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Involvement of MicroRNAs in Hydrogen Peroxide-mediated Gene Regulation and Cellular Injury Response in Vascular Smooth Muscle Cells*

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MicroRNAs (miRNAs) comprise a novel class of endogenous, small, noncoding RNAs that negatively regulate ~30% of genes in a cell via degradation or translational inhibition of their target mRNAs. However, the effects of reactive oxygen species (ROS) on miRNA expression and the roles of miRNAs in ROS-mediated gene regulation and biological functions of vascular cells are unclear. Using microarray analysis, we demonstrated that miRNAs are aberrantly expressed in vascular smooth muscle cells (VSMCs) after treatment with hydrogen peroxide (H_2O_2). H_2O_2-mediated up-regulation of microRNA-21 (miR-21) was further confirmed by quantitative real-time PCR. To determine the potential roles of miRNAs in H_2O_2-mediated gene regulation and cellular effects, miR-21 expression was down-regulated by miR-21 inhibitor and up-regulated by pre-miR-21. H_2O_2-induced VSMC apoptosis and death were increased by miR-21 inhibitor and decreased by pre-miR-21. Programmed cell death 4 (*PDCD4*) was a direct target of miR-21 that was involved in miR-21-mediated effects on VSMCs. Pre-miR-21-mediated protective effect on VSMC apoptosis and death was blocked via adenovirus-mediated overexpression of *PDCD4* without the miR-21 binding site. Moreover, activator protein 1 was a downstream signaling molecule of PDCD4 in miR-21-modulated VSMCs. The results suggest that miRNAs in VSMCs are sensitive to H_2O_2 stimulation. miRN-21 participates in H_2O_2-mediated gene regulation and cellular injury response through PDCD4 and the activator protein 1 pathway. miRNAs might play a role in vascular diseases related to ROS.

The current literature indicates an increasing body of evidence demonstrating that reactive oxygen species (ROS)[3] such as superoxide and hydrogen peroxide (H_2O_2) are involved in the pathogenesis of many vascular diseases by modulating expression of a large number of genes related to vascular cell differentiation, proliferation, migration, and apoptosis (1–5). In this respect, increased ROS are associated with a variety of vascular disorders such as atherosclerosis, hypertension, restenosis after angioplasty or bypass, diabetic vascular complications, transplantation arteriopathy, and vascular aneurysm (5–12). ROS-mediated gene expression regulation has recently been extensively studied at epigenetic and transcriptional levels (3–5, 13, 14). It is clear that exposure of vascular cells to ROS modulates oxidation-sensitive signaling pathways and transcription factors that could be an important mechanism responsible for ROS-mediated expression changes of multiple genes.

Recent studies reveal that post-transcriptional controls of gene expression such as translational regulation are as important as epigenetic and transcriptional controls (15, 16). However, the effects of ROS on gene expression regulation at the translational level are currently unclear.

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 (17–20). 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 (21) discovered the enzymatic activity of RNA, this recent discovery of miRNA and RNA interference may represent the second RNA revolution (22). Currently, ~700 miRNAs have been identified and sequenced in humans (23, 24), and the estimated number of miRNA genes is as high as 1000 in the human genome (23–25). As a group, miRNAs may directly regulate at least 30% of the genes in a cell (25, 26). It is not surprising that miRNAs are involved in the regulation of almost all major cellular functions, such as cell differentiation, proliferation/growth, mobility, and apoptosis. Therefore, miRNAs could be the pivotal regulators in normal development and physiology, as well as disease states, including vascular disease (24).

Although miRNAs represent a new layer of gene expression regulators at the translational level, the effects of ROS on miRNA expression and the potential roles of miRNAs in ROS-

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[3] The abbreviations used are: ROS, reactive oxygen species; miRNA, microRNA; VSMC, vascular smooth muscle cell; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PDCD4, programmed cell death 4; LNA, locked nucleic acid; FAM, 6-carboxyfluorescein; m.o.i., multiplicity of infection; UTR, untranslated region; Ad, adenovirus; GFP, green fluorescent protein; qRT, quantitative reverse transcription; CMV, cytomegalovirus; AP-1, Activator protein 1.

mediated gene regulation and biological functions of vascular cells are unclear. The objective of the current study is to determine the effect of an ROS, H_2O_2, on miRNA expression in cultured vascular smooth muscle cells (VSMCs) and to determine whether miRNAs play a role in H_2O_2-mediated effects on gene expression and cellular function.

EXPERIMENTAL PROCEDURES

Cell Culture—VSMCs were obtained from the aortic media of male Sprague-Dawley rats (5 weeks old) using an enzymatic dissociation method as described (27). VSMCs were cultured with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells between passage 3 and 6 were applied for the experiments.

Microarray Analysis of miRNA Expression—Cultured rat VSMCs were treated with either vehicle or H_2O_2 (200 μM) for 6 h. miRNAs were then isolated from the cultured cells using the mirVana miRNA isolation kit (Ambion, Inc.). miRNA expression was determined by miRNA microarray analysis as described (28, 29). The assay started with a 4- to 8-μg total RNA sample, which was size-fractionated using a YM-100 microcon centrifugal filter, and the small RNAs (<300 nucleotides) isolated were 3′-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a microfluidic chip using a micro-circulation pump (Atactic Technologies). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to 238 mature miRNAs (Chip ID miRRat, 9.2 version, LC Sciences) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by *in situ* synthesis using photogenerated reagent chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 μl of 6× saline-sodium phosphate-EDTA buffer (0.90 M NaCl, 60 mM Na_2HPO_4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34 °C. After RNA hybridization, tag-conjugating Cy3 and Cy5 dyes were circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were analyzed by subtracting the background and then normalizing the signals using a LOWESS filter (locally weighted regression). For two-color 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 < 0.05. Proprietary "spike-in" controls were used at each step of the process (30, 31).

Measurements of VSMC Apoptosis and Cell Death Induced by H_2O_2—Briefly, VSMCs cultured in 0.1% fetal bovine serum were treated with either vehicle or H_2O_2 (10–200 μM, Sigma) for 24 h. Afterward, cell death and cell apoptosis were measured by trypan exclusion and terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) staining as described previously (29, 32). For the trypan exclusion, the cells were harvested and stained with 0.25% trypan blue for 2 min, and live cells were counted using a hemocytometer (32). Then the total cells were counted, and the percentage of dead cells (% cell death) was calculated. For TUNEL analysis, VSMCs 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 Applied Science) according to the manufacturer's protocol. The number of TUNEL-positive cells was counted under a fluorescence microscope. A VSMC apoptosis index was calculated using the following formula: (number of TUNEL-positive cells/total cells) × 100.

Oligonucleotide Transfection, miR-21 Knockdown, miR-21 Overexpression, and Programmed Cell Death 4 (PDCD4) Gene Up-regulation in Cultured VSMCs—Oligonucleotide transfection was performed according to an established protocol (28, 29). Briefly, cells were transfected using a transfection reagent (Qiagen) 24 h after seeding into the well. Transfection complexes were prepared according to the manufacturer's instructions. For the miR-21 knockdown, the 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-tcagtctgataagcta-3′, and its control oligonucleotide, LNA-control, 5′-FAM-cgtcagtatgcgaatc-3′. For the miR-21 up-regulation, pre-miR-21 (Ambion, Inc.) was added directly to the complexes at final oligonucleotide concentration of 30 nM. *PDCD4* gene up-regulation was performed by adenovirus expressing *PDCD4* without miR-21 binding site at 3′-UTR (Ad-PDCD4) (30 m.o.i.) or with miR-21 binding site at 3′-UTR (Ad-PDCD4) (30 m.o.i.). The transfection medium was replaced 4 h post-transfection by the regular culture medium. Vehicle control, oligonucleotide control (Ambion, Inc.) and adenoviruses control (Ad-GFP) were applied.

RNA Levels Were Determined by qRT-PCR—Briefly, RNAs from VSMCs were isolated with an RNA isolation kit (Ambion, Inc.). qRT-PCR for miR-21 was performed on cDNA generated from 50 ng of total RNA using the protocol of the mirVana qRT-PCR miRNA detection kit (Ambion, Inc). 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 Applied Science). Amplification and detection of specific products were performed with a LightCycler 480 detection system (Roche Applied Science). As an internal control, U6 was used for miR-21 template normalization and glyceraldehyde-3-phosphate dehydrogenase was used for *PDCD4* template normalization. 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 or glyceraldehyde-3-phosphate dehydrogenase 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 $= 2^{-(\Delta Ctsample - \Delta Ctcontrol)}$ (28, 29).

Western Blot Analysis—Proteins isolated from cultured VSMCs 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). Glyceraldehyde-3-phosphate dehydrogenase antibody (1:5000 dilution, Cell Signaling) was used as a loading control.

Construction of the Adenovirus Expressing PDCD4 and Control Virus Expressing GFP—The adenovirus expressing *PDCD4* and control virus expressing GFP (Ad-GFP) were generated using the adeno-X™ expression systems 2 kit (Clontech) according to the manufacturer's protocols. Briefly, a 1410-bp fragment of the full-length coding sequence was amplified with primers tgaattcatggatgtagaaaacgagcagata and taagcttcagtagctctcaggtttaagacga by using RT-PCR and was inserted into pDNR-CMV donor vector (Clontech) at EcoRI and HindIII sites. This vector was named pDNR-CMC-PDCD4. The construct was sequenced to confirm the DNA sequence. The *PDCD4* fragment was then excised from the pDNR-CMC-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 PacI was used to transfect low passage HEK 293 cells to produce recombinant adenovirus with Lipofectamine 2000 according to the manufacturer's protocols (Invitrogen). Adenovirus-expressing GFP was generated as described before (33). 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), producing pENTR3C-GFP. pENTR3C-GFP was transformed into *Escherichia coli* DH5α, and the plasmids were amplified. These plasmids were recombined with pAd/CMV/V5-DEST as described by the manufacturer (Invitrogen), producing pAd-GFP plasmids, which were verified by DNA sequencing. The pAd-EGFP with PacI was 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. Ad-PDCD4, Ad-PDCD4-Lack, and Ad-GFP were titrated using a standard plaque assay.

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.

Activator protein 1 (AP-1) activity was measured using luciferase assay as described (34). Briefly, adenoviral vector (Ad-AP1-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 VSMCs pretreated with vehicle, control oligonucleotides (oligonucleotide control), LNA-anti-miR-21 (30 nM), pre-miR-21 (30 nM), Ad-GFP (30 m.o.i.), Ad-PDCD4 (30 m.o.i.), Ad-PDCD4-Lack (30 m.o.i.), 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. The luciferase expression was measured on a scintillation counter by using a dual luciferase reporter system.

Statistics—All data are presented as means ± S.E. For relative gene expression, the mean value of the vehicle control group is defined as 100% or 1. Two-tailed unpaired Student's *t* tests and analysis of variance were used for statistical evaluation of the data. The SigmaStat statistical analysis program was used for data analysis. A p value < 0.05 was considered significant.

TABLE 1
Aberrant expression of miRNAs in VSMCs treated with H_2O_2

Down-regulated miRNAs		Up-regulated miRNAs	
miR-290	miR-107	rno-miR-351	rno-miR-20a
miR-193	miR-103	rno-miR-30d	rno-let-7e
miR-181c	miR-328	rno-let-7b	rno-miR-26b
miR-29b	miR-34a	rno-miR-30b	rno-miR-10b
miR-30e	miR-181b	rno-let-7f	rno-miR-15b
miR-145	miR-19b	rno-miR-18	rno-miR-92
miR-181a	miR-324-5p	rno-let-7i	rno-miR-352
miR-199a	miR-101b	rno-miR-342	rno-miR-21
miR-22	miR-214	rno-let-7d	rno-miR-20b
miR-130a	miR-23b	rno-miR-361	rno-miR-10a
miR-30a-5p	miR-23a	rno-miR-424	rno-miR-98
miR-99b	miR-143	rno-miR-132	rno-miR-7
miR-101a	miR-151	rno-miR-30c	rno-miR-195
miR-301	miR-31	rno-miR-25	rno-miR-365
miR-99a			

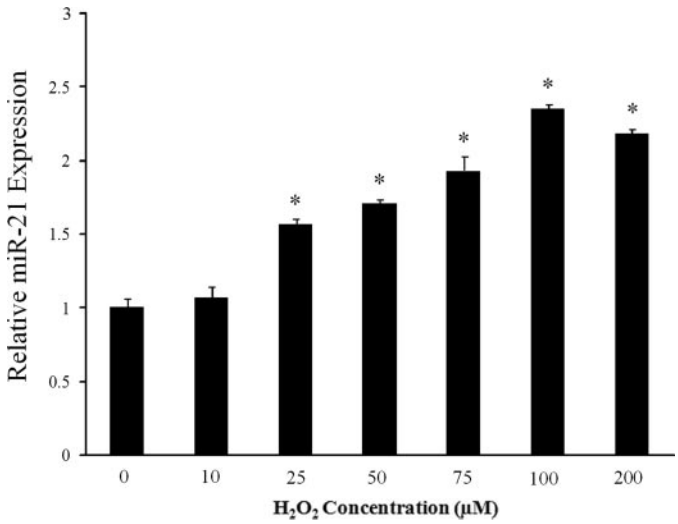


FIGURE 1. **The effect of H_2O_2 on miR-21 expression in cultured rat VSMCs.** Cultured rat VSMCs were treated with vehicle or H_2O_2 (10–200 μM) for 6 h. miR-21 levels were determined by qRT-PCR. Note: $n = 6$; *, $p < 0.05$ compared with vehicle control (0 μM).

RESULTS

The Effect of H_2O_2 on miRNA Expression in Cultured VSMCs—Overall, 143 miRNAs out of the 238 arrayed were found in VSMCs. After treatment with H_2O_2 (200 μM) for 6 h, microarray analysis demonstrated that 72 of the 143 miRNAs were differentially expressed with p value <0.05; 38 miRNAs were up-regulated, and 34 miRNAs were down-regulated. Fifty-seven miRNAs that are highly expressed and dysregulated in H_2O_2-treated VSMCs are listed in Table 1. Remarkably, miR-21, an

miRNA that we found has an anti-apoptotic effect on VSMCs induced by serum deprivation (29), was increased compared with the vehicle-treated control. The effect of H_2O_2 on miR-21 expression was further confirmed by qRT-PCR. As shown in Fig. 1, H_2O_2 (10–200 μM) increased expression of miR-21 in a dose-dependent manner.

The Effects of H_2O_2 on VSMC Apoptosis and Death—Although low concentrations of H_2O_2 had no effect on apoptosis and death, high concentrations (10–200 μM) increased VSMC cell death in a dose-dependent manner after 24-h treatment under our experimental condition (Fig. 2). Using the combination of trypan exclusion and TUNEL staining, we have confirmed that most of cell death in H_2O_2-treated VSMCs at 50 μM for 24 h was induced by apoptosis.

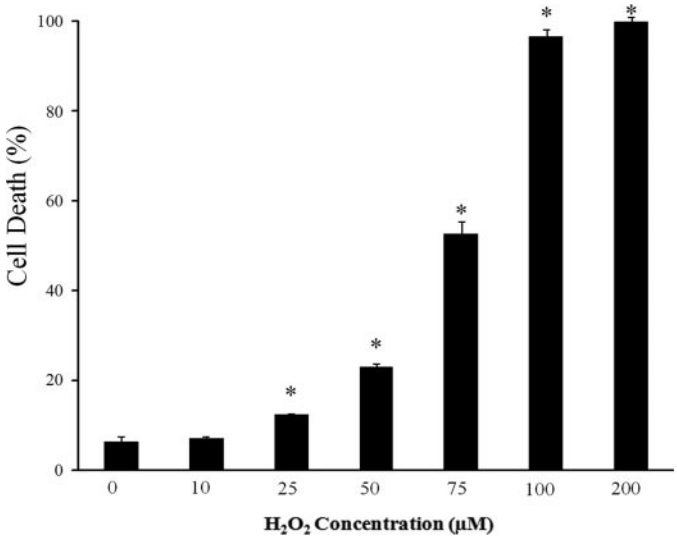


FIGURE 2. **Death of rat VSMCs induced by H_2O_2.** Cultured rat VSMCs were treated with vehicle or H_2O_2 (10–200 μM) for 24 h. Cell was measured by trypan exclusion. Note: $n = 6$; *, $p < 0.05$ compared with vehicle control (0 μM).

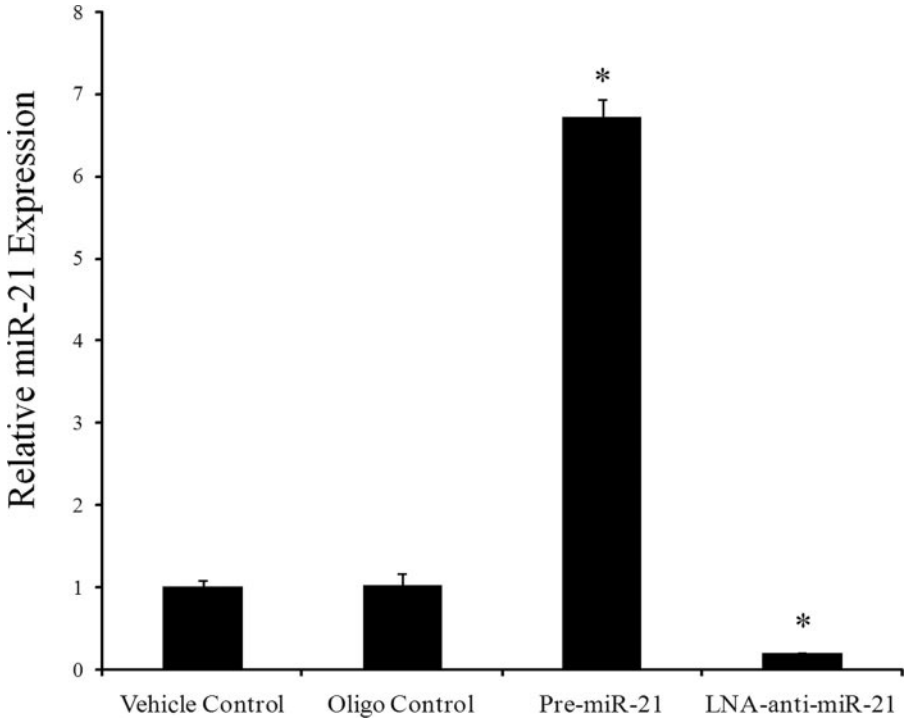


FIGURE 3. **Modulating miR-21 expression in cultured VSMCs.** Cultured VSMCs were treated with vehicle, miR-21 inhibitor (LNA-anti-miR-21, 30 nM) or pre-miR-21 (30 nM) for 4 h. miR-21 levels were determined 24 h later by qRT-PCR. Note: $n = 6$; *, $p < 0.05$ compared with vehicle control.

Modulating miR-21 Expression in Cultured VSMCs—To modulate miR-21 expression in cultured VSMCs, both gain-of-function and loss-of-function approaches were applied. As shown in Fig. 3, LNA-anti-miR-21 deceased, but pre-miR-21 increased miR-21 expression in VSMCs. The effects of both LNA-anti-miR-21 and pre-miR-21 on miR-21 expression were miR-21-specific, because no effects were found on other miRNAs such as miR-24 and miR-146 (data not shown).

The Effect of miR-21 on H_2O_2-induced VSMC Death and Apoptosis—Pre-miR-21 decreased H_2O_2-induced VSMC death as determined by trypan exclusion (Fig. 4*A*) and apoptosis as determined by terminal TUNEL staining (Fig. 4*B*). In contrast, VSMC apoptosis and death were increased after treatment with LNA-anti-miR-21 (Fig. 4, *A* and *B*). Representative TUNEL-stained photomicrographs from VSMCs treated with vehicle, control oligonucleotide, Pre-miR-21, and LNA-anti-miR-21 are shown in Fig. 4 (*C–F*). The results indicate that miR-21 has a protective effect on H_2O_2-induced VSMC injury responses (apoptosis and death).

PDCD4 Is a miR-21 Target Gene in VSMCs—Computational analysis indicates that *PDCD4* is a potential target gene of miR-21 (Fig. 5*A*). If it is a miR-21 target, H_2O_2 should decrease its expression in VSMCs, because miR-21 expression was up-regulated after H_2O_2 stimulation (Fig. 1). To confirm this, we incubated VSMCs with either vehicle or H_2O_2 (50 μM) for 24 h, and protein level of PDCD4 was determined by Western blot. As shown in Fig. 5*B*, H_2O_2 decreased *PDCD4* expression in a dose-dependent manner. The results suggest that *PDCD4* is a potential miR-21 target gene in VSMCs stimulated with H_2O_2.

To verify that *PDCD4* is a gene target of miR-21, both gain-of-function and loss-of-function approaches were applied. As shown in Fig. 5*C*, LNA-anti-miR-21 increased, whereas pre-miR-21 decreased PDCD4 expression in cultured VSMCs. The similar regulatory effect of miR-21 modulation on PDCD4 expression was also found in H_2O_2-stimulated VSMCs (Fig. 5*D*). The results suggest that *PDCD4* is a target gene of miR-21. 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

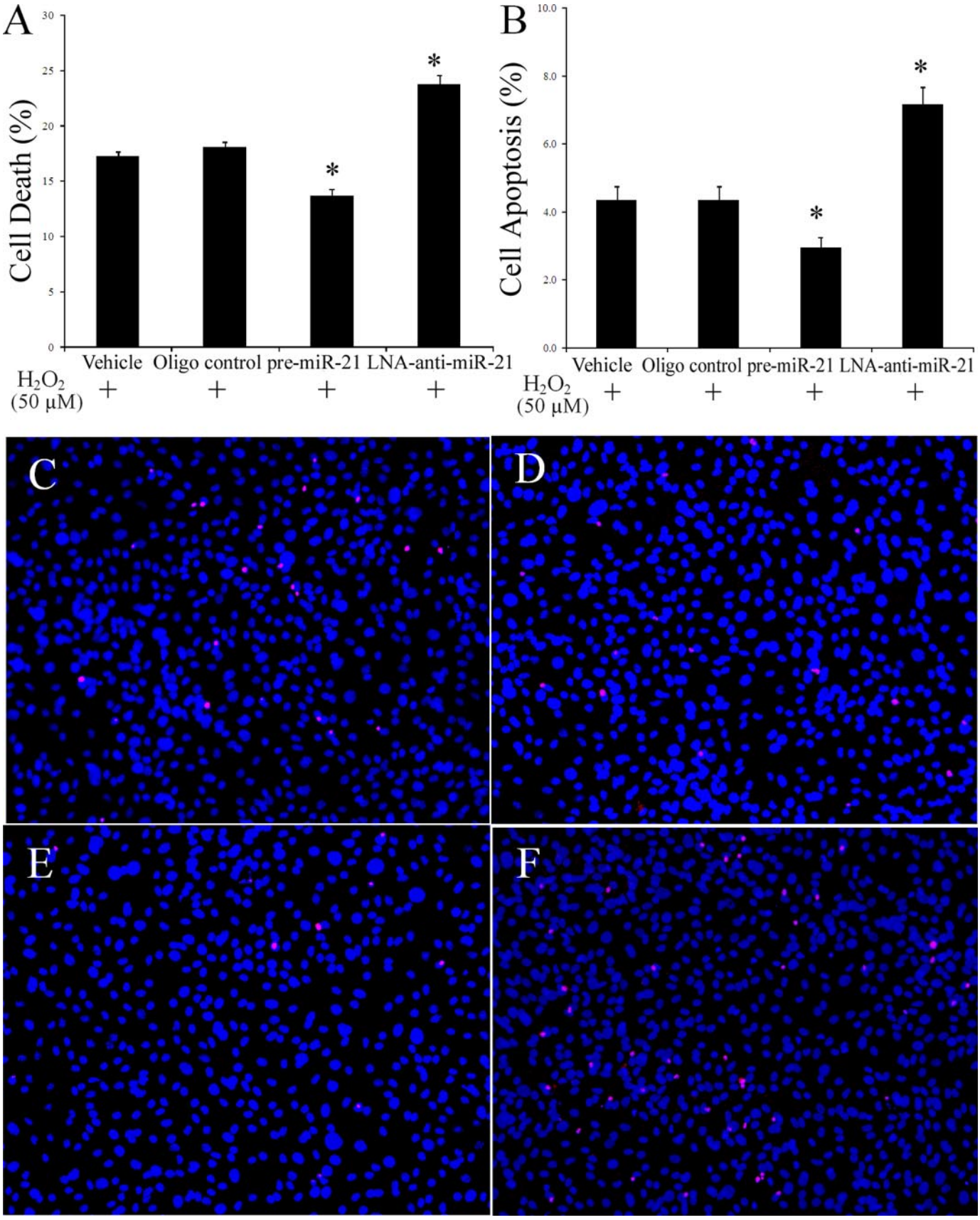


FIGURE 4. **The effect of miR-21 on H_2O_2-induced VSMC apoptosis and death.** Cultured rat VSMCs pre-treated with vehicle, miR-21 inhibitor (LNA-anti-miR-21, 30 nM) or pre-miR-21 (30 nM) were treated with H_2O_2 (50 μM) for 24 h. Then, cell death was determined by trypan exclusion (*A*) and cell apoptosis was determined by TUNEL staining (*B*). Representative TUNEL-stained photomicrographs from VSMCs treated with vehicle (*C*), control oligonucleotide (*D*), Pre-miR-21 (*E*), and LNA-anti-miR-21 (*F*). Note: $n = 6$; *, $p < 0.05$ compared with vehicle control treated with H_2O_2 (50 μM).

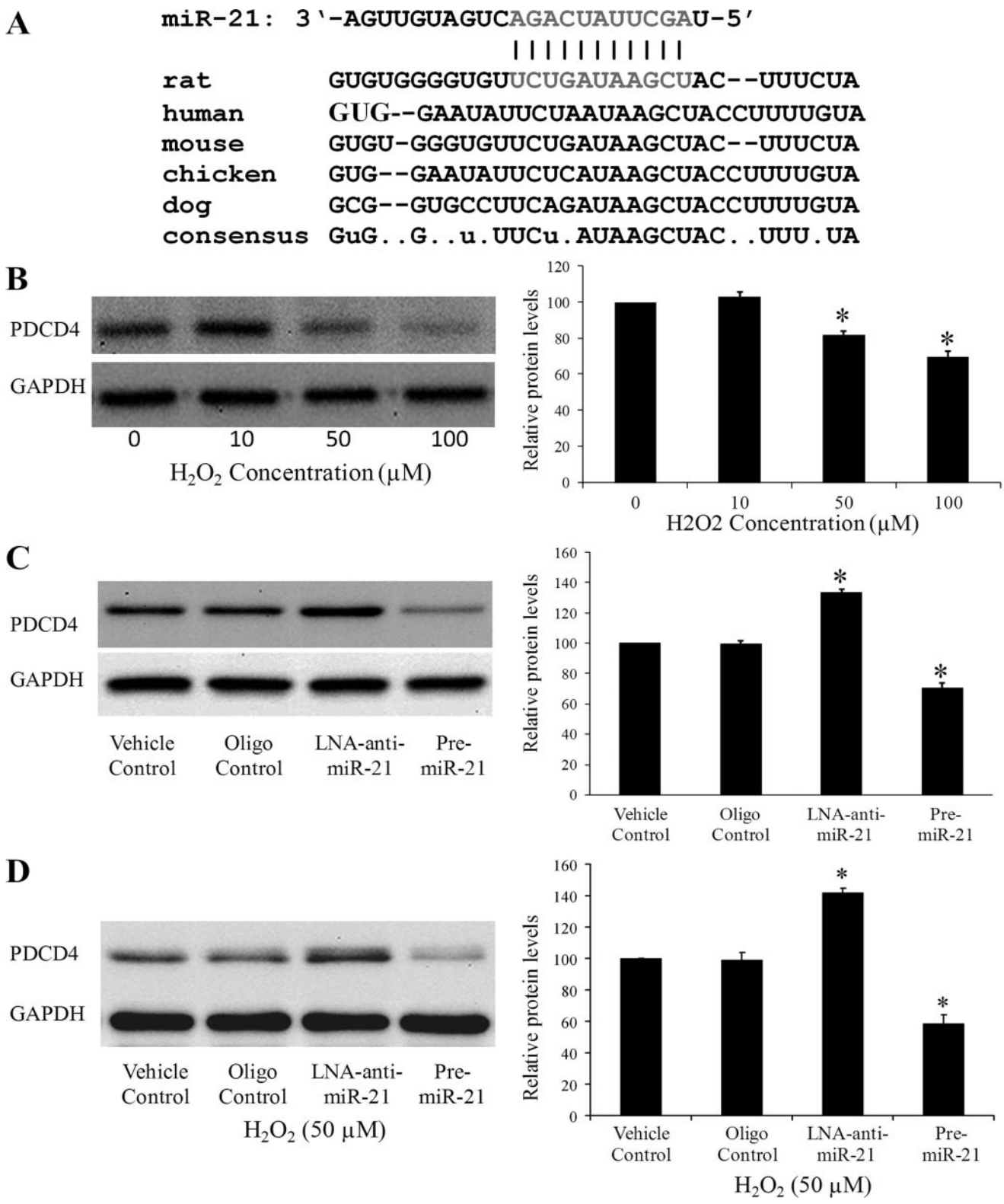


FIGURE 5. ***PDCD4* is a target gene of mi-21.** *A*, computational analysis indicates that *PDCD4* has a miR-21 binding site at nucleotides 228–249 of the *PDCD4*-3′-UTR, and is highly conserved in six species. *B*, H_2O_2 decreased PDCD4 expression in a dose-dependent manner. Rat VSMCs were treated with 0 μM, 10 μM, 50 μM, or 100 μM of H_2O_2 for 24 h, and the cell proteins were isolated for Western blot analysis of PDCD4. The *left panel* is the representative Western blot from five experiments, and the *right panel* is the quantification of PDCD4 protein. Note: $n = 5$; *, $p < 0.05$ compared with 0 μM control. *C*, miR-21 inhibitor. LNA-anti-miR-21 increased, whereas pre-miR-21 decreased, PDCD4 expression in cultured VSMCs. Rat VSMCs were treated with vehicle (*Vehicle Control*), control oligonucleotide (*Oligo Control*, 30 nM), miR-21 inhibitor (*LNA-anti-miR-21*, 30 nM), or pre-miR-21 (30 nM) for 4 h. 48 h later, proteins were isolated for Western blot analysis of PDCD4. The *left panel* is the representative Western blot from five experiments, and the *right panel* is the quantification of PDCD4 protein. Note: $n = 5$; *, $p < 0.05$ compared with vehicle control. *D*, in addition to the treatments in *C*, all the cells were treated with H_2O_2 (50 μM) for 24 h. 48 h after H_2O_2 treatment, proteins were isolated for Western blot analysis of PDCD4. The *left panel* is the representative Western blot from five experiments, and the *right panel* is the quantification of PDCD4 protein. Note: $n = 5$; *, $p < 0.05$ compared with vehicle control.

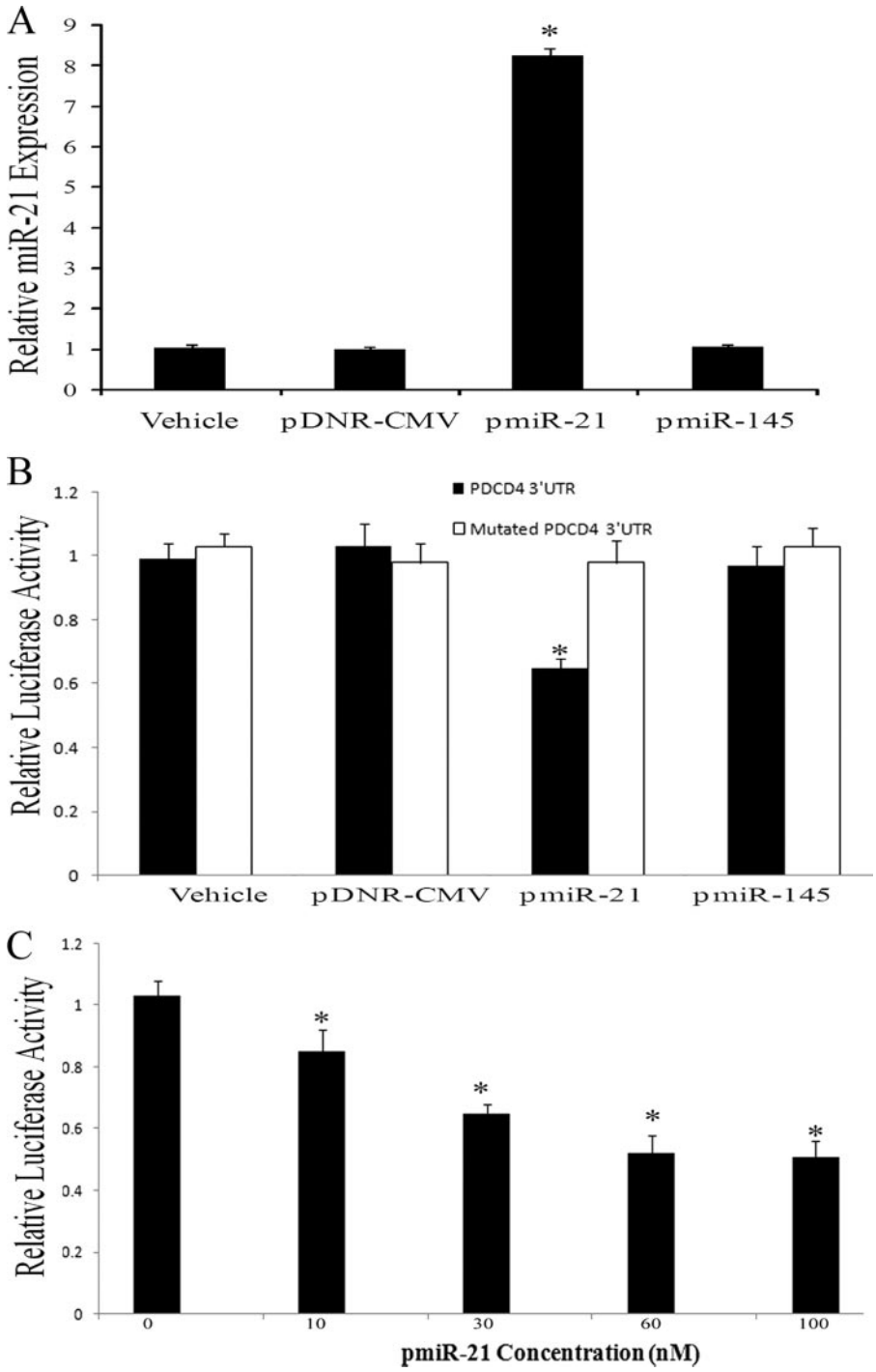
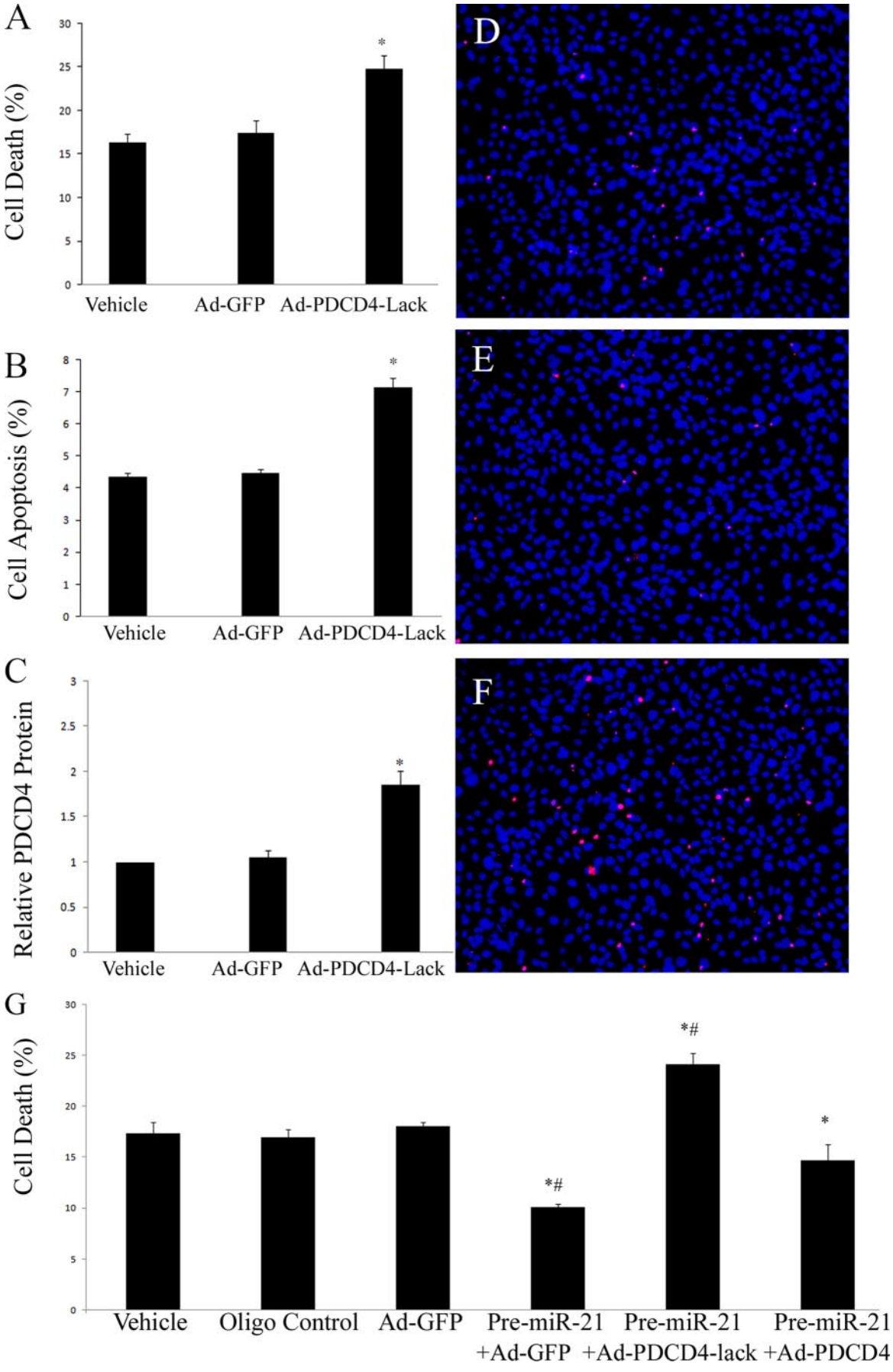


FIGURE 6. **miR-21 is able to bind to *PDCD4* and inhibit PDCD4 expression in HEK 293 cells.** A construct (1 μg/well) in which a fragment of the 3′-UTR of *PDCD4* mRNA with the putative miR-221 and miR-222 binding sequence was cloned into a firefly luciferase reporter construct and transfected into 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. *A*, pmiR-21, but not pmiR-145 or pDNR-CMV, increased miR-21 expression in HEK 293 cells. *B*, pmiR-21, but not pDNR-CMV or pmiR-145 inhibited luciferase activity. In the mutated control group, the inhibitory effect of pmiR-21 and pmiR-222 disappeared. *C*, dose-dependent effect of pmiR-21 on luciferase activity. The cells were treated with 0, 10, 30, 60, or 100 nM of pmiR-21. Note: $n = 5$; *, $p < 0.05$ compared with vehicle control.

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*A*). Accordingly, pmiR-21, but not pDNR-CMV or pmiR-145, inhibited luciferase activity (Fig. 6*B*). In the mutated control group, the inhibitory effect of pmiR-21 and pmiR-222 disappeared (Fig. 6*B*). The dose-dependent effect of pmiR-21 on luciferase activity is shown in Fig. 6*C*. The maximal inhibitory effect was ~50% inhibition that occurred between 30 and 60 nM pmiR-21. The results imply that miR-21 can bind to *PDCD4* directly and inhibit its expression.

PDCD4 Is a Functional Target Gene That Is Involved in miR-21-mediated Protective Effect on H_2O_2-induced VSMC Apoptosis and Death—The role of PDCD4 in H_2O_2-induced VSMC apoptosis and death is currently unknown. To determine the functional involvement of PDCD4 in miR-21-mediated cellular effect, we first determined the role of PDCD4 in H_2O_2-induced VSMC apoptosis and death. As shown in Fig. 7, overexpression of PDCD4 via ad-PDCD4 increased H_2O_2-induced VSMC apoptosis and death as determined by trypan exclusion (Fig. 7*A*) and terminal TUNEL staining (Fig. 7*B*). The relative expression of PDCD4 in these different treatment groups is shown in Fig. 7*C*. Representative TUNEL-stained photomicrographs from VSMCs treated with vehicle, Ad-GFP, and Ad-PDCD4 are shown in Fig. 7 (*D–F*). Interestingly, pre-miR-21-mediated protective effect on VSMC apoptosis and death was totally inhibited in H_2O_2-treated cells via adenovirus-mediated overexpression of *PDCD4* without miR-21 binding site (Ad-PDCD4-Lack) (Fig. 7*G*). In contrast, pre-miR-21 still had a protective effect on H_2O_2-induced VSMC apoptosis in Ad-PDCD4-treated cells, although the protective effect was lower than that in VSMCs without Ad-PDCD4 (Fig. 7*G*).

AP-1 Is a Downstream Signaling Molecule of PDCD4 That Might Be Involved in miR-21-mediated Protective Effect on VSMCs—As shown in Fig. 8*A*, overexpression of PDCD4 by Ad-PDCD4 or Ad-PDCD4-Lack inhibited AP-1 activity. In



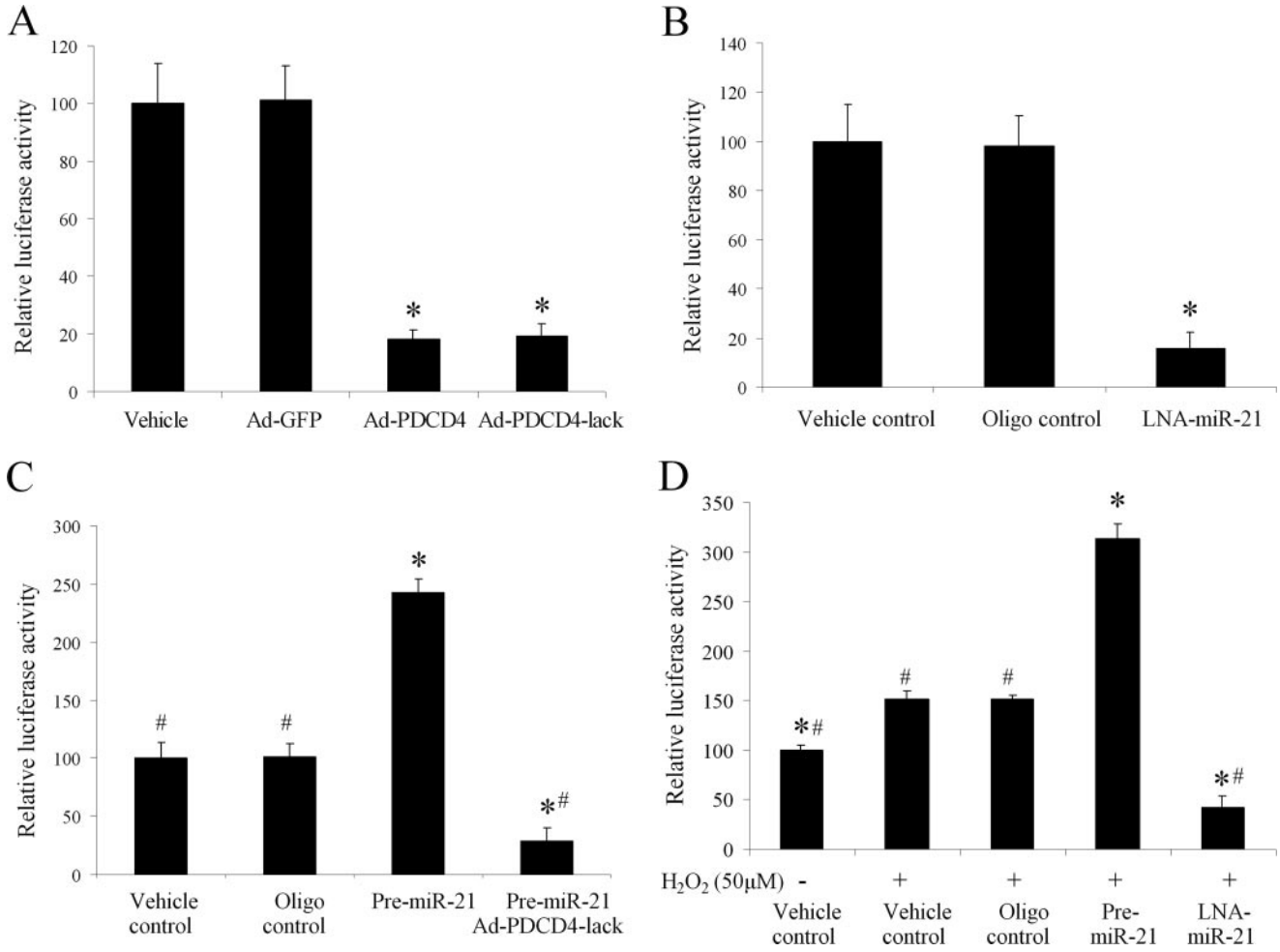


FIGURE 8. **miR-21 regulates AP-1 activity via its target gene *PDCD4*.** AP-1 was measured using luciferase assay. VSMCs pretreated with vehicle, control oligonucleotides (*Oligo Control*), LNA-anti-miR-21 (30 nM), pre-miR-21 (30 nM), ad-GFP (30 m.o.i.), Ad-PDCD4 (30 m.o.i.), Ad-PDCD4-Lack (30 m.o.i.), 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*, luciferase activity was decreased by LNA-anti-miR-21. *C*, luciferase activity was increased by pre-miR-21 (30 nM); 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*). Note: $n = 5$; *, $p < 0.05$ compared with vehicle control. #, $p < 0.05$ compared with pre-miR-21 group. *D*, the cell groups and treatments were the same as in *A–C* except for the additional treatment with H_2O_2 (50 μM) or vehicle for 24 h before the luciferase activity was measured. Note: $n = 5$; *, $p < 0.05$ compared with vehicle control treated with H_2O_2. #, $p < 0.05$ compared with pre-miR-21 group.

addition, increasing of PDCD4 expression via LNA-anti-miR-21 (Fig. 5*C*) resulted in decrease in AP-1 activity (Fig. 8*B*). In contrast, decreasing of PDCD4 expression via pre-miR-21 (Fig. 5*C*) resulted in increase in AP-1 activity (Fig. 8*C*). Moreover, 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*C*). In H_2O_2-stimulated VSMCs, AP-1 activity was increased (Fig. 8*D*). The regulatory effect of miR-21 on AP-1 activity was also verified in H_2O_2-stimulated VSMCs as demonstrated in Fig. 8*D*. The results suggest that AP-1 is a downstream signaling molecule of PDCD4 that might be involved in miR-21-mediated protective effect on VSMCs.

FIGURE 7. ***PDCD4* is a functional target gene that is involved in miR-21-mediated protective effect on H_2O_2-induced VSMC apoptosis and death.** Rat VSMCs were treated with vehicle, control adenovirus, Ad-GFP (30 m.o.i.) or adenovirus expressing *PDCD4* without miR-21 binding site (*Ad-PDCD4-Lack*, 30 m.o.i.) for 4 h. Cell death, cell apoptosis, and PDCD4 protein levels were determined 24 h after treatment with 50 μM of H_2O_2. *A*, overexpression of PDCD4 via Ad-PDCD4-Lack increased H_2O_2-induced VSMC death as determined by trypan exclusion. Note: $n = 6$; *, $p < 0.05$ compared with vehicle control. *B*, overexpression of PDCD4 via Ad-PDCD4-Lack increased H_2O_2-induced VSMC apoptosis as determined by TUNEL staining. Note; $n = 6$; *, $p < 0.05$ compared with vehicle control. *C*, Ad-PDCD4-Lack increased PDCD4 expression in VSMCs as determined by Western blot analysis. Note: $n = 5$; *, $p < 0.05$ compared with vehicle control. Representative TUNEL-stained photomicrographs from VSMCs treated with vehicle (*D*), Ad-GFP (*E*), and Ad-PDCD4 (*F*). *G*, cultured rat VSMCs pre-treated with vehicle (*Vehicle Control*), control oligonucleotide (*Oligo Control*, 30 nM), or pre-miR-21 (30 nM) for 4 h were treated with Ad-GFP (30 m.o.i.), Ad-PDCD4-Lack (30 m.o.i.), or adenovirus expressing *PDCD4* with miR-21 binding site (*Ad-PDCD4*, 30 m.o.i.). Then, the cells were treated with 50 μM of H_2O_2 for 24 h and cell apoptosis was determined. Pre-miR-21 had a protective effect on H_2O_2-induced VSMC apoptosis. However, the Pre-miR-21-mediated protective effect on VSMC apoptosis was totally inhibited in H_2O_2-treated cells via Ad-PDCD4-Lack. In contrast, pre-miR-21 still had a protective effect on H_2O_2-induced VSMC apoptosis in Ad-PDCD4-treated cells, although the protective effect was lower than that in VSMCs without Ad-PDCD4. Note: $n = 8$; *, $p < 0.05$ compared with vehicle control; #, $p < 0.05$ compared with Ad-PDCD4.

DISCUSSION

It is well established that ROS such as H_2O_2 play important roles in controlling cellular functions such as cell differentiation, proliferation, migration, apoptosis, and death (3). These cell functional controls are achieved via ROS-mediated gene expression regulation (3–5). For example, microarray analysis reveals that a large number of genes are regulated in cells treated with H_2O_2, and these regulated genes are responsible for H_2O_2-mediated cellular effects (4, 5).

Several investigators have studied the molecular mechanisms of ROS-mediated gene regulation. It is found that epigenetic regulation at the DNA level and transcription factors at the transcriptional level are two important mechanisms involved in H_2O_2-mediated expression changes of multiple genes (3–5, 13, 14). However, the role of post-transcriptional regulation in H_2O_2-mediated gene regulation is still unclear. Recently, there has been an important breakthrough in gene regulation in which miRNAs have been identified in mammalian cells (17–20). The new layer of gene expression regulators is found to regulate at least 30% of genes in a cell at the translational level. However, the role of miRNAs in H_2O_2-mediated gene regulation and its functional effects on the VSMCs are currently unidentified.

In the current study, we identified that miRNA expression is very sensitive to H_2O_2 stimulation. Six hours after treatment with H_2O_2, multiple miRNAs are either down- or up-regulated. The multiple aberrantly expressed miRNAs match the complex process of gene regulation via ROS such as H_2O_2, in which multiple genes have been dysregulated (3–5). The result of the current study indicates that miRNAs may participate in H_2O_2-mediated modulation of gene expression.

To test the potential roles of miRNAs in H_2O_2-mediated cellular effects, we selected an up-regulated miRNA, miR-21. We found H_2O_2 (10–100 μM) increased miR-21 expression in a dose-dependent manner. Interestingly, up-regulation of miR-21 expression inhibited H_2O_2-mediated VSMC apoptosis and death. In contrast, H_2O_2-mediated VSMC apoptosis and death were exacerbated after down-regulation of miR-21 expression. The results suggest that miR-21 had an anti-apoptotic effect in H_2O_2-mediated VSMC apoptosis and death. It should be noted that the effects of miR-21 modulations on VSMC apoptosis and death are modest in magnitude. We think that, although miR-21 is a novel regulator for apoptosis and death in H_2O_2-stimulated VSMCs, it is only one of multiple regulators for these cellular events.

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 experimental cells as the miRNA targets and functions are cell-specific (35). In the current study, computational analysis suggests that *PDCD4* is a miR-21 target. Moreover, PDCD4 regulation by miR-21 has been recently reported in cancer cells (36–38).

To test whether PDCD4 is an miR-21 target gene in VSMCs, we have first confirmed that H_2O_2 decreased PDCD4 expression in a dose-dependent manner. It is established that PDCD4 is a pro-apoptotic protein. The negative relationship between H_2O_2 and PDCD4 in H_2O_2-treated VSMCs indicates that the decreased expression of PDCD4 via H_2O_2 may be a defensive response of the VSMCs. In addition, PDCD4 expression in VSMCs was able to be regulated by miR-21 in both H_2O_2-stimulated and unstimulated cells as determined by both gain-of-function and loss-of-function approaches. However, the effects of miR-21 modulations on PDCD4 expression are modest in magnitude. In particular, even when we used miR-21 at a higher level than that in H_2O_2-stimulated cells, the magnitude of PDCD4 modulation was still smaller than that in H_2O_2-stimulated cells. We think that the modest effects on PDCD4 expression in VSMCs might be explained by the following two reasons: First, the modest effect on its multiple target gene expression *in vitro* is a characteristic of an individual miRNA in cardiovascular cells, based on our recent studies on miR-21, miR-145, miR-221, and miR-222. The observation has also been confirmed by a recent miRNA study using the proteomic approach. These investigators have found that a single miRNA can repress the production of hundreds of its target proteins but that this repression is typically relatively mild (39). Another potential reason is that PDCD4 expression in H_2O_2-stimulated cells is not only controlled by miR-21 but other unidentified regulators might also be involved.

We have confirmed that 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 has the putative miR-21 binding sequence. Furthermore, we have found that the miR-21 overexpression-mediated protective effect on VSMC apoptosis and death is blocked after overexpression of *PDCD4* without miR-21 binding sites. The results indicate that *PDCD4* is a functional target gene of miR-21, which is involved in the miR-21-mediated protective effect on VSMC injury elicited by H_2O_2.

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, although its final outcome on cell apoptosis is cell-specific (40, 41). Several recent studies suggest that AP-1 is a downstream signaling molecule of PDCD4 in other types of cells (42, 43). We thus hypothesize that AP-1 might be a downstream signaling molecule of PDCD4 that is involved in miR-21-mediated effects on VSMCs. The hypothesis is supported by the following findings in both H_2O_2-stimulated and unstimulated VSMCs. First, we found that increasing PDCD4 by Ad-PDCD4 or Ad-PDCD4-Lack inhibits AP-1 activity. Second, miR-21 inhibitor 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, a pre-miR-21-mediated increase in AP-1 activity is able to be blocked by adenovirus-expressing *PDCD4* without miR-21 binding sites.

In the current study, we have found that there is significant necrosis involved in the H_2O_2 response, and miR-21 also has a protective effect on VSMC necrosis. However, the mechanisms responsible for it are unclear and need to be investigated in future study. Several recent reports have suggested that the phosphatase and tensin homolog is a target gene for miR-21. Phosphatase and tensin homolog is an important molecule for

cell survival and might play a role in the miR-21-mediated protective effect on cell necrosis.

In summary, the current study reveals that the new layer of gene regulators, miRNAs, in VSMCs are sensitive to H_2O_2 stimulation. miR-21 participates in H_2O_2-mediated gene regulation and injury response 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 cardiovascular diseases related to ROS such as atherosclerosis, hypertension, restenosis after angioplasty or bypass, diabetic vascular complications, and transplantation arteriopathy.

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