construction of the adenovirus expressing miR-21, programmed cell death 4, or control adenovirus expressing GFP

The adenovirus expressing miR-21 (Ad-miR-21), programmed cell death 4 (Ad-PDCD4), or control adenovirus expressing GFP (Ad-GFP) were generated using the Adeno-X™ Expression Systems 2 kit (Clontech) according to the manufacturer's protocols.[10] These adenoviruses were purified by cesium chloride gradient ultracentrifugation and were titrated using a standard plaque assay.

2.5 Knockdown of cardiac miR-21 expression *in vivo* using antagomiR-21

AntagomiR is a novel class of chemically engineered antisense oligonucleotides targeting a specific miRNA. These antagomirs contain a hydroxyprolinol-linked cholesterol and 2′-*O*Me phosphoramidites to stabilize them in the presence of nuclease degradation and to promote their tissue uptake.[13] In the current study, antagomiR-21 was used to knockdown the cardiac miR-21 expression *in vivo*. The antagomir-21 was synthesized by Integrated DNA Technologies with the following sequence and structure: 5′mU*mC*mA*mAmCmAmUmCmAmGmUm CmUmGmAmUmAmAmGmC*mU* mA*/3CholTEG/′. The control oligo was also synthesized by Integrated DNA Technologies. AntagomiR-21 was delivered into hearts using the local delivery method as described in our recent study.[10] Briefly, rats were anaesthetized with ketamine (80 mg/kg ip) and xylazine (5 mg/kg ip). The pericardium was opened via the third intercostal space. The aorta and pulmonary artery were identified. A 23 G catheter containing 200 μL of PBS solution with antagomiR-21 (80 mg /kg) was advanced from the apex of the left ventricle to the aortic root. The aorta and pulmonary arteries were clamped distal to the site of the catheter and the solution was injected. The clamp was maintained for 10 s when the heart pumped against a closed system (isovolumically). This procedure allows the solution that contains the adenovirus to circulate down the coronary arteries and perfuse the heart. After 10 s, clamps on the aorta and pulmonary artery were released and the chest was closed. Twenty-hours later, cardiac IP and I/R were performed in these rats and the heart tissues were isolated for miR-21 assay.

2.6 Cardiac myocyte culture and cell ischaemia injury model

Primary cultures of neonatal rat cardiac ventricular myocytes were performed as described previously.[10,12,13] In brief, hearts from 1- to 2-day-old Sprague-Dawley rats were placed in ice-cold 1× phosphate-buffered saline solution. After repeated rinsing, the atria were cut off, and the ventricles were minced with scissors. The minced tissue and ventricular cells were dispersed by digestion with collagenase type IV (0.45 mg/mL), 0.1% trypsin, and 15 μg/mL DNase I. Cardiomyocytes (0.33×10^6 cells/mL) were cultured in cardiac myocyte culture medium containing DMEM/F-12 supplemented with 5% horse serum, 4 μg/mL transferrin, 0.7 ng/mL sodium selenite, 2 g/L bovine serum albumin, 3 mmol/L pyruvic acid, 15 mmol/L HEPES, 100 μmol/L ascorbic acid, 100 μg/mL ampicillin, 5 μg/mL linoleic acid, 1% penicillin, 1% streptomycin, and 100 μmol/L 5-bromo-2′-deoxyuridine and seeded into six-well plates. Cell ischaemic injury, induced by hypoxia in a serum- and glucose-free medium, and reoxygenation (H/R) was performed as described.[10] Hypoxia was achieved by placing the cells in a hypoxia chamber filled with 5% CO_2 and 95% N_2 at 37°C for 4 h. Following hypoxia exposure, the cells were reoxygenated with 5% CO_2 and 95% O_2 for 3 h in DMEM containing 5% serum and normal glucose.

2.7 Oligo transfection, miR-21 knockdown, and PDCD4 gene upregulation in cultured cardiac myocyte

Oligo transfection was performed according to an established protocol.[14,15] For the miR-21 knockdown, miR-21 inhibitor (LNA-anti-miR-21) (Exiqon, Inc.) was added to the culture medium at final oligonucleotide concentration of 30 nM. For the miR-21 upregulation, pre-miR-21 (Ambion, Inc.) was added directly to the complexes at final oligonucleotide concentration of 30 nM. PDCD4 gene upregulation was performed by Ad-PDCD4 (30 MOI). The transfection medium was replaced 4 h post-transfection by the regular culture medium. Vehicle control, oligo controls for LNA-anti-miR-21 and pre-miR-21 (Ambion, Inc.), and adenovirus control (Ad-GFP) were applied.

2.8 Quantitative reverse transcriptase–polymerase chain reaction and Northern blot analysis

miRNA expression in culture cardiac cells and in rat hearts was determined by quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) as described.[14] In brief, qRT–PCR was performed on cDNA generated from 50 ng of total RNA using the protocol of the mirVana qRT–PCR miRNA Detection Kit (Ambion, Inc.). Amplification and detection of specific products were performed with a Roche Lightcycler 480 Detection System. As an internal control, U6 was used for template normalization. The primers used were provided by Ambion, Inc. Fluorescent signals were normalized to an internal reference, and the threshold cycle (C_t) was set within the exponential phase of the PCR. The relative gene expression was calculated by comparing cycle times for each target PCR. The target PCR C_t-values were normalized by subtracting the U6 C_t-value, which provided the ΔC_t-value. The relative expression level between treatments was then calculated using the following equation: relative gene expression $= 2^{-(\Delta Ctsample - \Delta Ctcontrol)}$. Northern blot analysis of miR-21 was performed as described.[12] The probe sequence of miR-21 used was: 5′-TCAACATCAGTCTGATAAGCTA-3′.

2.9 Western blot analysis

Proteins isolated from cultured cardiac myocytes were determined by western blot analysis. Equal amounts of protein were subjected to SDS–PAGE. A standard western blot analysis was conducted using PDCD4 antibody (Santa Cruz Biotechnology).[15] GADPH antibody (1:5000 dilution; Cell Signaling) was used as the loading control.

2.10 Luciferase assay

A construct in which a fragment of the 3′-UTR of PDCD4 mRNA containing the putative miR-21 binding sequence was cloned into a firefly luciferase reporter construct and transfected into HEK 293 cells with either vehicle (vehicle control), an empty plasmid (pDNR-CMV) (0.2 μg/mL), a plasmid expressing miR-21 (pmiR-21) (0.2 μg/mL), or a control plasmid expressing an unrelated miRNA, miR-145 (pmiR-145), following the transfection procedures provided by Invitrogen. The construct with mutated targeting fragment (AUAAGCUA) at the 3′-UTR of PDCD4 without the putative miR-21 binding sequence was used as a mutated control.

2.11 Detection of apoptosis

Cultured cardiac myocyte apoptosis and apoptosis in heart sections were measured by terminal deoxynucleotide transferase dUTP nick end labelling (TUNEL) staining as described previously.[10,14] Briefly, cardiac myocytes cultured on coverslips in 24-well plates were fixed in 4% paraformaldehyde. The numbers of TUNEL-positive cells and the total cells in infarcted and border areas were counted under a fluorescence microscope. In heart tissue, TUNEL staining was performed in frozen heart sections (8 μM). The TUNEL staining was done using the *in situ* cell death detection kit (Roche) according to the manufacturer's protocol. The numbers of TUNEL-positive cells and the total cells in heart sections were counted under a fluorescence microscope.

2.12 Statistical analysis

All data are presented as mean ± standard error. For relative gene expression, the mean value of the vehicle control group was defined as 100%. Two-tailed unpaired Student's *t*-tests and ANOVA were used for statistical evaluation of the data. Sigma stat statistical analysis program was used for data analysis. A $P < 0.05$ was considered significant.

3. Results

3.1 Rat cardiac miRNA expression is regulated by IP

Among the 341 arrayed miRNAs, 40 miRNAs were differentially expressed (21 up and 19 down) in rat hearts with IP, compared with those in the sham group. miRNAs that were highly expressed in rat hearts with a 30% or greater change in their expression after IP are shown in *Table 1*. Remarkably, miR-21 was one of most upregulated miRNAs in hearts by IP and its expression was increased more than threefold compared with the control.

Table 1 miRNAs that are highly expressed in rat heart and over 30% change in their expression after IP

miRNA	Sham control	Ischaemic preconditioning	Expression change (%)
miR-21	1646	5812	353.10
miR-15b	349	816	233.81
miR-320	1186	2333	196.71
let-7e	2370	4211	177.68
miR-214	491	812	165.38
miR-16	2634	4203	159.57
miR-92a	400	622	155.50
let-7b	12129	18306	150.93
miR-151	886	1336	150.79
miR-361	727	1055	145.12
miR-195	1771	2483	140.20
let-7c	16935	23254	137.31
miR-22	739	993	134.37
miR-30a	1709	1193	69.81
miR-27b	3483	2401	68.93
miR-27a	1896	1269	66.93
miR-126	28702	18420	64.18
miR-26a	25592	16046	62.70
miR-30c	5445	3388	62.22
miR-125a-5p	1757	1036	58.96
miR-30b-5p	2366	1359	57.44
miR-26b	4373	2301	52.62
miR-352	711	316	44.44
miR-499	4171	913	21.89
miR-322	620	134	21.61

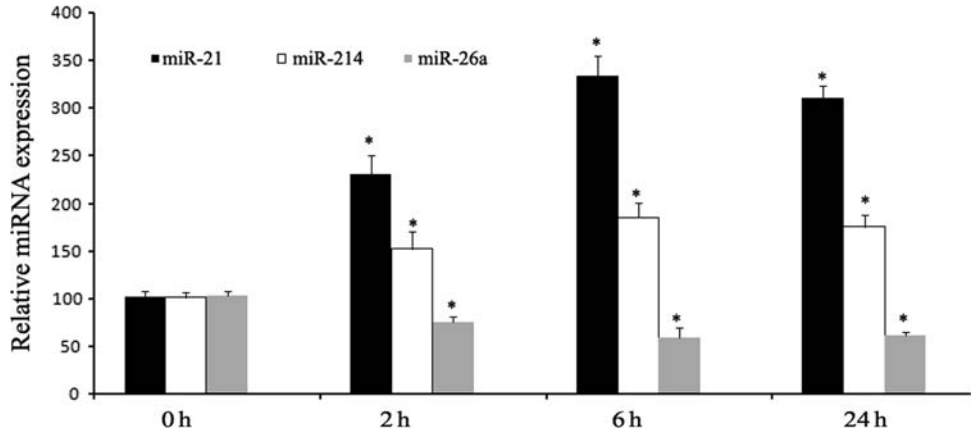

Figure 1 Verification of IP-regulated miRNAs in rat hearts. The expression of miR-21, miR-214, and miR-26a in sham-opened rat hearts (0 h), and in rat hearts at 2, 6, and 24 h after IP was determined by qRT–PCR. Note $n = 6$; $^*P < 0.05$ compared with the 0 h group.

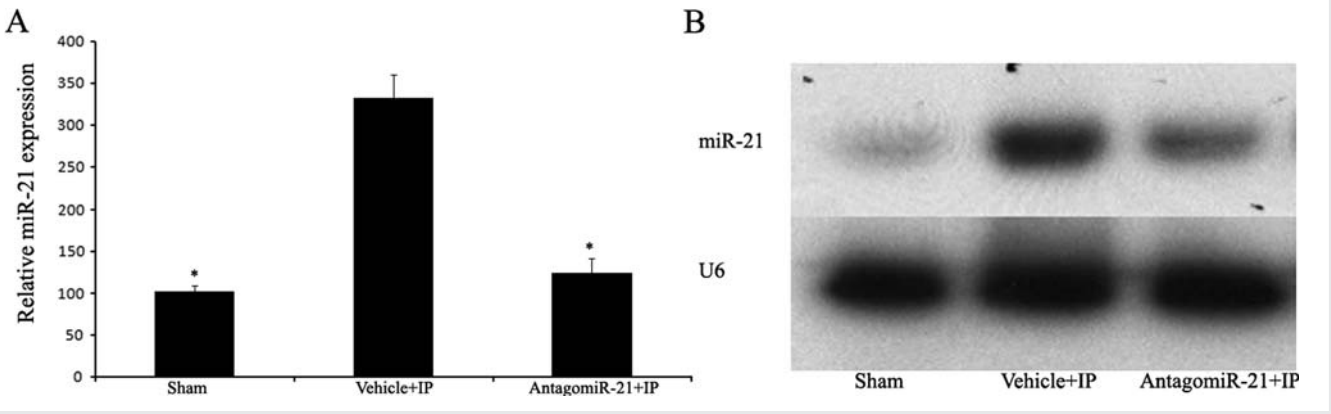

Figure 2 Inhibition of IP-upregulated miR-21 via antagomiR-21. AntagomiR-21 (80 mg/kg) or vehicle control was delivered into the rat hearts at 24 h before IP using the local delivery method described in Methods section. miR-21 expression was determined at 6 h after IP. (*A*) IP-induced upregulation of cardiac miR-21 was inhibited by antagomiR-21 as determined by qRT–PCR. (*B*) The inhibitory effect of antagomiR-21 on miR-21 expression was verified by Northern blot analysis. Note $n = 6$; $^*P < 0.05$ compared with the Vehicle + IP group.

3.2 Verification of miRNA expression via qRT–PCR

Some IP-regulated miRNAs identified by microarray analysis were further verified via qRT–PCR. In this experiment, RNAs were isolated from rat hearts at 2, 6, and 24 h after IP. The expression of miR-21, miR-214, and 26a was then determined. As shown in *Figure 1*, compared with sham-opened group (0 h), the expression of miR-21 and miR-214 in IP hearts was significantly upregulated at all the detected time points. In contrast, the expression of 26a was downregulated by IP. The results were consistent with those from microarray analysis.

3.3 Knockdown of miR-21 expression inhibits IP-induced cardiac protection against I/R in rat hearts

To determine the biological role of IP-induced miR-21 in IP-mediated cardiac protection, miR-21 expression was blocked by its antagomiR. In this experiment, antagomiR-21 or vehicle control was delivered into the rat hearts at 24 h before IP using the local delivery method described in Methods section. As shown in *Figure 2A*, IP-induced upregulation of cardiac miR-21 was successfully inhibited by antagomiR-21. The inhibition of miR-21 was further verified by Northern blot analysis (*Figure 2B*).

To determined the role of IP-induced miR-21 in IP-mediated cardiac protection, the rats were divided into the following four groups as demonstrated in *Figure 3A*. Group 1 was I/R rats without IP treated with vehicle (vehicle + I/R); Group 2 was I/R rats treated with vehicle and IP (Vehicle + IP + I/R); Group 3 was I/R rats treated with control oligo (scramble) and IP (scramble + IP + I/R); Group 4 was I/R rats treated with anagomiR-21 and IP (AnagomiR-21 + IP + I/R). Myocardial infarct size was determined in heart slices as shown in *Figure 3B*. The results showed that myocardial infarct size was significantly by IP (Group 2 vs. Group 1) (*Figure 3C*). Although the control oligo (scramble) (Group 3) had no effect on IP-induced cardiac protection, IP-induced cardiac

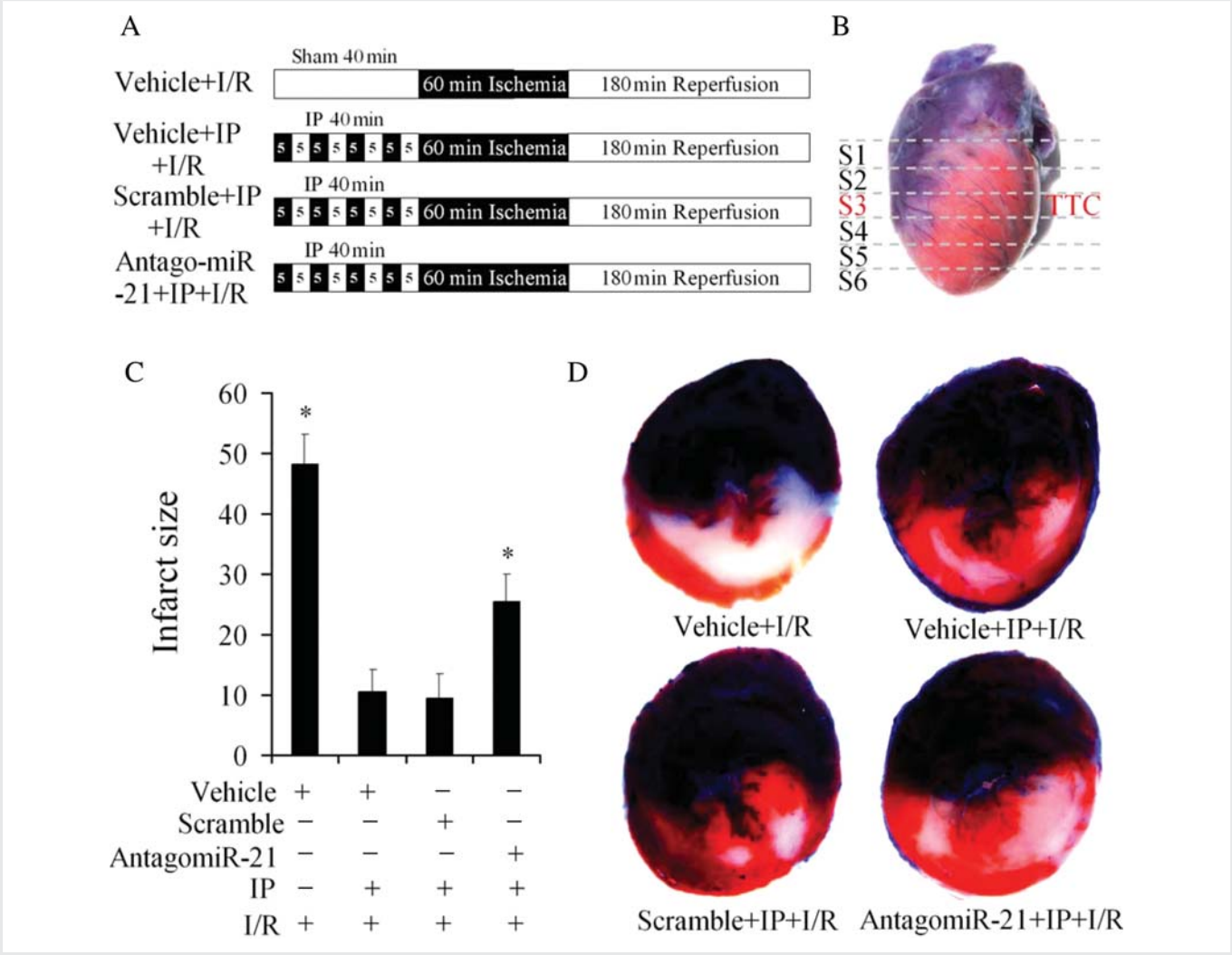

Figure 3 The role of miR-21 in IP-mediated protective effect on myocardial infarct size induced by I/R. (*A*) Schematic diagram showing the four groups of rats with different treatments. (*B*) Schematic diagram showing the six-heart slices for histology analysis. (*C*) The effects of IP and miR-21 inhibition on myocardial infarct size. (*D*) Representative TTC and Evans blue stained heart slices from rats treated with vehicle + I/R, vehicle + IP + I/R, scramble + IP + I/P, and antagomiR-21 + IP + I/P. Note: blue colour is Evans blue; red colour is TTC staining showing the uninfarcted area; write colour is infarcted area. $n = 9$, $^*P < 0.05$ compared with the group treated with vehicle + IP + I/R in (*C*).

protection on myocardial infarct size was significantly attenuated in antagomiR-21-treated animals (Group 4) (*Figure 3C*). Representative TTC and Evans blue stained heart slices from these four groups were shown in *Figure 3D*.

3.4 The effect of miR-21 inhibition on cardiac cell apoptosis induced by H/R injury *in vitro*

In order to determine the potential cellular mechanism in miR-21-mediated effect on cardiac I/R, a cell H/R model was applied, in which the cultured cell injury was induced by hypoxia in a serum- and glucose-free medium followed by reoxygenation in normal culture medium. We found that miR-21 expression was successfully inhibited by its inhibitor, LNA-anti-miR-21 (30 nM) in cultured cardiac cells (*Figure 4A*). As expected, H/R resulted in an increase in apoptosis (*Figure 4B*). Notably, cardiac myocyte apoptosis was exacerbated after treatment with LNA-anti-miR-21 (*Figure 4B*). In contrast, control oligo (scramble) had no effect on H/R-mediated cell apoptosis. Representative TUNEL-stained photomicrographs from uninjured cardiac myocytes and cardiac myocytes treated with vehicle, control oligos (scramble control), or LNA-anti-miR-21 were shown in *Figure 4C*. The results indicated that IP-upregulated miR-21 had a protective effect against the ischaemia-induced cardiac myocyte apoptosis *in vitro*.

3.5 The effect of miR-21 inhibition on cardiac cell apoptosis induced by I/R injury *in vivo*

To further confirm the cellular mechanism of IP-upregulated miR-21-mediated cardiac protection *in vivo*, apoptosis was determined in heart sections from these four groups of rats by immunoflorescence with TUNEL staining. The myocytes were identified by immunofluorescence with myosin heavy chain antibody. As shown in *Figure 5A*, cardiac cell apoptosis was significantly decreased by IP. Although

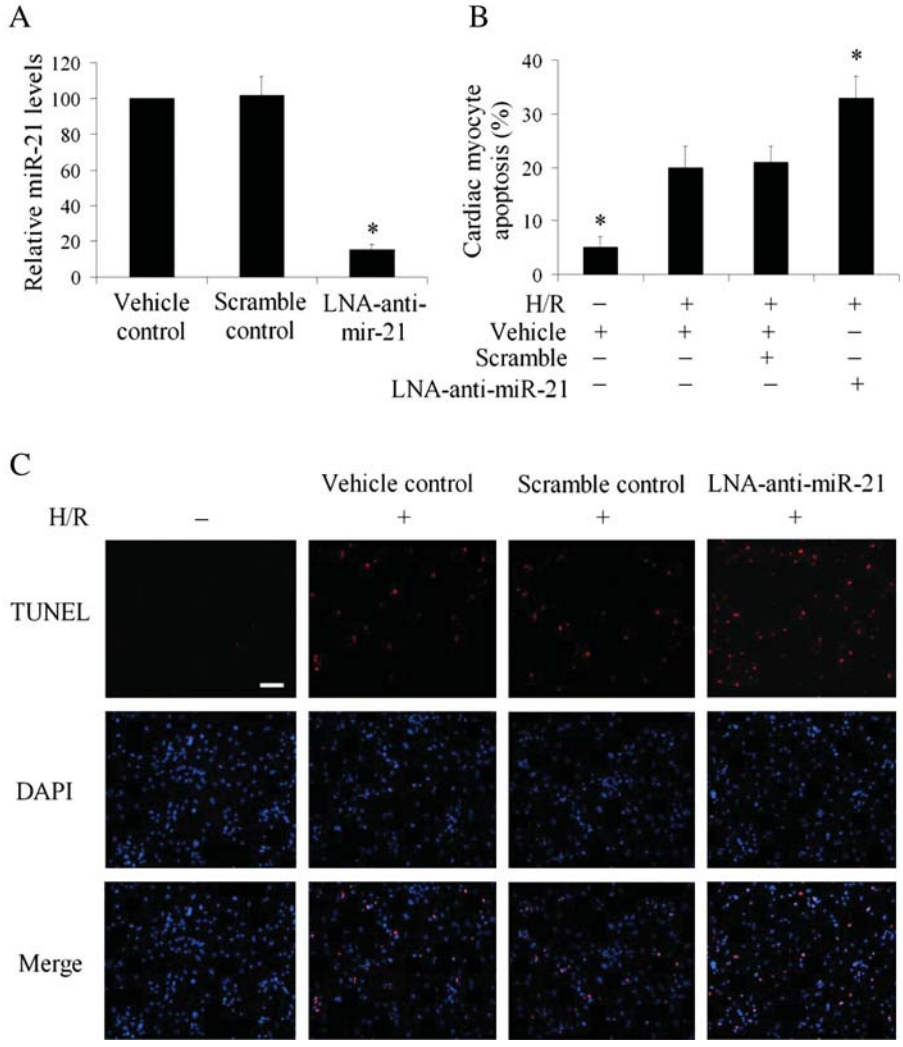

Figure 4 The effect of miR-21 inhibition on H/R induced cardiac cell apoptosis *in vitro*. Cultured cardiac cell injury was induced by hypoxia for 4 h in a serum- and glucose-free medium followed by reoxygenation for 3 h in normal culture medium. Cell apoptosis was determined by TUNEL staining. (*A*) miR-21 inhibitor, LNA-anti-miR-21 (30 nM) decreased miR-21 expression in cultured cardiac cells. Note: $n = 6$, $^*P < 0.05$ compared with the vehicle control. (*B*) H/R resulted in an increase in apoptosis. Cardiac myocyte apoptosis was exacerbated after treatment with LNA-anti-miR-21. Note: $n = 6$, $^*P < 0.05$ compared with H/R treated with the vehicle. (*C*) Representative TUNEL-stained photomicrographs from cells without I/R, and cardiac myocytes with I/R treated with vehicle, control oligo (scramble), or LNA-anti-miR-21. Note: red colour is TUNEL staining representing apoptotic cell; blue colour is the cell nucleus stained by 4′,6-diamidino-2-phenylindole. Scale bar in (*C*) is 100 μm.

the control oligo (scramble) had no effect on IP-induced cardiac cell apoptosis, IP-induced cardiac protection on cardiac cell apoptosis was significantly attenuated in antagomiR-21-treated animals (*Figure 5A*). Representative TUNEL-stained photomicrographs in heart sections from different treatment rats were displayed in *Figure 5B*. The results indicate that the miR-21-mediated protective effect against myocardial damage is related to its anti-apoptotic effect on cardiac cells *in vivo*.

3.6 PDCD4 is an miR-21 target gene that is involved in miR-21-mediated anti-apoptotic effects on cardiac cells

Computational analysis indicates that PDCD4 is a potential target gene of miR-21 as described in our recent study.[15] The role of PDCD4 in cardiac cell apoptosis is currently unclear. To test it, we overexpressed PDCD4 by Ad-PDCD4 in cultured cardiac myocytes. As shown in *Figure 6A*, PDCD4 expression was increased by Ad-PDCD4 in these cardiac cells. Indeed, consistent with the PDCD4 expression, Ad-PDCD4 increased cardiac myocyte apoptosis induced by H/R (*Figure 6B*). In addition, PDCD4 expression in cardiac cells was upregulated by LNA-anti-miR-21 (30 nM), but downregulated by pre-miR-21 (30 nM) (*Figure 6C* and *D*). The results suggest that PDCD4 is an miR-21 target gene that is involved in miR-21-mediated anti-apoptotic effects on cardiac cells. To further confirm that miR-21 is able to directly bind to PDCD4 and inhibit PDCD4 expression, a construct in which a fragment of the 3′-UTR of PDCD4 mRNA with the putative miR-21 binding sequence was cloned into a firefly luciferase reporter construct and transfected into HEK 293 cells with either vehicle (vehicle control), an empty plasmid (pDNR-CMV), a pmiR-21, or a control plasmid expressing an unrelated miRNA, miR-145 (pmiR-145), following the transfection

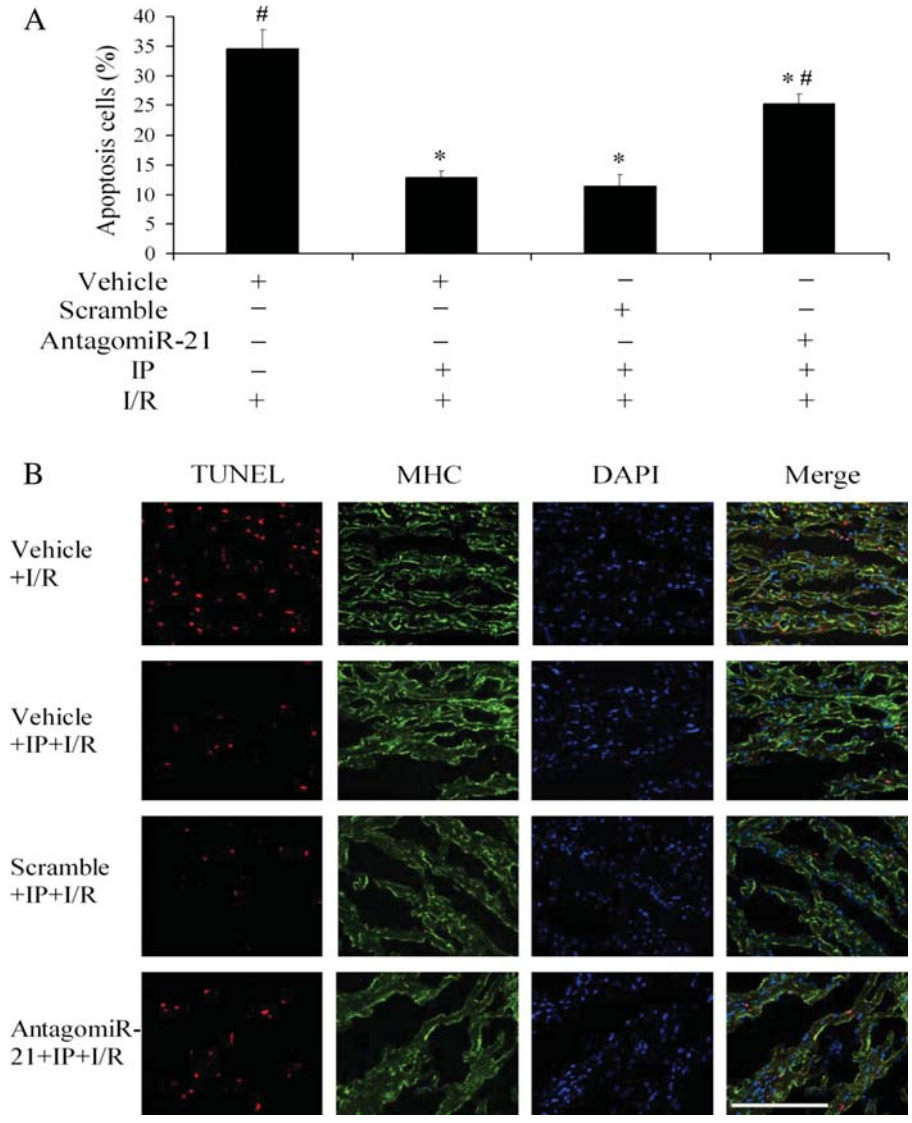

Figure 5 The effect of miR-21 inhibition on the cardiac cell apoptosis in rat hearts after I/R. (*A*) Quantitative analysis of the apoptotic cells in heart sections. Note: $n = 6$, $^{*}P < 0.05$ compared with I/R treated with vehicle (Vehicle + I/R); $^{\#}P < 0.05$ compared with I/R treated with IP (vehicle + IP + I/R). (*B*) Representative TUNEL- and myosin heavy chain (MHC)-stained photomicrographs OF cardiac myocytes in heart sections from rats treated with vehicle + I/R, vehicle + IP + I/R, scramble + IP + I/P, and antagomiR-21 + IP + I/P. Note: red colour is TUNEL staining representing apoptotic cells; green colour is the MHC staining representing cardiac myocytes; blue colour is the cell nucleus stained by 4′,6-diamidino-Z-phenylindole. Scale bar in (*C*) is 500 μm.

procedure provided by Invitrogen. As expected, pmiR-21, but not pDNR-CMV or pmiR-145 inhibited luciferase activity (*Figure 6E*). In the mutated control group, the inhibitory effect of pmiR-21 disappeared (*Figure 6E*). The results imply that miR-21 can bind to PDCD4 directly and inhibit its expression.

4. Discussion

In the current study, the expression profile of miRNAs in cardiac IP was identified. The results revealed that multiple miRNAs were regulated by IP (*Table 1*). The multiple IP-regulated miRNAs found in this study match the complexity of IP, in which many genes are modulated.[2] It is well established that an individual miRNA is functionally important as a transcription factor because of the ability to regulate the expression of its multiple target genes. Thus, miRNAs may play important roles in IP-mediated cardiac protection against ischaemic injury via regulating protection- or injury-related genes.

Recent studies revealed that miRNAs participated in ischaemic injury of the hearts, which provided indirect evidence that miRNAs may have potential roles in IP-mediated cardiac protection. First, multiple miRNAs are deregulated in human hearts with coronary artery disease.[16] The potential involvement of miRNAs in acute myocardial infarction (AMI) is suggested in a study using miR-126 null mice, in which Wang *et al.*[17] have found that the survival rate in miR-126 deficient mice following AMI is significantly reduced compared with that in wild-type mice. The expression signature in the late phase of AMI has just been identified by an excellent study reported by van Rooij *et al.*[18] These investigators found that miR-29 plays an important

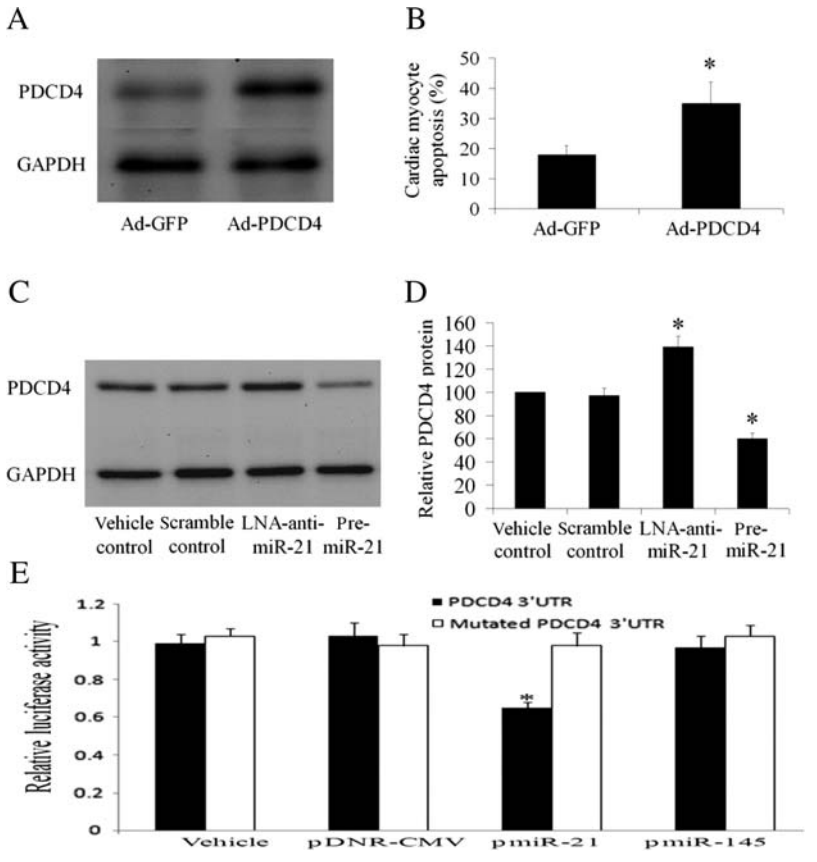

Figure 6 PDCD4 is an miR-21 target gene that is related to miR-21-mediated anti-apoptotic effect on cardiac cells. (*A*) Overexpression of PDCD4 by Ad-PDCD4 (30 MOI) in cultured cardiac myocytes. (*B*) Ad-PDCD4 (30 MOI) increased cardiac myocyte apoptosis induced by H/R as determined by TUNEL staining. Note: $n = 5$, $^*P < 0.05$ compared with the Ad-GFP control. (*C*) Modulation of PDCD4 expression in cardiac myocytes by LNA-anti-miR-21 (30 nM) and pre-miR-21 (30 nM). (*D*) Quantitative analysis of PDCD4 protein. Note: $n = 6$, $^*P < 0.05$ compared with the vehicle control. (*E*) miR-21 is able to bind to PDCD4 and inhibit PDCD4 expression in HEK 293 cells. A construct (1 μg/well) in which a fragment of the 3′-UTR of PDCD4 mRNA with the putative miR-21 binding sequence was cloned into a firefly luciferase reporter construct and transfected into HEK 293 cells with either vehicle (vehicle), an empty plasmid (pDNR-CMV, 30 nM), a plasmid expressing miR-21 (pmiR-21, 30 nM), or a control plasmid expressing an unrelated miRNA, miR-145 (pmiR-145, 30 nM). The construct (1 μg/well) with mutated fragment of the 3′-UTR of PDCD4 mRNA without the putative miR-21 binding sequence was used as the mutated control. pmiR-21, but not pDNR-CMV or pmiR-145, inhibited luciferase activity. In the mutated control group, the inhibitory effect of pmiR-21 disappeared. Note; $n = 5$; $^*P < 0.05$ compared with vehicle control.

role in cardiac fibrosis during the repair process after AMI. In an *in vitro* I/R injury model, Yin *et al.*[19] demonstrated that, in mouse hearts pre-injected with heat-shock (HS)-induced miRNAs including miR-21, myocardial infarct size after I/R injury *in vitro* is reduced. More recently, using miR-320 transgenic mice, Fan and colleagues found that miR-320 was involved in the regulation of cardiac I/R by targeting Hsp20.[20] In our recent study, miRNA expression signature in the early phase of AMI was identified. Moreover, we found that overexpression of miR-21 inhibited infarct size in rat hearts with AMI.[10]

The direct evidence showing that miRNAs are involved in cardiac IP was from recent two reports,[8,9] in which the expression of miR-1, miR-21, miR-24, and miR-199a was regulated by IP. Moreover, injection of IP-miRNAs protected the hearts against I/R in a Langendorff mouse model.[8] In the current study, we demonstrated in an *in vivo* cardiac IP model that miR-21 was quickly upregulated by IP. Although miR-1, miR-24, and miR-199a were also mildly regulated by IP, the difference did not reach the 30% limitation compared with the controls. Notably, IP-mediated cardiac protection in rat heart *in vivo* was inhibited by knockdown of cardiac miR-21 expression. The protective effect of miR-21 on ischaemic injury was further verified in an *in vitro* cardiac cell H/R model. It should be noted that IP-mediated cardiac protection in rat hearts was only partially blocked by miR-21, suggesting that other mechanisms should be identified in future studies.

miR-21 is found to be an anti-apoptotic miRNA. However, its anti-apoptotic is cell specific. For example, miR-21 has a strong anti-apoptotic effect on many cancer cells; however, it has no effect on HeLa cell apoptosis.[21] In our recent studies, we have found that vascular smooth muscle cell apoptosis is controlled by miR-21.[11] In addition, miR-21 also has an apoptotic effect on cardiac cells induced by hydrogen peroxide (H_2O_2).[14] In the current study, we identified that miR-21 was involved in IP-induced cardiac protection both in cultured cells *in vitro* and in rat heart with I/R *in vivo*.

PDCD4 is a critical mediator for cancer cell apoptosis. Our recent studies have revealed that PDCD4 is an important target gene of miR-21 that is related to miR-21-mediated anti-apoptotic effect on

vascular smooth muscle cells.[15] In the current study, the roles of PDCD4 as a target gene of miR-21 and a mediator in miR-21-mediated cardiac cell protection were further verified in cultured cardiac cells and in cardiac cell I/R model. It is still unclear that how quickly the PDCD4 could be regulated by miR-21 at the protein level. However, in a PDGF-stimulated rat cell model and in balloon-injured rat carotid arteries, we have found that both miR-21 and PDCD4 are able to be modulated quickly (data not shown). Thus, PDCD4 could be an active molecule under disease conditions.

In summary, miRNA signature in rat hearts with IP has revealed that multiple miRNAs are regulated by IP. Among them, miR-21 has a protective effect on I/R by reducing cardiac cell apoptosis via its target gene PDCD4. Identifying the roles of IP-regulated miRNAs in cardiac protection may provide novel therapeutic and preventive targets for ischaemic heart diseases.

Conflict of interest: none declared.

Funding

The author's research was supported by National Institutes of Health Grants (HL080133, HL095707) and by a grant from the American Heart Association (09GRNT2250567).

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