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MicroRNA-21 protects against the H₂O₂-induced injury on cardiac myocytes via its target gene PDCD4

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Abstract

Reactive oxygen species (ROS)-induced cardiac cell injury via expression changes of multiple genes plays a critical role in the pathogenesis of numerous heart diseases. MicroRNAs (miRNAs) comprise a novel class of endogenous, small, noncoding RNAs that negatively regulate about 30% of the genes in a cell via degradation or translational inhibition of their target mRNAs. Currently, the effects of ROS on miRNA expression and the roles of miRNAs in ROS-mediated injury on cardiac myocytes are uncertain. Using quantitative real-time RT-PCR (qRT-PCR), we demonstrated that microRNA-21 (miR-21) was upregulated in cardiac myocytes after treatment with hydrogen peroxide (H₂O₂). To determine the potential roles of miRNAs in H₂O₂-mediated gene regulation and cellular injury, miR-21 expression was downregulated by miR-21 inhibitor and upregulated by pre-miR-21. H₂O₂-induced cardiac cell death and apoptosis were increased by miR-21 inhibitor and was decreased by pre-miR-21. Programmed cell death 4 (PDCD4) that was regulated by miR-21 and was a direct target of miR-21 in cardiac myocytes. Pre-miR-21-mediated protective effect on cardiac myocyte injury was inhibited in H₂O₂-treated cardiac cells via adenovirus-mediated overexpression of PDCD4 without miR-21 binding site. Moreover, Activator protein 1 (AP-1) was a downstream signaling molecule of PDCD4 that was involved in miR-21-mediated effect on cardiac myocytes. The results suggest that miR-21 is sensitive to H₂O₂ stimulation. miR-21 participates in H₂O₂-mediated gene regulation and functional modulation in cardiac myocytes. miR-21 might play an essential role in heart diseases related to ROS such as cardiac hypertrophy, heart failure, myocardial infarction, and myocardial ischemia/reperfusion injury.

Keywords

MicroRNAs; Cardiac myocytes; Apoptosis; Cell death; Reactive oxygen species

1. Introduction

It is well known that high levels of reactive oxygen species (ROS)-induced cardiac cell injury plays an important role in the pathogenesis of many heart diseases including cardiac hypertrophy, heart failure, myocardial infarction, and myocardial ischemia/reperfusion injury [1–3]. Recent studies have revealed that ROS such as superoxide and hydrogen peroxide (H₂O₂)-elicited expression changes of multiple genes are responsible for ROS-mediated cardiac cell injury responses such as cell death and apoptosis [3–6].

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ROS-mediated gene expression regulation has recently been extensively studied at epigenetic and transcriptional levels [7–9]. It is clear that exposure of cardiac myocytes to ROS modulates oxidation-sensitive signaling pathways and transcription factors, which could be an important mechanism responsible for ROS-mediated expression changes of multiple genes [3–6]. More recently, post-transcriptional controls of gene expression such as translational regulation have been proven to be as important as epigenetic and transcriptional controls [10,11]. However, the effects of ROS on gene expression regulation at the translational level in heart cells are currently uncertain.

MicroRNAs (miRNAs) comprise a novel class of endogenous, small, noncoding RNAs that negatively regulate gene expression via degradation or translational inhibition of their target mRNAs [12–15]. Functionally, an individual miRNA is as important as a transcription factor because it is able to regulate the expression of its multiple target genes. Analogous to the first RNA revolution in the 1980s when Cech discovered the enzymatic activity of RNA [16], this recent discovery of miRNA and RNA interference (RNAi) may represent the second RNA revolution [17]. Currently, about 700 miRNAs have been identified and sequenced in humans [17–19], and the estimated number of miRNA genes is as high as 1000 in the human genome [18–20]. As a group, miRNAs may directly regulate at least 30% of the genes in a cell [19–21]. It is not surprising that miRNAs may be involved in the regulation of almost all major cellular functions, such as cell differentiation, growth, mobility and death (apoptosis and necrosis). Therefore, miRNAs could be the pivotal regulators in development, physiology, and disease [19].

Although miRNAs characterize a new layer of gene expression regulators at the translational level, the effects of ROS on miRNA expression and the roles of miRNAs in ROS-mediated gene regulation and biological functions of cardiac myocytes are unclear. We have recently identified that microRNA-21 (miR-21) is upregulated in hypertrophic cardiac cells and is involved in the pathogenesis cardiac hypertrophy [22]. The objective of the current study is to determine the effect of a ROS, H₂O₂, on miR-21 expression in cultured cardiac myocytes and to determine whether miR-21 plays a role in ROS-mediated gene expression regulation and cellular injury responses in heart cells.

2. Materials and methods

2.1. Cell culture

Primary cultures of neonatal rat cardiac ventricular myocytes were performed as described [22]. In brief, the hearts from 1- to 2-day-old Sprague–Dawley rats were removed after hypothermia anesthesia immersion in ice water and 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; Sigma, St. Louis, MO), 0.1% trypsin (Life Technologies, Inc., Grand Island, NY), and 15 µg/ml DNase I. Cardiomyocytes (0.33×10⁶ cells/ml) were cultured in the cardiac myocyte culture medium containing Dulbecco's modified Eagle's medium supplemented with 10% serum, 4 µg/ml transferrin, 0.7 ng/ml sodium selenite, 2 g/L bovine serum albumin (fraction V), 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 and 1% streptomycin, and 100 µmol/L 5-bromo-2'-deoxyuridine, and seeded into six-well plates.

2.2 Treatments with H₂O₂ for miR-21 expression

Cultured rat cardiac myocytes were treated with either vehicle or H₂O₂ (10–100 µM) for 6 h. miRNAs were then isolated from the cultured cells using the mirVana miRNA isolation

kit (Ambion, Inc., Austin, TX). miR-21 expression was determined by quantitative real-time RT-PCR (qRT-PCR) via a kit provided by RNA Bioscience.

2.3 Measurements of cardiac myocyte death and apoptosis induced by H₂O₂

Briefly, rat cardiac myocytes cultured in 0.1% FBS were treated with either vehicle or H₂O₂ (10–200 μ M) for 24 h. Afterwards, cell death (apoptosis and necrosis) was measured by double labeling with fluorescent dyes (SYTOX green staining, Invitrogen) and cell apoptosis was measured by terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) staining as described previously [23,24]. SYTOX green is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes but cannot cross the membranes of live cells. For SYTOX green nucleic acid staining, the cultured rat cardiac myocytes were incubated with 500 nM SYTOX green for 10 min after treatment with different concentrations of H₂O₂. Dead and live cells were visualized with an invert fluorescence microscope (Nikon), equipped with a CCD digital camera and processed with image analysis software (NIS-Elements BR 3.0). For TUNEL analysis, cardiac myocytes 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.

2.4. Oligo transfection, miR-21 knockdown, miR-21 overexpression, and programmed cell death 4 (PDCD4) gene upregulation in cultured cardiac myocytes

Oligo transfection was performed according to an established protocol [22,24]. Briefly, cells were transfected using a transfection reagent (Qiagen, Chatsworth, CA) 24 h after seeding into the well. Transfection complexes were prepared according to the manufacturer's instructions. For the miR-21 knockdown, miR-21 inhibitor (LNA-anti-miR-21) was added to the culture media at final oligonucleotide concentration of 30 nM. The locked nucleic acid (LNA)-anti-miR molecules were synthesized as unconjugated and fully phosphorothioated mixed LNA/DNA oligonucleotides with a 6-carboxyfluorescein (FAM) moiety at the 5' end. The following sequences were synthesized by Exiqon: LNA-anti-miR-21, 5'-FAM-tcagtcgtgataagcta-3', and its control oligo, LNA-scramble, 5'-FAM-cgtcagtcgtatgcgaatc-3'. For the miR-21 upregulation, pre-miR-21 (Ambion, Inc.) was added directly to the complexes at final oligonucleotide concentration of 100 nM. PDCD4 gene upregulation was performed by adenoviruses expressing PDCD4 with (Ad-PDCD4) (30 MOI) or without (Ad-PDCD4-lack) (30 MOI) the miR-21 binding site at 3'-UTR. The transfection medium was replaced 4 h post-transfection by the regular culture medium. Vehicle control, oligo control for LNA-anti-miR-21 (LNA-scramble), oligo control for pre-miR-21 (pre-scramble, Ambion, Inc.), and adenovirus control (Ad-GFP) were applied.

2.5. RNA levels were determined by qRT-PCR

Briefly, RNAs from cardiac myocytes were isolated with a RNA Isolation Kit (Ambion, Inc.). qRT-PCR for miR-21 was performed on cDNA generated from 50 ng of total RNA according to the manufacturer's protocol. qRT-PCR for PDCD4 was performed on cDNA generated from 200 ng of total RNA using the protocol of a qRT-PCR mRNA detection kit (Roche). Amplification and detection of specific products were performed with a Roche Lightcycler 480 Detection System. As an internal control, U6 was used for miR-21 template normalization and GAPDH was used for PDCD4 template normalization. Fluorescent signals were normalized to an internal reference, and the threshold cycle (Ct) 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 Ct values were normalized by subtracting the U6 or GAPDH Ct value, which provided the Δ Ct value. The relative

expression level between treatments was then calculated using the following equation:

$$\text{relative gene expression} = 2^{-(\Delta C_{\text{t sample}} - \Delta C_{\text{t control}})}$$
 [22,24].

2.6. 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, CA). GAPDH antibody (1:5000 dilution; Cell Signaling, MA) was used as a loading control.

2.7. Construction of the adenovirus expressing PDCD4 and control virus expressing GFP

The adenovirus expressing PDCD4 (Ad-DCD4), PDCD4 without miR-12 binding site (Ad-PDCD4-lack), or control adenovirus expressing GFP (Ad-GFP) was generated using the Adeno-X™ Expression Systems 2 kit (Clontech, CA) according to the manufacturer's protocols. Briefly, a 1410-bp fragment of the full length coding sequence was amplified with primers tgaattcatggatgtagaaaacgagcagata and taagcttcagtagctctcagggttaagacga by using RT-PCR and was inserted into pDNR-CMV donor vector (Clontech, CA) at EcoR I and Hind III sites. This vector was named pDNR-CMV-PDCD4. The construct was sequenced to confirm the DNA sequence. The PDCD4 fragment was then excised from the pDNR-CMV-PDCD4 and was inserted into the pLP-Adeno-X-CMV vector using cre recombinase, which was then termed pLP-Adeno-X-CMV-PDCD4. The pLP-Adeno-X-CMV-PDCD4 plasmid digested by Pac I was used to transfect low-passage HEK 293 cells to produce recombinant adenovirus with Lipofectamine 2000 according to the manufacturer's protocols (Invitrogen, CA). Adenovirus expressing GFP was generated as described [25]. The GFP DNA fragment was excised from pGFP-N3 (Clontech) by digestion of the plasmid with SalI and NotI and subcloned into an entry vector, pENTR3C (Invitrogen, CA), producing pENTR3C-GFP. pENTR3C-GFP was transformed into *E. coli* DH5, and the plasmids were amplified. These plasmids were recombined with pAd/CMV/V5-DEST as described by the manufacturer (Invitrogen, CA), producing pAd-GFP plasmids, which were verified by DNA sequencing. The pAd-EGFP was linearized with PacI and transfected into HEK293A cells. The resulting adenoviruses (Ad-PDCD4, Ad-PDCD4-Lack, and Ad-GFP) were further amplified by infection of HEK293A cells and purified by cesium chloride gradient ultracentrifugation. The Ad-PDCD4, Ad-PDCD4-Lack, and Ad-GFP were titrated using a standard plaque assay.

2.8. 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.

Activator protein 1 (AP-1) activity was measured using luciferase assay as described [26]. Briefly, adenoviral vector (Ad-API-Luc) containing *Photinus pyralis* (firefly) gene that is controlled by a synthetic promoter with direct repeats of the transcription recognition sequences for the AP-1 was purchased from Vector Biolabs. Cultured cardiac myocytes pretreated with vehicle, control oligos (LNA-scramble and pre-scramble), LNA-anti-miR-21 (30 nM), pre-miR-21 (30 nM), Ad-GFP (30 MOI), Ad-PDCD4 (30 MOI), Ad-PDCD4-Lack (30 MOI), or pre-miR-21 plus Ad-PDCD4-Lack for 4 h were transfected with Ad-API-Luc for 5 h with 10 PFU/cell. Luciferase activity was measured after 24 h.

Luciferase expression was measured on a scintillation counter by using a dual luciferase reporter system. Luciferase activity was normalized by *Renilla* luciferase signal in HEK 293 cells and was normalized by protein level for AP-1 activity in cardiac myocytes.

2.9. Statistics

All data are presented as mean $\pm$ standard error. For relative gene expression, the mean value of the vehicle control group is defined as 1 or 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* value <0.05 was considered significant.

3. Results

3.1. The effect of H₂O₂ on miR-21 expression in cultured cardiac myocytes

As shown in Fig. 1, short-time exposure (6 h) of cardiac myocytes to H₂O₂ resulted in the increased expression of miR-21. H₂O₂-mediated increase in miR-21 expression is in a dose-dependent manner with a peak at approximately 50 μ M.

3.2. The effect of H₂O₂ on cardiac myocyte injury

Although low concentrations of H₂O₂ had no effect on cell death, high concentrations (30–200 μ M) of H₂O₂ increased cardiac myocyte death in a dose dependent manner after 24 h treatment under our experimental condition as shown in Fig. 2(A). Representative SYTOX green-stained cell photomicrographs, their corresponding total cell photomicrographs (bright field, BF), and their merged photomicrographs (merge) from cells treated with vehicle (0 μ M), 50 μ M or 200 μ M of H₂O₂ were displayed in Fig. 2(B). We had confirmed that cell death was a mixture of cell apoptosis and necrosis with the majority of dead cells being apoptotic cells, especially at the doses lower than 50 μ M of H₂O₂.

3.3. Modulating miR-21 expression in cultured cardiac myocytes

To modulate miR-21 in cultured cardiac myocytes, both gain-of-function and loss-of-function approaches were applied. As shown in Fig. 3, LNA-anti-miR-21 decreased, but pre-miR-21 increased miR-21 expression in cardiac myocytes. In contrast, no effects of their control oligos (scrambled oligos) on miR-21 expression were found. In addition, the effects of both LNA-anti-miR-21 and pre-miR-21 on miR-21 expression were miR-21 specific, as no effects were found on other miRNAs such as miR-24 and miR-146 (data not shown).

3.4. The effect of miR-21 on H₂O₂-induced cardiac myocytes death and apoptosis

Pre-miR-21 decreased H₂O₂-induced cardiac myocyte death and apoptosis as determined by SYTOX green staining (Fig. 4(A)) and TUNEL staining (Fig. 5(A)). In contrast, cardiac myocyte death and apoptosis were increased after treatment with LNA-anti-miR-21 (Figs. 4(A) and 5(A)). Representative SYTOX green-stained and TUNEL-stained photomicrographs from cardiac myocytes treated with vehicle, control oligo, pre-miR-21 and LNA-anti-miR-21 were shown in Figs. 4(B) and 5(B). The results indicated that miR-21 had a protective effect against the H₂O₂-induced cardiac myocyte death and apoptosis.

3.5. PDCD4 is a miR-21 target gene in cardiac myocytes

Computational analysis indicates that PDCD4 is a potential target gene of miR-21 (Fig. 6(A)). If it is a miR-21 target, H₂O₂ should decrease its expression in cardiac myocytes because miR-21 expression was upregulated after H₂O₂ stimulation (Fig. 1). To confirm this, we incubated cardiac myocytes with either vehicle or H₂O₂ (50 μ M) for 24 h and protein level of PDCD4 was determined by western blot. As shown in Fig. 6(B), H₂O₂

decreased PDCD4 expression. The results suggested that PDCD4 might be a potential miR-21 target gene in cardiac myocytes stimulated with H₂O₂.

To verify PDCD4 as a target gene of miR-21 in cardiac myocytes, both gain-of-function and loss-of-function approaches were applied. As shown in Fig. 6(C) and (D), LNA-anti-miR-21 increased, whereas pre-miR-21 decreased PDCD4 expression in cultured cardiac myocytes. The results suggested that PDCD4 was a target gene of miR-21. To further confirm that miR-21 was able to directly bind to PDCD4 and inhibit PDCD4 expression, 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), a plasmid expressing miR-21 (pmiR-21), or a control plasmid expressing an unrelated miRNA, miR-145 (pmiR-145), following the transfection procedure provided by Invitrogen. As expected, we found that pmiR-21, but not pmiR-145 and pDNR-CMV, increased miR-21 expression in HEK 293 cells (Fig. 6(E)). Accordingly, pmiR-21, but not pDNR-CMV or pmiR-145 inhibited luciferase activity (Fig. 6(F)). The results implied that miR-21 was able to bind to PDCD4 directly and inhibited its expression.

3.6. PDCD4 is a functional gene target that is involved in miR-21-mediated protective effect on H₂O₂-induced injury on cardiac myocytes

To verify the functional involvement of PDCD4 in miR-21-mediated cellular effect, we determined the role of PDCD4 in H₂O₂-induced cardiac myocyte apoptosis. As shown in Fig. 7(A), pre-miR-21 had a protective effect on H₂O₂-induced cardiac myocyte apoptosis. Overexpression of PDCD4 via Ad-PDCD4 increased H₂O₂-mediated cell apoptosis. Furthermore, the pre-miR-21-mediated protective effect on cardiac myocytes apoptosis was inhibited via adenovirus-mediated overexpression of PDCD4 without miR-21 binding site (Ad-PDCD4-lack). Representative TUNEL-stained photomicrographs from cardiac myocytes treated with vehicle, Ad-GFP, pre-miR-21, Ad-PDCD4, and pre-miR-21 plus Ad-PDCD4-lack were displayed in Fig. 7(B). Representative western blots PDCD4 for each group were showed in Fig. 7(C).

3.7. AP-1 is a downstream signaling molecule of PDCD4 that is involved in miR-21-mediated effect on cardiac myocytes

As shown in Fig. 8(A), overexpression of PDCD4 by Ad-PDCD4 or Ad-PDCD4-lack inhibited AP-1 activity. In addition, increasing of PDCD4 expression via LNA-anti-miR-21 (Fig. 6(C)) resulted in a decrease in AP-1 activity (Fig. 8(B)). In contrast, decreasing of PDCD4 expression via pre-miR-21 (Fig. 6(D)) resulted in an increase in AP-1 activity (Fig. 8(B)); however, the pre-miR-21-induced increase in luciferase activity was totally blocked after transfection of adenovirus expression PDCD4 without miR-21 binding site (Ad-PDCD4-Lack) (Fig. 8(B)). The results suggested that AP-1 was a downstream signaling molecule of PDCD4 that was involved in miR-21-mediated effect on cardiac myocytes.

4. Discussion

In the current study, we have identified that miR-21 expression is sensitive to H₂O₂ stimulation in cardiac myocytes. Six hours after treatment with H₂O₂, miR-21 is upregulated in a dose-dependent manner. The expression changes of miRNAs after ROS stimulation could be very important in ROS-mediated modulations of multiple gene expression and signaling transduction pathways, because at least 30% of genes in a cell are directly regulated by this new layer of gene expression regulators. In this respect, the expression changes other miRNAs induced by ROS stimulation in cardiac cells should be investigated in future studies.

Our recent studies have found that miR-21 plays critical roles in cardiac cell hypertrophy and in vascular smooth muscle cell apoptosis-induced by serum deprivation [22,24]. To test the potential role of miR-21 in H₂O₂-mediated cardiac myocyte injury, miR-21 expression is modulated by miR-21 inhibitor and pre-miR-21. Interestingly, upregulation of miR-21 expression inhibits H₂O₂-mediated neonatal rat cardiac myocyte apoptosis and death. In contrast, H₂O₂-mediated cardiac myocyte apoptosis and death is exacerbated after downregulation of miR-21 expression. The results suggest that miR-21 has an anti-apoptotic effect in H₂O₂-mediated neonatal rat cardiac myocyte apoptosis and death.

miR-21 expression in the heart cells is development and age-dependent. For example, the adult cardiac myocytes have relative lower expression level of miR-21 than that in neonatal cardiac myocytes [27]. In addition, non-myocytes in the heart such as fibroblasts also have high miR-21 expression level. The biological significance of miR-21 in adult cardiac myocytes has not been understood completely. Recently, Thum T et al. have just reported their excellent work on the biological role of miR-21 in adult hearts [28]. These investigators have revealed that the upregulation of miR-21 in cardiac hypertrophy is mediated by an increase in miR-21 expression in cardiac fibroblasts but not cardiomyocytes. In addition, this group has also generated a cardiomyocyte-specific miR-21 transgene but has not found any cardiac phenotype under basal or cardiac hypertrophy-inducing conditions. However, they have found that miR-21 has an anti-apoptotic effect on adult cardiac fibroblasts. It should be noted that, in the study, they have not determined the role of miR-21 in adult cardiac myocytes under an apoptosis-inducing condition. The following observations indicate that a biological significance of miR-21 might be applied in adult cardiac myocytes. First, miR-21 expression in the normal mouse heart ventricles, in which the majority of cells are cardiac myocytes, is ranked at top 50 among the 157 detected mature miRNAs [22]. Thus, miR-21 is still an abundant miRNA in adult heart. Second, miR-21 was significantly increased in adult heart with hypertrophy [22]. Third, our unpublished data demonstrated that, in adult rat hearts with acute myocardial infarction, the expression of miR-21 is downregulated in infarcted area and is upregulated in border area at 6 h after coronary ligation. Overexpression of miR-21 via adenovirus decreases infarcted sizes by reducing cardiac myocyte apoptosis (death) in these adult animals in vivo. Fourth, another recent report has demonstrated that miR-21 is a regulator for cardiac myocyte growth in cultured adult cardiac myocytes [29]. Even in the study performed by Thum T et al., they have also found that downregulation of miR-21 in adult heart by an antagomir approach is able to reduce interstitial fibrosis, cardiomyocyte size and the heart weight under the hypertrophy-inducing condition [28]. Nevertheless, the physiological and pharmacological significance of miR-21 in H₂O₂-mediated injury responses in cultured adult cardiac myocytes should be verified in future study.

miRNAs modulate their biological functions via their multiple target gene mRNAs. Although their potential gene targets can be predicted by computational analysis, these targets must be experimentally verified in investigatory cells because miRNA-mediated effects on gene expression and cellular functions are cell specific. In the current study, computational analysis suggests that PDCD4 may be a miR-21 target. Moreover, PDCD4 regulation by miR-21 has been recently reported in cancer cells [30–32]. To test whether PDCD4 is a miR-21 target gene in heart cells, we have first confirmed that H₂O₂ decreases PDCD4 expression in cultured cardiac myocytes. In addition, PDCD4 expression in cardiac myocytes is able to be regulated by miR-21 as determined by both gain-of-function and loss-of-function approaches. Furthermore, miR-21 is able to bind to PDCD4 and regulate its expression directly using a construct in which a fragment of the 3'-UTR of PDCD4 mRNA with the putative miR-21 binding sequence. Finally, we have found pre-miR-21-mediated protective effect on H₂O₂-mediated cardiac myocyte apoptosis is blocked after overexpression of PDCD4 without the miR-21 binding site. This result indicates that

PDCD4 is indeed a functional target gene of miR-21 that is involved in miR-21-mediated protective effect on cardiac myocyte injury elicited by H₂O₂.

It is well established that AP-1 is a key signaling molecule that determines life or death cell fates in response to extracellular stimuli including ROS [33,34]. Several recent studies suggest that AP-1 is a downstream signaling molecule of PDCD4 in other types of cells [35,36]. We thus hypothesize that AP-1 might be a downstream signaling molecule of PDCD4 that is involved in miR-21-mediated effect on cardiomyocyte cell death. The hypothesis is supported by the following findings: First, we found that increasing PDCD4 by Ad-PDCD4 inhibits AP-1 activity. Second, LNA-anti-miR-21 increases PDCD4 expression and results in a decrease in AP-1 activity. In contrast, pre-miR-21 decreases PDCD4 expression and results in an increase in AP-1 activity. Third, pre-miR-21-mediated increase in AP-1 activity is able to be blocked by Adenovirus-expressing PDCD4 without the miR-21 binding site.

In summary, the current study reveals that the miR-21 in cardiac myocytes is sensitive to H₂O₂ stimulation. MiR-21 protects against the H₂O₂-induced injury on cardiac myocytes via its target gene PDCD4 and AP-1 pathway. These novel findings may have extensive implications for the diagnosis and therapy of a variety of heart diseases related to ROS such as cardiac hypertrophy, heart failure, myocardial infarction, and myocardial ischemia/reperfusion injury.

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Fig. 1.

The effect of H₂O₂ on miR-21 expression in cultured rat cardiac myocytes. Cultured neonatal rat cardiac myocytes were treated with vehicle or H₂O₂ (10–100 μM) for 6 h. miR-21 levels were determined by qRT-PCR. Note: $n=5$; * $P<0.05$ compared with vehicle control (0 μM).

Fig. 2.

The effect of H₂O₂ on rat cardiac myocyte death. Cultured neonatal rat cardiac myocytes were treated with vehicle or H₂O₂ (10–200 μM) for 24 h. Cell death was measured by SYTOX green staining. (A) The effect of H₂O₂ on rat cardiac myocyte death. Note: $n=5$; $*P<0.05$ compared with vehicle control (0 μM). (B) Representative SYTOX green-stained cell photomicrographs, their corresponding total cell photomicrographs (bright field, BF), and their merged photomicrographs (merge) from cells treated with vehicle (0 μM), 50 μM or 200 μM of H₂O₂.

Fig. 3.

Modulating miR-21 expression in cultured cardiac myocytes. Cultured cardiac myocytes were treated with vehicle, miR-21 inhibitor scramble (LNA-scramble, 30 nM), miR-21 inhibitor (LNA-anti-miR-21, 30 nM), pre-miR-21scramble (30 nM), or pre-miR-21 (30 nM) for 4 h. miR-21 levels were determined 24 h later by qRT-PCR. Note: $n=5$; $*P<0.05$ compared with vehicle control.

Fig. 4.

The effect of miR-21 on H₂O₂-induced cardiac myocyte death. Cultured rat cardiac myocytes pre-treated with vehicle, miR-21 inhibitor scramble (LNA-scramble, 30 nM), miR-21 inhibitor (LNA-anti-miR-21, 30 nM), pre-miR-21 scramble (30 nM), or pre-miR-21 (30 nM) were treated with H₂O₂ (50 μ M) for 24 h. (A) The effect of miR-21 on H₂O₂-induced cardiac myocyte death. Note: $n=6$; * $P<0.05$ compared with vehicle control. (B) Representative SYTOX green-stained cell photomicrographs, their corresponding total cell photomicrographs (bright field, BF), and their merged photomicrographs (merge) from cells treated with vehicle, LNA-scramble, LNA-anti-miR-21, pre-miR-21scramble, or pre-miR-21.

Fig. 5.

The effect of miR-21 on H₂O₂-induced cardiac myocyte apoptosis. Cultured rat cardiac myocytes pre-treated with vehicle, miR-21 inhibitor scramble (LNA-scramble, 30 nM), miR-21 inhibitor (LNA-anti-miR-21, 30 nM), pre-miR-21 scramble (30 nM) or pre-miR-21 (30 nM) were treated with H₂O₂ (50 μ M) for 24 h. (A) The effect of miR-21 on H₂O₂-induced cardiac myocyte apoptosis. Note: $n=6$; * $P<0.05$ compared with vehicle control. (B) Representative TUNEL-stained cell photomicrographs, their corresponding total cell photomicrographs (DAPI), and their merged photomicrographs (merge) from cells treated with vehicle, LNA-scramble, LNA-anti-miR-21, pre-miR-21scramble, or pre-miR-21. Note: Blue color was cell nuclear staining by DAPI that reflected the total cells.

Fig. 6.

PDCD4 is a gene target of mi-21. (A) Conserved miR-21 binding site in 3'-untranslated region (3'-UTR) of PDCD4. (B) H₂O₂ decreased PDCD4 expression in cultured cardiac myocytes. (C) miR-21 inhibitor, LNA-anti-miR-21 (30 nM) increased PDCD4 expression in cardiac myocytes compared with vehicle or miR-21 inhibitor scramble (LNA-scramble, 30 nM). (D) pre-miR-21 (30 nM) decreased PDCD4 expression in cardiac myocytes compared with vehicle or pre-miR-21 scramble (30 nM). (E) pmiR-21, but not pmiR-145 and pDNR-CMV, increased miR-21 expression in HEK 293 cells. (F) pmiR-21, but not pDNR-CMV or pmiR-145 inhibited luciferase activity. Note: $n=6$; * $P<0.05$ compared with vehicle control.

Fig. 7. PDCD4 is a functional target gene that is involved in miR-21-mediated protective effect on H_2O_2 -induced cardiac cell apoptosis. (A) H_2O_2 (50 μM)-induced cardiac myocyte apoptosis was inhibited by pre-miR-21 (30 nM). Overexpression of PDCD4 via Ad-PDCD4 increased the H_2O_2 -cardiac myocyte apoptosis. The pre-miR-21-mediated protective effect on cardiac myocyte apoptosis was inhibited in H_2O_2 -treated cells via adenovirus-mediated overexpression of PDCD4 without miR-21 binding site (Ad-PDCD4-lack). Note: $n=5$; * $P<0.05$ compared with vehicle control. (B) Representative TUNEL-stained photomicrographs from H_2O_2 (50 μM)-treated cardiac myocytes pre-treated with vehicle,

Ad-GFP, pre-miR-21, Ad-PDCD4, and pre-miR-21 plus Ad-PDCD4-Lack. (C)
Representative western blot of PDCD4 from different treatment groups.

Fig. 8.

miR-21 regulates AP-1 activity via its target gene PDCD4. Activator protein 1 (AP-1) was measured using luciferase assay. Cultured cardiac myocytes pretreated with vehicle, control oligos (LNA-scramble and pre-scramble), LNA-anti-miR-21 (30 nM), pre-miR-21 (30 nM), ad-GFP (30 MOI), Ad-PDCD4 (30 MOI), Ad-PDCD4-Lack (30 MOI), or pre-miR-21 plus Ad-PDCD4-Lack for 4 h, were transfected with Ad-AP1-Luc for 5 h with 10 PFU/cell. Luciferase activity was measured after 24 h. (A) Luciferase activity was decreased by overexpression of PDCD4 via either Ad-PDCD4 or Ad-PDCD4-Lack. (B) LNA-anti-miR-21 decreased luciferase activity. In contrast, the luciferase activity was increased by pre-miR-21; however, the pre-miR-21-induced increase in luciferase activity was totally blocked after transfection of adenovirus expressing PDCD4 without the miR-21 binding site (Ad-PDCD4-Lack). Note: $n=5$; * $P<0.05$ compared with vehicle control. # $P<0.05$ compared with pre-miR-21 group.