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Antagonist of microRNA-21 Improves Balloon Injury-Induced Rat Iliac Artery Remodeling by Regulating Proliferation and Apoptosis of Adventitial Fibroblasts and Myofibroblasts

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ABSTRACT

Molecular pathways involved in adventitial fibroblasts (AFs) and myofibroblasts (MFs) proliferation and apoptosis contribute to vascular remodeling. MicroRNA-21 (miR-21) plays an important role in regulating cellular proliferation and apoptosis of many cell types; however, the effect of miR-21 on AFs and MFs is still unknown. In this study, we found that miR-21 was expressed in AFs and overexpressed in MFs. Inhibition of miR-21 decreased proliferation and increased apoptosis of AFs and MFs, and overexpression of miR-21 with pre-miR-21 had the reverse effect. Programmed cell death 4 (PDCD4), related to cell proliferation and apoptosis, was validated as a direct target of miR-21 by dual-luciferase reporter assay and gain and loss of function of miR-21 in AFs and MFs. PDCD4 knockdown with siRNA partly rescued the reduced proliferation with miR-21 inhibition and alleviated the increased apoptosis induced by miR-21 inhibition in AFs and MFs. Moreover, increasing PDCD4 expression by miR-21 inhibition significantly decreased JNK/c-Jun activity. In contrast, decreasing PDCD4 expression by pre-miR-21 treatment increased JNK/c-Jun activity, while the effect of miR-21 inhibition on JNK/c-Jun activity could be rescued by PDCD4 siRNA. Moreover, miR-21 inhibition could regulate proliferation and apoptosis of vascular AFs and MFs in vivo. Furthermore, miR-21 inhibition reversed vascular remodeling induced by balloon injury. In summary, our findings demonstrate that miR-21 may have a critical role in regulating proliferation and apoptosis of AFs and MFs, and PDCD4 is a functional target gene involved in the miR-21-mediated cellular effects in vascular remodeling by a miR-21/PDCD4/JNK/c-Jun pathway. J. Cell. Biochem. 113: 2989–3001, 2012.

KEY WORDS: microRNA-21; ADVENTITIAL FIBROBLAST; MYOFIBROBLAST; PROLIFERATION; APOPTOSIS; PDCD4; JNK

Vascular remodeling is the pathological endpoint of diverse cardiovascular diseases such as coronary heart diseases, post-angioplasty restenosis, atherosclerosis, and hypertension [Shi et al., 1996; Sartore et al., 2001; Heeneman et al., 2007]. Increasing evidence suggests that the adventitia is a critical regulator of vessel wall function in health and disease [Sartore et al., 2001; Michel et al.,

Abbreviations: AF, adventitial fibroblast; MF, myofibroblast; miR-21, MicroRNA-21; miRNA, microRNA; ECM, extracellular matrix; PDCD4, Programmed cell death 4; TGF-β1, transforming growth factor β1; EdU, 5-ethynil-2′-deoxyuridine; BrdU, 5-bromo-2′-deoxyuridine; 3′-UTR, 3′-untranslated region; VSMC, vascular smooth muscle cell.

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2007]. The balance in adventitial fibroblasts (AFs) proliferation and apoptosis is decisive for the physiology of the vasculature. Under pathological stimuli such as injury and cytokines, AFs, the principal cell type in the adventitia, are activated and undergo phenotypic changes, including proliferation and differentiation to myofibroblasts (MFs) [Sartore et al., 2001; Maiellaro and Taylor, 2007]. Increased proliferative capacity and increased secretion of the extracellular matrix (ECM) proteins are key hallmarks of MFs. Transient appearance of MFs is a feature of normal wound repair; however, when the MF life cycle is not regulated properly, absence of apoptosis leads to overproduction of ECM proteins deposition and pathological vascular remodeling [Das et al., 2001; Thannickal et al., 2003]. Moreover, these processes are accompanied by adventitial cells migration toward the lumen, thereby contributing to neointimal formation [Sartore et al., 2001; Siow et al., 2003]. Thus, uncontrolled cell proliferation and suppression of apoptosis are critical cellular events in vascular remodeling [Pollman et al., 1999; Das et al., 2001; Wernig and Xu, 2002]. Vascular AFs and MFs might be two potential target cells for anti-vascular remodeling therapeutics. However, little is known about the regulatory mechanisms controlling their proliferation and apoptosis.

As a new layer of gene regulation mechanism, MicroRNAs (miRNAs) have been implicated in a multitude of important cellular processes, including regulation of cellular proliferation, apoptosis, and differentiation [Trang et al., 2008; Chen et al., 2010]. Deregulation of miRNA expression has been associated with various disorders. Because cell apoptosis, growth, and differentiation are important cellular events in the development of cancer, most previous research into the role of miRNAs has focused on cancer. The role of miRNAs in the context of the cardiovascular system has only recently begun to be explored, and good progress has been made in the vascular area, but no studies have been performed in the adventitial area.

Numerous studies have demonstrated some characteristics of proliferative vascular disease are similar to those in cancer in cellular events and molecular pathways [Ross et al., 2001]. Because proliferation and apoptosis are two important cellular events in vascular remodeling and cancers, miRNAs may play important roles in vascular remodeling. Multiple studies have identified miR-21 overexpressed in a wide variety of cancer cell lines and causally linked to cancer-related processes such as proliferation and apoptosis [Meng et al., 2007; Frankel et al., 2008; Ali et al., 2010]. We recently found miR-21 expressed in AFs and overexpressed in MFs dedifferentiated from AFs after treatment with transforming growth factor β1 (TGF-β1). The objective of the current study was to determine whether miR-21 has a role in regulating proliferation and apoptosis of vascular AFs and MFs and subsequently affecting vascular remodeling.

MATERIALS AND METHODS

CELL CULTURE

AFs were isolated from thoracic aortas of 4- to 6-week-old male Wistar–Kyoto rats weighing 120–180 g and cultured by the tissue adherence method [An et al., 2007]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Gibco) and maintained in humidified atmosphere of 5% CO_2 at 37°C. AFs at 80–90% confluence were passaged with 0.125% trypsin-EDTA. Cells from passage three to six were used. All experimental protocols complied with the guidelines of the Committee on Care and Use of Laboratory Animals of Shandong University.

MYOFIBROBLAST MODEL

Subconfluent AFs were incubated with different concentrations of TGF-β1 (PeproTech EC, London, UK) in serum-free medium. The MF phenotype in TGF-β1–treated fibroblasts was identified by determining the expression of α-smooth muscle actin (α-SMA) by RT-PCR, western blot analysis, and fluorescent immunocytochemistry.

FLUORESCENT IMMUNOCYTOCHEMISTRY

Fibroblasts with or without TGF-β1 treatment were grown on glass coverslips, rinsed with phosphate buffered saline (PBS), fixed with 3% paraformaldehyde for 20 min, permeabilized with 0.5% Triton-X100 for 15 min and blocked with 2% bovine serum albumin (BSA) for 30 min. Cells were incubated overnight with the primary antibodies mouse anti-α-SMA (1:200) (Boster, Wuhan, China) and rabbit anti-vimentin (1:200) (Bios, Beijing). Immunofluorescent staining for a-SMA and vimentin involved incubation with FITC-conjugated anti-mouse IgG (1:100) and TRITC-conjugated anti-rabbit IgG (1:100) (Zhongshan Golden Bridge Biological Technology, Beijing), respectively. Cellular nuclei were stained with DAPI. Images were examined under a Zeiss laser scanning confocal microscope (Zeiss, Germany).

miRNA AND siRNA TRANSFECTION

Cells were transfected with pre-miR-21 and miR-21 inhibitor (details below) for miR-21 overexpression and knockdown, respectively. The samples were assayed after 48 h. Before large-scale transfection, the transfection condition was optimized with cells cultured in 6- or 96-well plates in Opti-MEM-I serum-reduced medium, negative control oligonucleotide with FAM moiety at the 5′ end, and Lipofectamine 2000 Reagent (Invitrogen) following the manufacturer's procedure. The transfection efficiency was determined by use of the negative control oligonucleotide with 6-FAM at the 5′ end. After optimization, transfection complexes were added to cells at a final oligonucleotide concentration of 50 nM. This study involved a vehicle control and pre-miR and miR inhibitor negative controls. All oligonucleotides, including pre-miR-21 (Cat#AM17100), miR-21 inhibitor (Cat#AM17000), and their respective negative controls (FAM™-labeled Pre-miR™ Negative Control [Cat#AM17121] and FAM™-labeled Anti-miR™ Negative Control [Cat#AM17012]) were purchased from Ambion. All transfections were carried out in triplicate.

For Programmed cell death 4 (PDCD4) knockdown, PDCD4 siRNA was chemically synthesized by GenePharma (Shanghai) and transfected into cells by use of Lipofectamine 2000 reagent (Invitrogen) as per the manufacturer's instructions. The final PDCD4 siRNA concentration was 80 nM.

REAL-TIME QUANTITATIVE RT-PCR

Total RNA was isolated from cultured cells 48 h post-transfection with use of the mirVanaTM miRNA Isolation Kit (CAT#AM1560, Ambion) or Trizol (Invitrogen) according to the manufacturer's protocol. cDNA synthesis involved carried out in a thermal cycler (Eppendorf, Hamburg, Germany). PCR reactions for miRNA and mRNA involved the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) and iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA), respectively. Real-time RT-PCR analyses of miRNA expression involved use of TaqMan miRNA assays (Applied Biosystems). Reverse transcription (RT) involved the Taqman microRNA RT Kit (Applied Biosystems) with an miR-21 stem-loop primer (Ambion). Total RNA of 10 ng was used in each reaction (15 μl) and was mixed with the RT primer (3 μl). The RT reaction conditions were 16°C for 30 min; 42°C for 30 min; 85°C for 5 min; and then held at 4°C. Real-time PCR involved the Taqman gene expression master mix (Applied Biosystems). The cDNA products were diluted at 150×, and 1.33 μl of the diluted cDNA was used for PCR reaction with TaqMan primers (2 μl). The PCR reaction conditions were 10 min at 95°C, then 40 cycles of 15 s at for 30 min, and 1 min at 60°C. RT-PCR analyses of the mRNA expression of α-SMA and β-actin involved PrimeScript® RT-PCR kits (TaKaRa Biotechnology, Dalian, China). The sequences of PCR primers were for α-SMA (accession no. NM_031004.2), forward: 5′-AGC CAG TCG CCA TCA GGA AC-3′; reverse 5′-CCG GAG CCA TTG TCA CAC AC-3′; and β-actin (accession no. NM_031144.2), forward: 5′-GTG TGA CGT TGA CAT -3′; reverse: 5′-ACA TCT GCT GGA AGG TG-3′. Analysis of relative miRNA and mRNA expression involved the ΔΔCT method, with U6 and β-actin used as endogenous controls, respectively, according to the manufacturer's guidelines. All RT-PCR tests were performed at least three times.

RAT ILIAC ARTERY BALLOON INJURY MODEL AND ADVENTITIAL OLIGO TRANSFER

Iliac artery balloon injury was performed according to Gabeler et al. [2002]. Briefly, Wistar-Kyoto rats (320–350 g) were generally anesthetized with sodium pentobarbital (50 mg/kg, IP). A 2F Fogarty catheter (Medtronic) was inserted to the right iliac artery. To produce iliac artery injury, the balloon was inflated to 3–4 atm and withdrawn three times. The left iliac arteries were not injured and served as controls. All experimental protocols complied with the guidelines of the Committee on Care and Use of Laboratory Animals of Shandong University.

Two hundred microlitres of 20% (w/v) F-127 pluronic gel (Sigma) containing 0.24 mg antagomir against miR-21 (miR-21 antagomir, RIBOBIO, Guangzhou, China) or PBS was applied to the adventitial around injured artery segments. Animals were sacrificed, at 1 day, 3 days, and 21 days after injury. Animals were divided into three groups: miR-21 antagomir group (treated with miR-21 antagomir dissolved in F-127 pluronic gel), vehicle group (treated with PBS dissolved in F-127 pluronic gel), and uninjured group.

PROLIFERATION ASSAY

Cell proliferation in vitro was determined by 5-ethynil-2′-deoxyuridine (EdU). After 48 h of transfection, cell proliferation was quantified by the uptake of 5-ethynil-2′-deoxyuridine (EdU) into DNA by use of a Cell-Light™ EdU DNA Cell Proliferation Kit (RIBOBIO, Guangzhou, China). 5-bromo-2′-deoxyuridine (BrdU) is used for identifying cells in DNA synthesis (S-phase) of the cell cycle; however, detection of BrdU is accomplished immunologically and requires harsh treatments to denature double-stranded DNA for the specific anti-BrdU antibody to bind BrdU. Recently, EdU, an alternative to BrdU, was developed by replacing the methyl group at position 5 of deoxyuridine with an alkyne group. In contrast to BrdU incorporation, EdU can be directly conjugated to fluorescent azide, and the assay does not require DNA denaturation and permits good structural preservation. All procedures for EdU incorporation experiments followed the kit's protocol [Salic and Mitchison, 2008; Limsirichaikul et al., 2009]. Briefly, cells were treated in 96-well plates. Before fixation, cells were incubated with EdU for 2 h, permeabilized in 1.0% Triton-X100 for 25 min, and underwent EdU staining. Cell nuclei were stained with Hoechst 33342 for 10 min. The proportion of nucleated cells incorporating EdU was determined by fluorescence microscopy. The assay was performed in triplicate and repeated three times. Cell proliferation rate was calculated as the percentage of EdU-positive nuclei to total nuclei in five high-power fields per well.

To detect AF and MF proliferation in vivo, BrdU (30 mg/kg, IP; Sigma) dissolved in PBS, was injected 12 and 24 h before euthanasia. BrdU-incorporating nuclei were detected with a specific anti-BrdU antibody (1:50, Boster, Wuhan, China) according to the manufacturer's instructions. Cell proliferation rate was calculated as the percentage of BrdU-positive nuclei to total nuclei in five high-power fields per each section. Measurements were performed on five sections per animal.

APOPTOSIS ASSAY

The level of apoptosis in cultured cells was evaluated after 48 h of cell transfection by flow cytometry (FACS) (FACSCalibur flow cytometer, Becton Dickinson, San Jose, CA) of annexin-V-stained cells with use of the phycoerythrin (PE) Annexin V Apoptosis Detection Kit I (BD Biosciences) and measurement of caspase-3 activity by use of the Caspase-3 Colorimetric Assay Kit (Keygen, Nanjing, China). For FACS analysis, the detached cells were pooled with the trypsinized adherent cells, resuspended in binding buffer and incubated with PE-conjugated annexin V and 7-amino-actinomycin (7-AAD) for 15 min at room temperature in the dark. A total of 10,000 events were acquired and analyzed by flow cytometry. Data were analyzed by use of CellQuest software (Becton Dickinson). For detection of caspase-3 activity, cells were plated in triplicate in a 96-well plate and transfected with oligos as described previously. The protein concentration was determined by use of the Enhanced BCA Protein Assay Kit (Beyotime, Shanghai) by the Lowry method. Caspase-3 activity for all samples was normalized to that of an equal protein amount and measured by use of a multiplate reader (Thermo Scientific Varioskan Flash). Relative luminescence intensity was presented as fold change as compared with untreated controls.

Detection of apoptotic cells in vivo was performed by the TUNEL procedure with the In Situ Cell Death Detection Kit (Roche). Cell apoptotic rate was calculated as the percentage of TUNEL-positive

nuclei to total nuclei in five high-power fields per well section. Measurements were performed on five sections per animal.

IMMUNOCYTOCHEMISTRY

To detect MF differentiation in vivo, immunohistochemistry was performed in paraffin-embedded vessel sections. The mouse anti-α-SMA (1:50, Boster, Wuhan, China) was used. Immunostaining was detected using a SABC immunohistochemistry kit (Jingmei Biotech, Beijing). The secondary antibody was the anti-mouse IgG coupled with horseradish peroxidase. Counterstaining was performed with hematoxylin. Measurements were performed on five sections per animal.

WESTERN BLOT ANALYSIS

After transfection for 48 h, cells were harvested with lysis buffer. Lysates were collected, and protein concentration was determined by use of the Enhanced BCA Protein Assay Kit (Beyotime, Shanghai). Protein samples were separated on 10% SDS–PAGE gels, transferred to polyvinylidene fluoride membrane (Millipore, MA), and blocked with 5% nonfat milk/TBST for 2 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies. Membranes washed with TBST three times, were incubated with secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) (Millipore), and density of bands was quantified with use of Quantity One image analysis software (Bio-Rad). Antibodies against PDCD4, JNK, phospho-JNK (Thr183/Tyr185) (p-JNK), PTEN, AKT, and phospho-AKT (Ser473) (p-AKT) were from Cell Signalling Technology (1:1,000, Danvers, MA); antibodies against c-Jun and phospho-c-Jun (Ser63) (p-c-Jun) were from Bioworld Technology (1:500, USA); antibodies against α-SMA was from Boster Technology (1:500, Wuhan, China). Antibody against β-actin (1:500; Santa Cruz Biotechnology) was used as a loading standard. Secondary antibodies were horseradish peroxidase-conjugated sheep anti-mouse IgG and goat anti-rabbit IgG (1:10,000; Santa Cruz Biotechnology). β-actin was used to normalize the western blot bands. For target proteins with a similar molecular weight to β-actin, we duplicated the sample set in either the same gel or in two gels that underwent electrophoresis in the same tank. After transfer, the two identical blots were randomly selected for either β-actin or target protein detection. Equal protein loading for different lanes was achieved by quantifying protein concentrations of the samples.

DUAL-LUCIFERASE REPORTER ASSAY

A construct of a fragment of the 3′ untranslated region (UTR) of PDCD4 mRNA containing the putative miR-21 binding sequence cloned into pMIR-REPORT Luciferase vector (a firefly luciferase reporter vector) (GeneChem Co. Ltd, Shanghai) was co-transfected (800 ng/well) with vehicle control, pre-miR-21 (50 nM), and pre-miR-21 negative control (50 nM) into 293T cells in 24-well plates by use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The vector without the putative miR-21 binding sequence was the negative control. The samples were co-transfected with pRL-TK plasmid expressing Renilla luciferase to determine the transfection efficiency. At 24 h after transfection, firefly and renilla luciferase activities were measured by use of the dual-luciferase reporter assay system (Promega, Madison, WI) on a luminometer.

MORPHOMETRIC ANALYSIS

Vascular remodeling was evaluated using morphometric analysis. Neointimal formation is expressed as the neointimal to medial area ratio (N/M). Morphometric analysis of N/M and lumen size was performed in sections stained with hematoxylin–eosin (H–E) using the Image Pro Plus (Media Cybernetics) computer software. Measurements were performed on five sections per animal.

STATISTICAL ANALYSIS

All data are presented as mean ± SEM from at least three independent experiments. Differences between two groups were analyzed by Student's *t*-test and between multiple groups by one-way ANOVA. Data analysis involved use of GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

DIFFERENTIATION OF AFs TO MFs

The expression of α-SMA, a key marker of MF differentiation, is low in AFs. TGF-β1 is well known to induce differentiation of fibroblasts to MFs by stimulating α-SMA expression both in vitro and in vivo [Thannickal et al., 2003; Michel et al., 2007]. Therefore, we established the cell model of MFs by inducing AF phenotypic transition with TGF-β1 treatment. In cultured AFs, TGF-β1 induced a time- and dose-dependent steady increase in α-SMA mRNA expression (Fig. 1A). Immunoblotting revealed α-SMA protein expression significantly increased with TGF-β1 treatment (Fig. 1B). AFs without TGF-β1 treatment were uniformly positive for vimentin (Fig. 1C) and negative stained for α-SMA (Fig. 1D). After TGF-β1 treatment (10 ng/ml, 24 h), more than 90% of the total cells per high-power field were positive for α-SMA (Fig. 1E, left) and formed numerous actin stress fibers (Fig. 1E, right), characteristic of MFs. Double immunofluorescent staining revealed that AFs with TGF-β1 treatment co-expressed vimentin and α-SMA (Fig. 1F). Therefore, in all subsequent experiments, we incubated fibroblasts with TGF-β1 (10 ng/ml) for 24 h.

MIR-21 COULD BE MODULATED BOTH IN AFs AND MFs

We used real-time RT-PCR to detect and quantify miRNAs expression of miR-21. MiR-21 was found endogenously expressed in AFs and markedly increased in MFs: The expression of miR-21 expression was about 4.8 times higher in MFs than in AFs ($P < 0.01$) (Supplementary Fig. 1A). We used TaqMan probes that will fluoresce only with amplified mRNA expression; therefore, the data represent true miR-21 expression and not background noise.

To determine whether the expression of miR-21 in AFs and MFs was regulated, we used both gain- and loss-of-function approaches. Negative control oligos labeled with FAM were successfully transfected into AFs and MFs (Supplementary Fig. 1B). The transfection efficiency was approximately 90%. As expected, the ectopic expression of miR-21 was confirmed by real-time quantitative RT-PCR. Pre-miR-21 significantly increased but

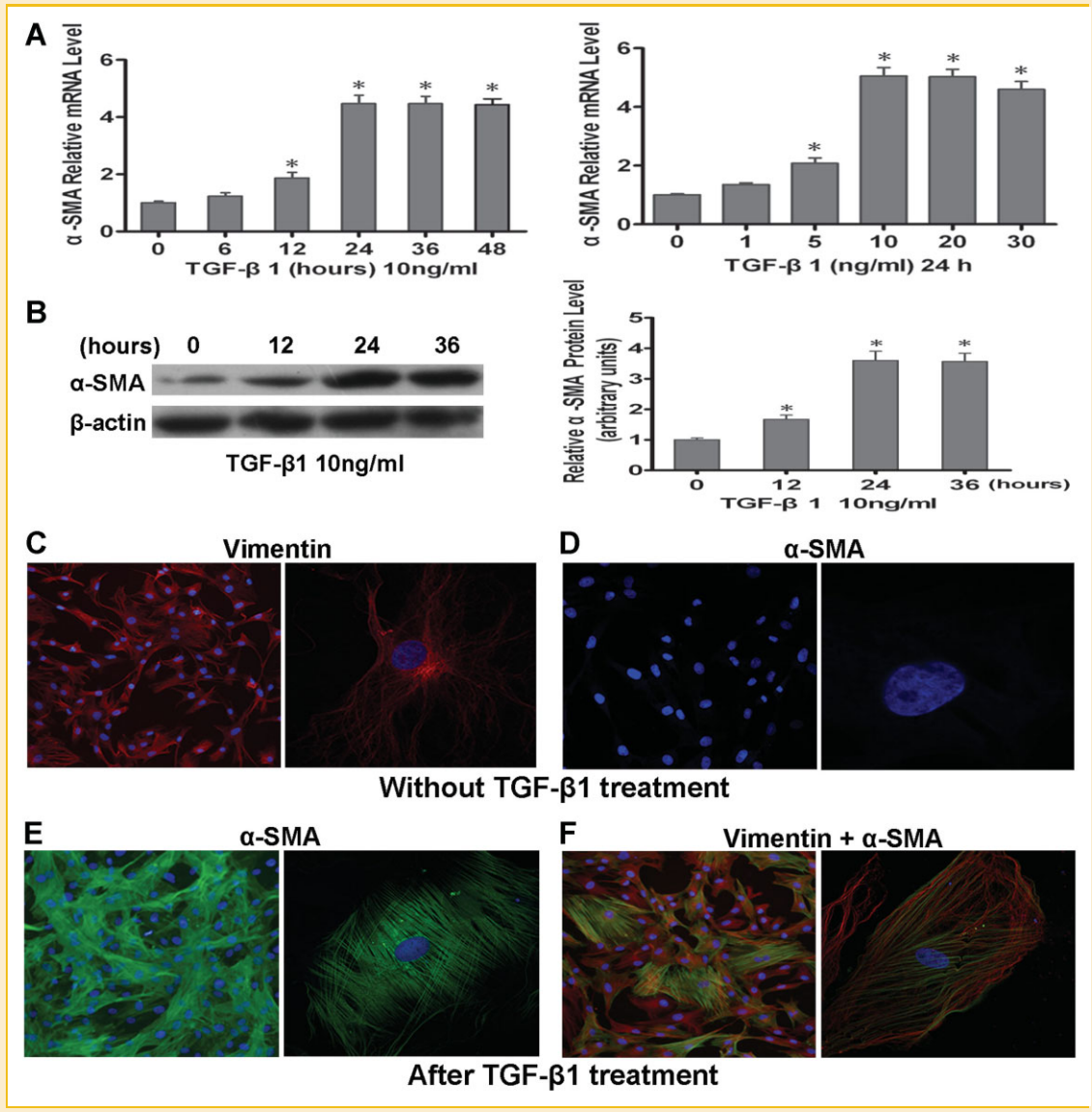

Fig. 1. Transforming growth factor β1 (TGF-β1) induced differentiation from adventitial fibroblasts (AFs) to myofibroblasts (MFs). A: TGF-β1 induced a time- and dose-dependent increase in α-smooth muscle actin (α-SMA) mRNA expression. (*$P < 0.05$ vs. control, n = 3). B: Immunoblotting analysis of α-SMA protein expression with TGF-β1 treatment. For loading control, β-actin was used. A representative figure is included. Because of the size of the β-actin band being close to that of the test band, duplicate gels were run and one hybridized for β-actin, while the other was probed for α-SMA protein. (*$P < 0.05$ vs. AF [time 0], n = 3). C–F: MF phenotype revealed by a-SMA staining on laser scanning confocal microscopy. AFs without TGF-β1 treatment were uniformly positive for vimentin (C) and negative stained for a-SMA (D). After TGF-β1 treatment (10 ng/ml, 24 h), AFs were positive for a-SMA (E, left) and formed numerous actin stress fibers (E, right). Double labeling of AFs with TGF-β1 treatment showed co-expression of vimentin and a-SMA (F). Vimentin (red), α-SMA (green). Nuclei were visualized by DAPI staining (blue).

miR-21 inhibition decreased miR-21 expression after 48 h in AFs and MFs ($P < 0.05$) (Supplementary Fig. 1C, D). Control oligos had no effect on miR-21 expression.

MIR-21 PROMOTED PROLIFERATION OF AFs AND MFs

To determine the role of miR-21 in the proliferation of AFs and MFs, EdU incorporation assay was used. EdU-stained photomicrographs and corresponding photomicrographs of total cells are in Figure 2A. The proportion of cells with EdU-positive nuclei was determined. MiR-21 inhibitor decreased EdU incorporation while pre-miR-21 significantly increased EdU incorporation in AFs ($P < 0.05$), which indicated miR-21 promotes the proliferation of AFs (Fig. 2A,B). Moreover, miR-21 also promotes the proliferation of MFs (Fig. 2C). In contrast, the vehicle control and pre-miR and miR-inhibitor negative controls had no effect on cell proliferation in each type of cell. Furthermore, MFs showed higher proliferation than did AFs both at basal condition (34.1 vs. 23.8%, $P < 0.05$) and with pre-miR-21 treatment (42.7 vs. 33.4%, $P < 0.05$) (Fig. 2B,C). These results demonstrated that the expression of miR-21 was upregulated in proliferating AFs.

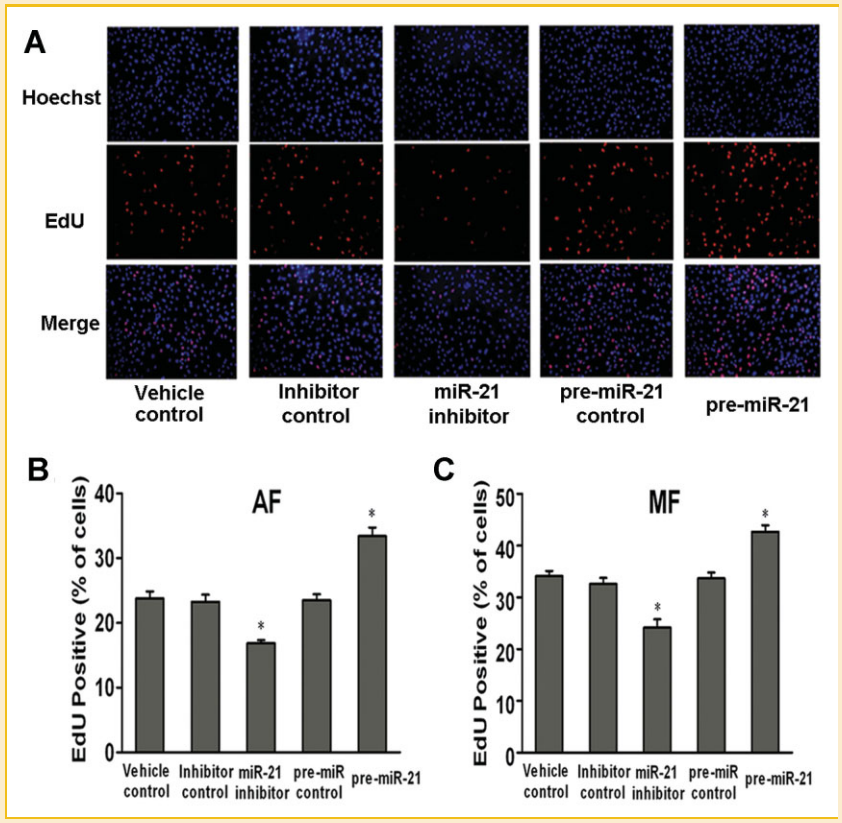

Fig. 2. Effect of miR-21 overexpression and miR-21 inhibition on proliferation of MFs and AFs. A: Representative photomicrographs of 5-ethynil-2′-deoxyuridine (EdU) staining (middle panel) and corresponding total cell photomicrographs (top panel). Blue, Hoechst 33342 labeling of cell nuclei; red, EdU labeling of nuclei of proliferative cells. B,C: Effect of miR-21 on AFs and MFs proliferation as determined by EdU staining. Quantitative data showing percentage of EdU-positive cells (number of red vs. blue nuclei). ($^*P < 0.05$ vs. corresponding vehicle control).

MIR-21 DECREASED APOPTOSIS OF AFs AND MFs

We further evaluated the effect of miR-21 on the apoptosis of AFs and MFs by use of FACS analysis of annexin-V-stained cells. Transfection with the miR-21 inhibitor increased the percentage of annexin V-positive cells by threefold in AFs and by 3.5-fold in MFs as compared with the vehicle control and miR-inhibitor negative control (Fig. 3A,C). However, pre-miR-21 decreased the apoptosis of AFs and MFs induced by serum deprivation for 24 h (Fig. 3B,C) Thus, miR-21 had an anti-apoptotic effect in cultured AFs and MFs.

Caspase-3 is known to play a key role in apoptosis. To determine whether caspase-3 was involved in the process of anti-apoptosis of miR-21, caspase-3 activity was detected. MiR-21 inhibitor increased caspase-3 enzymatic activities about 1.8-fold both in AFs and MFs compared with vehicle control or the miR-inhibitor control. In contrast, caspase-3 enzymatic activities were reduced by 40% in cells treated with pre-miR-21 compared with pre-miR control both in AFs and MFs (Fig. 3D). Thus, the anti-apoptosis effect of miR-21 was via a caspase-3 dependent pathway.

PDCD4 IS A DIRECT TARGET OF miR-21 AND DIRECTLY PARTICIPATES IN miR-21-MEDIATED PROLIFERATION AND APOPTOSIS IN AFs AND MFs

Bioinformatics analysis predicted PDCD4 to have miR-21 binding sites in the 3'-UTR and to be a potential target gene of miR-21 (Fig. 4A). Previous reports showed that miR-21 targeted PDCD4 and decreased its expression in several cancer cells [Asangani et al., 2008; Frankel et al., 2008]. However, whether PDCD4 is a target of miR-21 in AFs and MFs was unknown. If a target of miR-21, PDCD4 should express lower in MFs than in AFs because miR-21 expression was upregulated in MFs. As expected, western blot analysis revealed PDCD4 protein level significantly lower in MFs than in AFs (Fig. 4B). To confirm that miR-21 can directly bind to PDCD4, we cloned a construct with a fragment of the 3′-UTR of PDCD4 mRNA with the putative miR-21 binding sequence into a firefly luciferase reporter vector for co-transfection with vehicle control, pre-miR-21, and pre-miR-21 negative controls into 293T cells. As expected, pre-miR-21 significantly inhibited luciferase activity as compared with control treatments (Fig. 4C). Therefore, miR-21 could directly bind to PDCD4. To further confirm PDCD4 as a target gene of miR-21, we used gain- and loss-of-function approaches. The miR-21 inhibitor increased but pre-miR-21 decreased PDCD4 protein level in AFs and MFs (Fig. 4D). Therefore, PDCD4 is a direct target gene of miR-21 in AFs and MFs.

Because miR-21 regulates proliferation and apoptosis in AFs and MFs and PDCD4 was found a direct target of miR-21, we wondered whether PDCD4 directly participates in miR-21-mediated cellular effects in AFs and MFs. First, we knocked down PDCD4 protein by siRNA in AFs or MFs, then we studied the effect of PDCD4 siRNA on

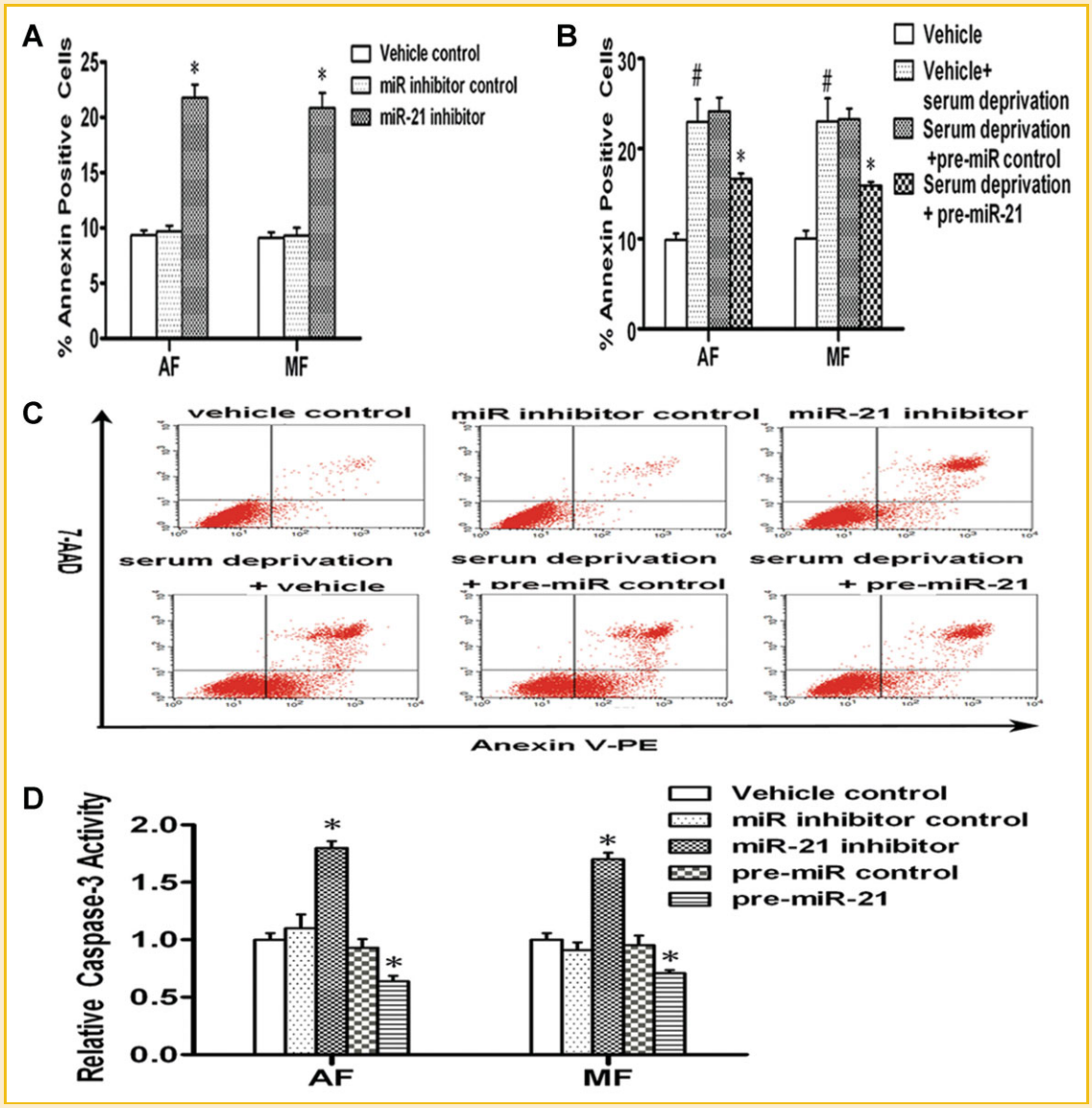

Fig. 3. Cell apoptosis in response to miR-21 gain- and loss-of-functions was determined by FACS analysis of annexin-V-stained cells and caspase-3 activity. A: Effect of miR-21 inhibition on apoptosis of MFs and AFs. ($^{*}P < 0.05$ vs. vehicle, n = 4). B: Effect of miR-21 overexpression with pre-miR-21 on apoptosis of MFs and AFs. ($^{*}P < 0.05$ vs. corresponding vehicle, $^{\#}P < 0.05$ vs. corresponding vehicle plus serum deprivation, n = 4). C: Representative flow cytometry results of apoptosis. Bottom left quadrant, PE-annexin V- and 7-amino-actinomycin (7-AAD-) (viable) cells; bottom right quadrant, PE-annexin V+ and 7-AAD- (early apoptotic) cells; top right quadrant, PE-annexin V+ and 7-AAD + (late apoptotic and necrotic) cells. D: Cell apoptosis was determined by caspase-3 activity. ($^{*}P < 0.05$ vs. vehicle control, n = 3).

regulating miR-21 by EdU cell proliferation and caspase-3 apoptosis assays. PDCD4 siRNA decreased PDCD4 protein level significantly (Supplementary Fig. 2). Knockdown of PDCD4 by siRNA increased proliferation as determined by EDU proliferation assay (Fig. 5A,B) and decreased apoptosis as determined by caspase-3 activity (Fig. 5C). Importantly, PDCD4 siRNA partly rescued the reduced cellular proliferation with miR-21 inhibition in AFs and alleviated the increase in apoptosis with mir-21 inhibition to a certain degree (Fig. 5B,C). According with above result, in the molecular level, the relative expression of PDCD4 in these different treatment groups was shown in Figure 5D. Similar results were found in MFs (Fig. 5B–D). The results suggest an essential role for PDCD4 as a mediator of the biological effects of miR-21 in AFs and MFs.

Recent study showed that miR-21 targeted Fas Ligand (FasL) and enhanced AKT phosphorylation in cardiac myocytes [Sayed et al., 2010]. However, we did not detect FasL expression, and miR-21 inhibition was not sufficient to induce its expression in vascular AFs, probably there might be cell-type dependent for FasL expression. However, in cultured vascular AFs, miR-21 could also increase AKT phosphorylation which might be attributed to the down-regulation of PTEN, a direct target of miR-21 and negative regulator of AKT, by pre-miR-21 (Supplementary Fig. 3).

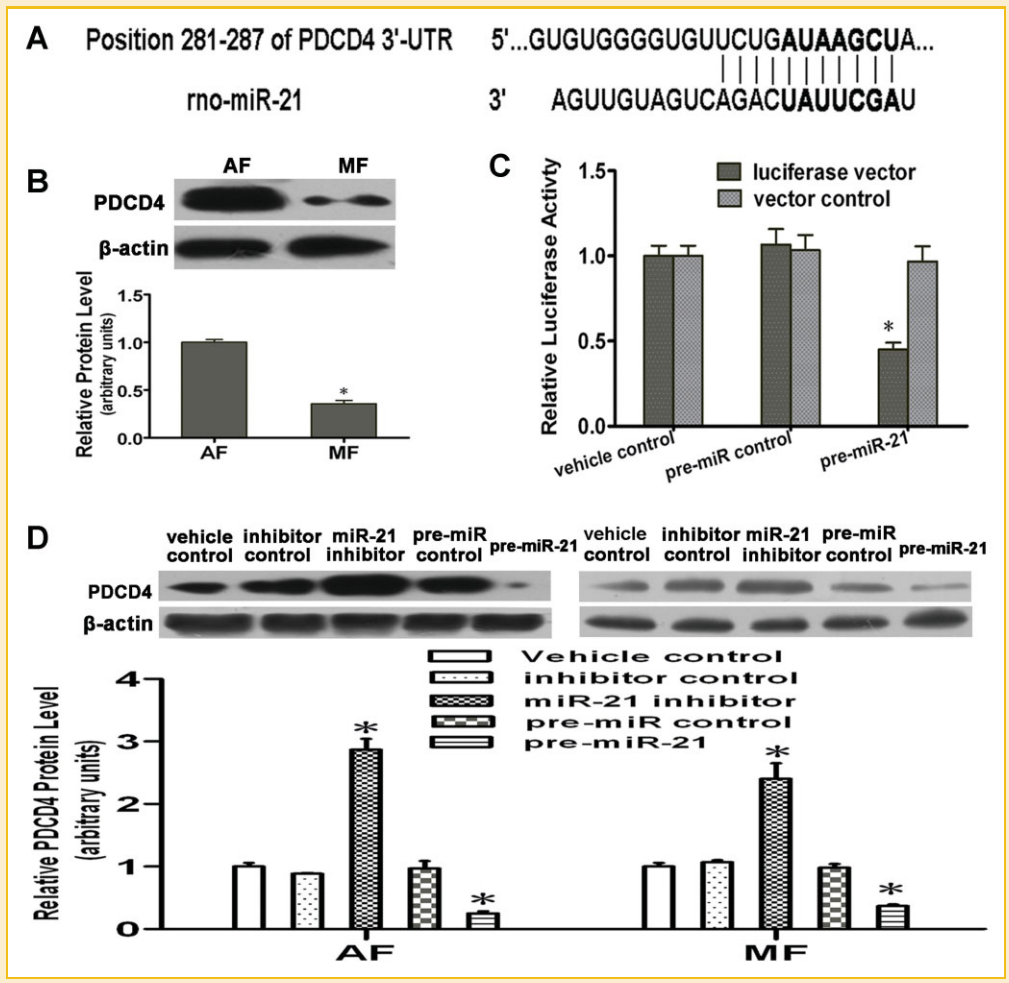

Fig. 4. Programmed cell death 4 (PDCD4) is a direct target gene of miR-21 in AFs and MFs. A: Predicted miR-21 binding sites within the 3′-UTR of PDCD4 mRNA. B: Western blot analysis of PDCD4 in MFs and AFs. (*$P < 0.05$ vs. AF, n = 3). C: Validation of predicted binding sites by luciferase reporter assay. 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 293T cells, with vehicle control and pre-miR-21 and pre-miR negative controls. Firefly luciferase activity was normalized to Renilla luciferase expression. (*$P < 0.05$ vs. corresponding vehicle control, n = 4). D: miR-21 inhibition increased and pre-miR-21 decreased PDCD4 expression in AFs and MFs. (*$P < 0.05$ vs. vehicle control, n = 3).

JNK/c-Jun IS A DOWNSTREAM SIGNALING PATHWAY OF PDCD4 IN THE miR-21-MEDIATED CELLULAR EFFECT IN AFs AND MFs

Although PDCD4 has an important role in AF and MF proliferation and apoptosis, we wondered how PDCD4 exerts its function. We found that knockdown of PDCD4 by siRNA significantly increased the JNK/c-Jun activity, the protein levels of phosphorylation of JNK (p-JNK) and phosphorylation of c-Jun (p-c-Jun) about twofold as compared with control siRNA, whereas the levels of bound JNK protein and c-Jun were similar (Supplementary Fig. 4A). JNK/c-Jun is a key signaling pathway that determines cell life or death [Sabapathy et al., 2001; Bogoyevitch et al., 2010]. We wondered whether c-Jun is a downstream signaling molecule of PDCD4 involved in the miR-21-medaited effect on AFs and MFs. The phosphorylation of JNK was relatively lower in AFs than in MFs (Supplementary Fig. 4B). The level of miR-21 was higher in MFs than AFs (Supplementary Fig. 1A) and the expression of PDCD4 was lower in MFs than AFs (Fig. 4B), JNK/c-Jun may be a downstream molecule of PDCD4 involved in the miR-21-mediated effect on AFs and MFs. To confirm our assumptions, increasing PDCD4 expression by miR-21 inhibition decreased JNK/c-Jun activity. MiR-21 inhibitor increased PDCD4 expression in AFs and MFs about 2–3-folds, while decreased the level of p-JNK and p-c-Jun by 55% and 47–70%, respectively (Fig. 6A). In contrast, decreasing PDCD4 expression via pre-miR-21 increased JNK/c-Jun activity. Pre-miR-21 decreased PDCD4 expression by 55–72%, while increased the level of p-JNK and p-c-Jun about twofolds (Fig. 6B). To further determine whether the decreased JNK/c-Jun activity was consequent to the increased PDCD4 expression by miR-21 inhibition, we measured JNK/c-Jun activity with miR-21 inhibition along with PDCD4 siRNA. The effect of miR-21 inhibition on JNK/c-Jun activity could be rescued in part by PDCD4 siRNA treatment (Fig. 6A). Therefore, PDCD4/JNK/c-Jun may be a downstream signaling pathway in the miR-21-mediated effect on AFs and MFs.

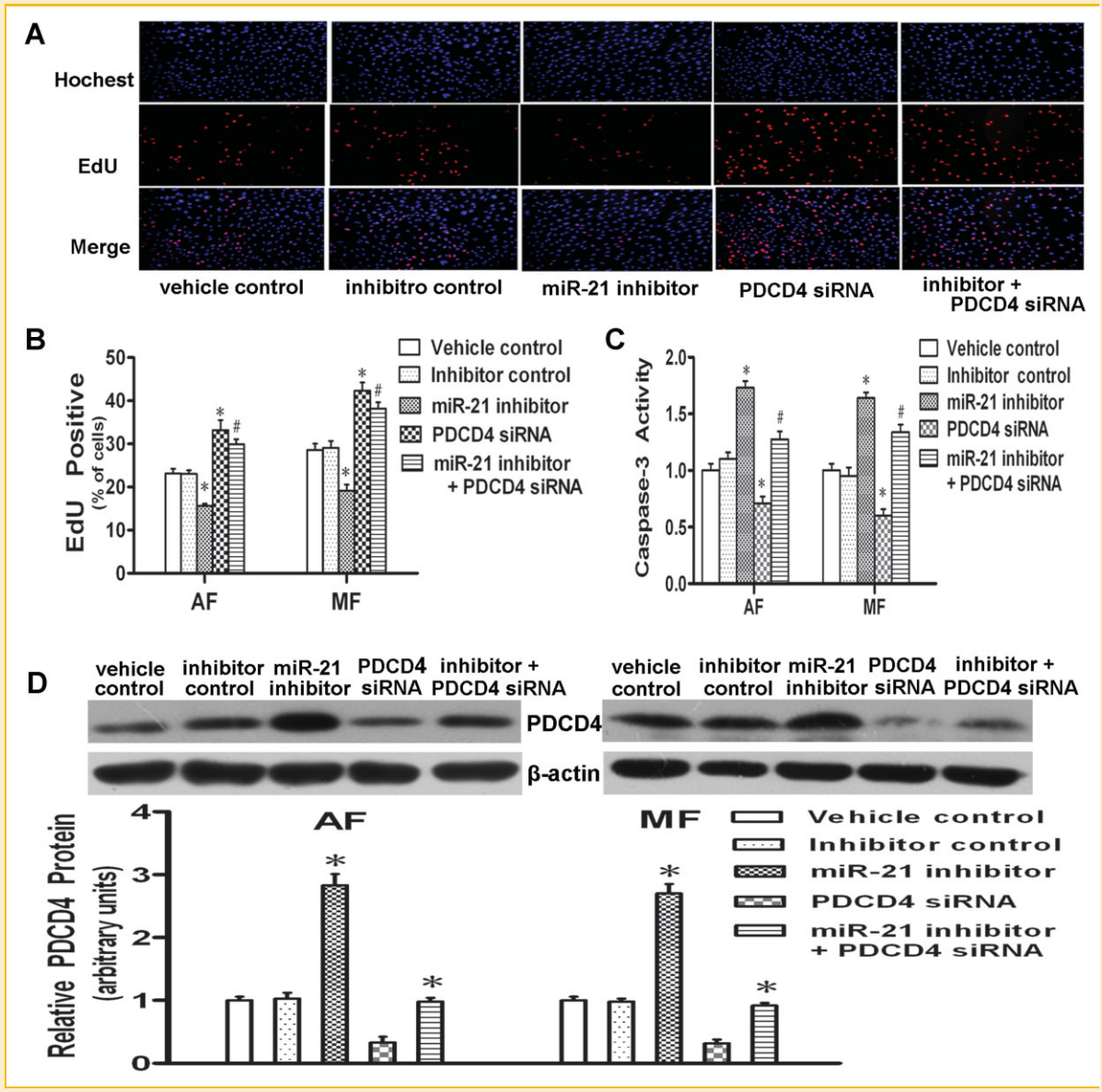

Fig. 5. PDCD4 directly participates in miR-21-mediated proliferation and apoptosis in AFs and MFs. A: Representative photomicrographs of 5-ethynil-2′-deoxyuridine (EdU) staining (middle panel) and corresponding total cell photomicrographs (top panel). B: Effect of knockdown of PDCD4 by siRNA on proliferation as determined by EdU proliferation assay. ($^{*}P < 0.05$ vs. corresponding vehicle, $^{\#}P < 0.05$ vs. miR-21 inhibition, n = 4). C Effect of knockdown of PDCD4 by siRNA on apoptosis as determined by caspase-3 activity. ($^{*}P < 0.05$ vs. corresponding vehicle, $^{\#}P < 0.05$ vs. miR-21 inhibition, n = 4). D: The relative expression of PDCD4 in different treatment groups. ($^{*}P < 0.05$ vs. corresponding vehicle, $^{\#}P < 0.05$ vs. miR-21 inhibition, n = 4).

ANTAGONIST OF miR-21 REGULATED PROLIFERATION AND APOPTOSIS OF AFs AND MFs IN INJURED ARTERY

To further confirm the cellular effects of miR-21, we determined the cellular proliferation and apoptosis in the adventitia. Three days after balloon injury, α-SMA positive cells were visible in the adventitia, which indicated some AFs differentiation into MFs (Supplementary Fig. 5). Three days after injury, the adventitia cells in the injured arteries treated with vehicle showed higher proliferation rate compared with the uninjured arteries; however, treatment with miR-21 antagomir led to a significant reduction in proliferation rate compared with vehicle group (30.2 vs. 50.8%, $P < 0.05$) (Fig. 7A,C). For apoptosis assay, rare positive cells were detectable in the adventitia from uninjured vessels. At 1 day after injury, many apoptotic cells could be seen in the adventitia from vehicle group. At 3 days after injury, numbers of apoptotic cells in adventitia from vehicle group were significantly reduced and showed no significant differences compared with uninjured group. However, the apoptosis level were significantly increased in miR-21 antagomir group compared with the uninjured group (34.3 vs. 9.2%, $P < 0.05$) and vehicle group (3 days after injury) (34.3 vs. 11.0%, $P < 0.05$) (Fig. 7B,D).

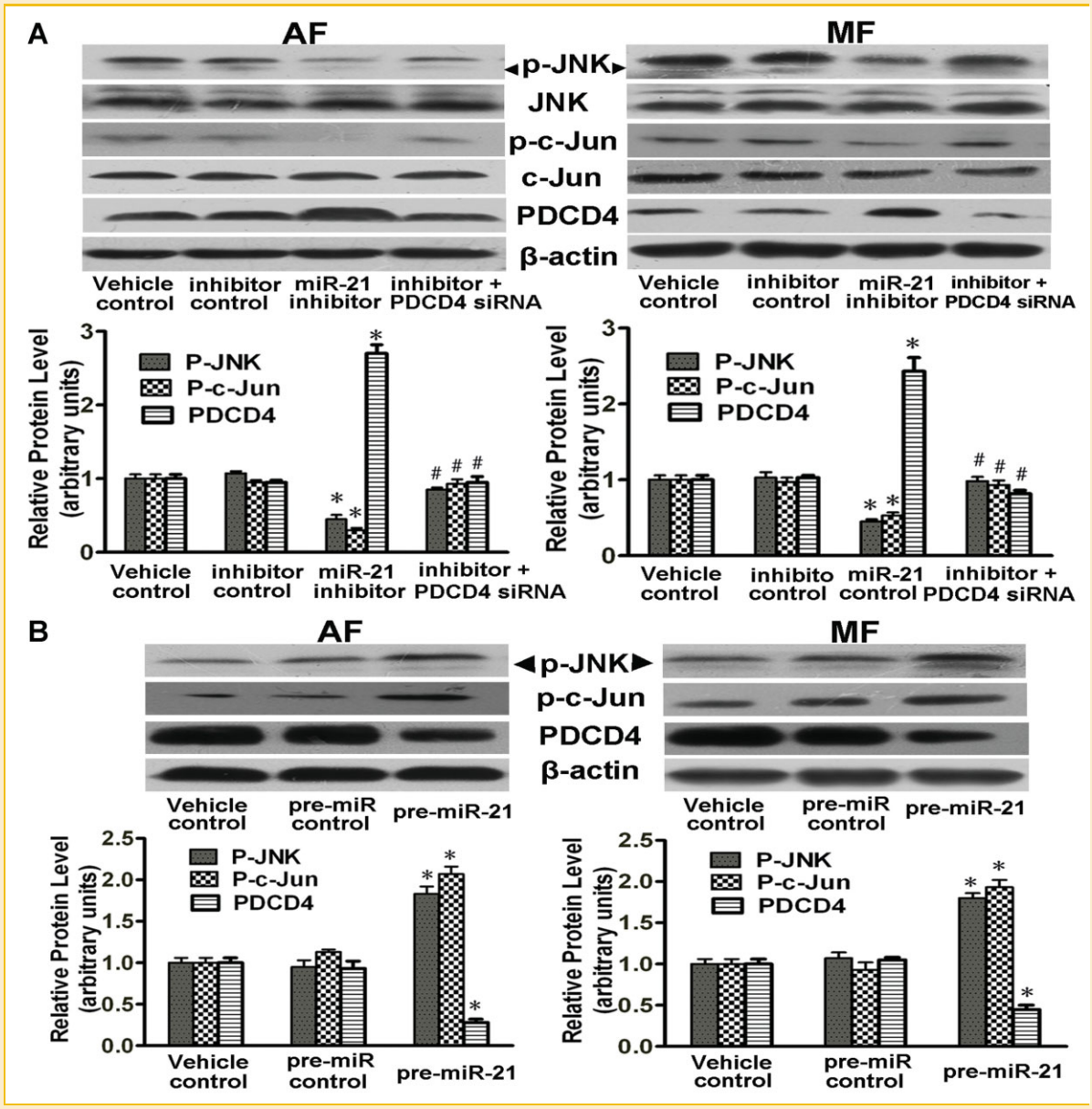

Fig. 6. JNK/c-Jun is a downstream signaling pathway of PDCD4 involved in miR-21-mediated effect on AFs and MFs. A: Effect of alteration of PDCD4 expression with miR-21 inhibition and PDCD4 siRNA on JNK/c-Jun activity. ($^{*}P < 0.05$ vs. corresponding vehicle, $^{\#}P < 0.05$ vs. miR-21 inhibition, n = 4). B: Effect of alteration of PDCD4 expression with pre-miR-21 on JNK/c-Jun activity. ($^{*}P < 0.05$ vs. corresponding vehicle). For loading control, β-actin was used. A representative figure is included. Because of the size of the β-actin band being close to that of the test band, duplicate gels were run and one hybridized for β-actin, while the other was probed for c-Jun and p-c-Jun protein.

ANTAGONIST OF miR-21 IMPROVED VASCULAR REMODELING INDUCED BY BALLOON INJURY

To determine the effect of miR-21 inhibition on vascular remodeling, morphometric analyses were carried out on the 21 days balloon-injured arteries. The injured arteries treated with vehicle showed extensive neointimal formation (Fig. 8A) and dramatic reduction in lumen size compared with the uninjured arteries (Fig. 8A,C). N/M of the miR-21 antagomir group was significantly lower than that of the vehicle group ($P < 0.05$), while lumen size of the miR-21 antagomir group was significantly larger than that of the vehicle group ($P < 0.05$) (Fig. 8).

DISCUSSION

In the present study, we investigated the function of miR-21 in regulating the proliferation and apoptosis of vascular AFs and MFs in vitro and in vivo. MiR-21 was expressed in AFs and significantly upregulated in MFs. Inhibition of miR-21 decreased the proliferation and increased apoptosis of AFs and MFs, while overexpression of miR-21 increased the proliferation and reduced the apoptosis of AFs and MFs. PDCD4 knockdown partly rescued the reduced proliferation, and alleviated the increased apoptosis induced by miR-21 inhibition. Moreover, miR-21 could affect the activity of JNK by

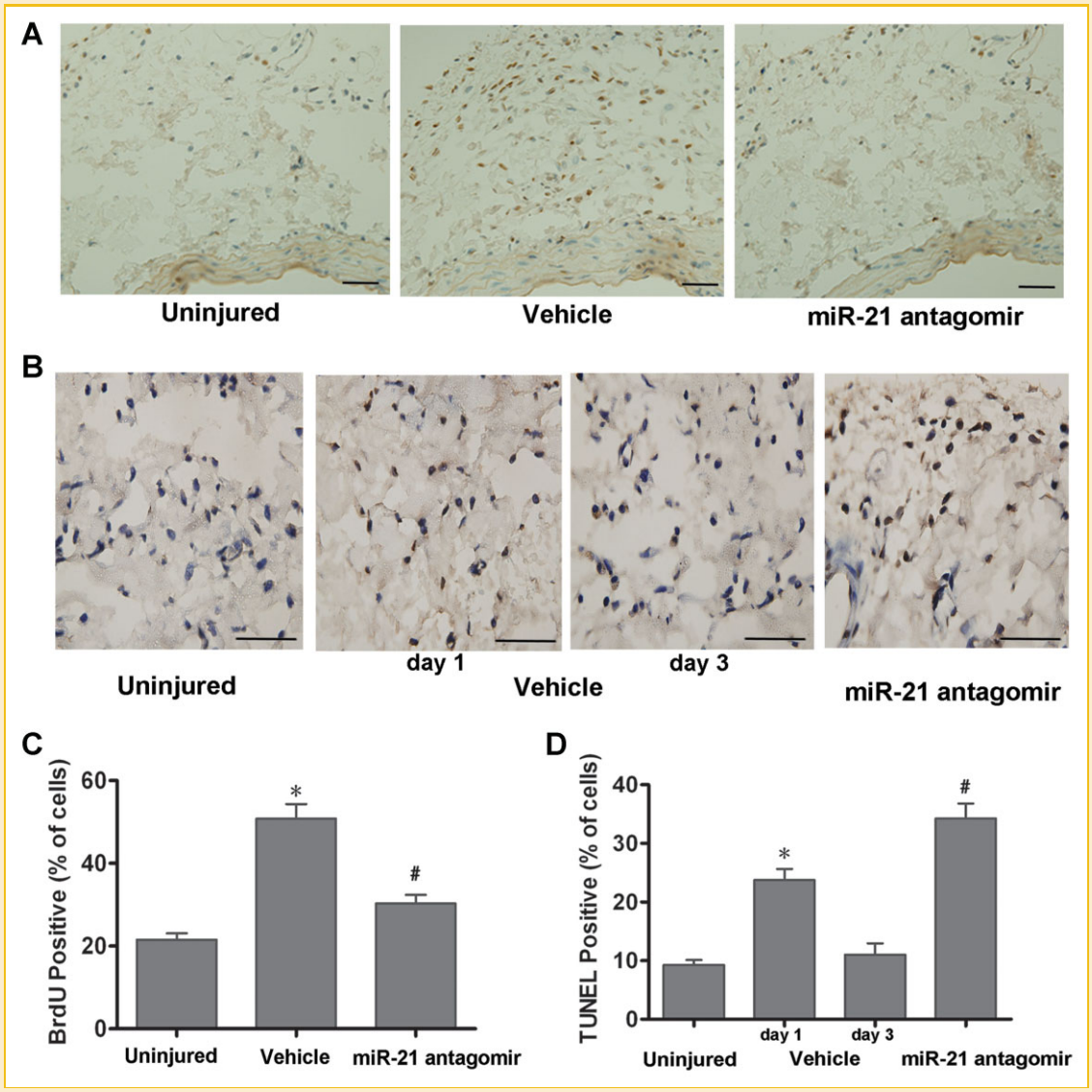

Fig. 7. Antagonist of miR-21 regulated proliferation and apoptosis of AFs and MFs in injured artery. A: BrdU stainning of uninjured, vehicle-treated and miR-21 antagomir-treated groups. BrdU-positive cells were visualized as brown color staining. (Scale bars: 50 μm.) (B) TUNEL stainning of uninjured, vehicle-treated and miR-21 antagomir-treated groups. TUNEL-positive cells were stained black-brown. (Scale bars: 50 μm). C: Quantification analysis of (A). ($^{*}P < 0.05$ vs. uninjured group, $^{\#}P < 0.05$ vs. vehicle group, n = 5). D: Quantification of (B). ($^{*}P < 0.05$ vs. uninjured group or vehicle group on day 3, $^{\#}P < 0.05$ vs. uninjured group or vehicle group on day 3, n = 5).

regulating PDCD4 expression. Furthermore, PDCD4 knockdown could rescue the miR-21 inhibition effect on JNK activity. Thus, PDCD4 is a functional target gene of miR-21 and PDCD4/JNK/c-Jun may be a downstream signaling pathway in the miR-21-mediated effect on AFs and MFs. Moreover, miR-21 inhibition suppressed neointimal formation by regulating the proliferation and apoptosis of vascular AFs and MFs. Dysregulation of miR-21 may alter targets involved in cell life and their downstream molecules, then disrupt the balance in cell proliferation and apoptosis, thus leading to suppressed apoptosis, unrestricted cell proliferation, neointimal formation, and ultimately vascular remodeling (Supplementary Fig. 6). In this study, we provide the first evidence that miR-21 inhibition improves vascular remodeling induced by arterial injury, at least in part, by regulating the proliferation and apoptosis of vascular AFs and MFs.

MiR-21 was reported to be strongly overexpressed in a wide variety of cancer cell lines and has been causally related to cellular proliferation and apoptosis [Frankel et al., 2008; Krichevsky and Gabriely, 2009; Ali et al., 2010]. Several studies have revealed miR-21 aberrantly expressed in many types of cardiovascular diseases such as in balloon injury of vascular walls and myocardial disease [Ji et al., 2007; Thum et al., 2008; Cheng et al., 2010]. miR-21 was found strongly overexpressed in vascular walls after balloon angioplasty of the rat carotid artery and plays a role in regulating proliferation and apoptosis of vascular smooth muscle cells (VSMCS) [Ji et al., 2007; Lin et al., 2009]. We found that expression of miR-21 was significantly upregulated in MFs. So miR-21 is usually upregulated in many pathophysiological processes in the vascular cells. Moreover, our study reveals that miR-21 plays a critical role in regulating proliferation and apoptosis

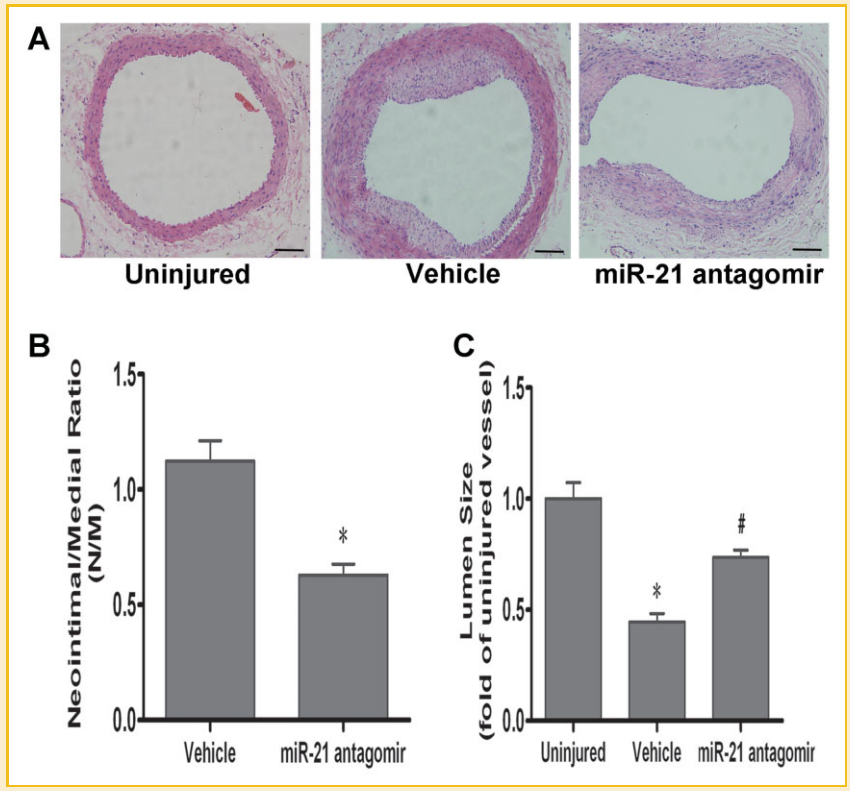

Fig. 8. Antagonist of miR-21 improved vascular remodeling induced by balloon injury. A: HE staining of uninjured, vehicle-treated and miR-21 antagomir-treated groups. (Scale bars: 100 μm). B: Neointimal to medial area ratio (N/M) of vehicle-treated and miR-21 antagomir-treated groups ($^{*}P < 0.05$ vs. vehicle group, n = 5). C: Lumen size quantification analysis of uninjured, vehicle-treated and miR-21 antagomir-treated groups ($^{*}P < 0.05$ vs. uninjured group, $^{\#}P < 0.05$ vs. vehicle group, n = 5).

of vascular AFs and MFs and subsequently affects vascular remodeling.

PDCD4, a tumor suppressor gene, regulates various aspects of cancer progression and acts as a potential target for anticancer therapies [Lankat-Buttgereit and Goke, 2009]. PDCD4 has been linked to cardiovascular biology by regulating the apoptosis of VSMCs and cardiac cells [Lin et al., 2009; Cheng et al., 2010], but no report exists of PDCD4 in AFs and MFs. miRNAs exert their biological functions by regulating target gene expression. PDCD4 has been experimentally validated as an miR-21 target in several cancer cells [Asangani et al., 2008; Frankel et al., 2008]. However, the target must be experimentally verified in specific cells because miRNA-mediated effects on gene expression and cellular functions are cell-specific [Jazbutyte and Thum, 2010]. In our study, we confirmed that PDCD4 was a direct target of miR-21 in AFs and MFs. We showed that PDCD4 expression in AFs was much higher than that of in MFs and miR-21 could regulate PDCD4 expression both in AFs and MFs. Moreover, we showed at the cellular and molecular level that regulation of miR-21 expression could modulate the proliferation and apoptosis of AFs and MFs through PDCD4, and the change in PDCD4 expression could affect miR-21-mediated cellular effects. Therefore, PDCD4 is a functional target gene involved in miR-21-mediated vascular alteration.

JNK/c-Jun pathway has essential roles in cell proliferation and apoptosis. JNK/c-Jun can be pro-apoptotic or anti-apoptotic, depending on the cellular context [Yasumoto et al., 2001; Du et al., 2004; Ho and Li, 2010]. JNK/c-Jun acts as pro-proliferative and an anti-apoptotic factor in VSMCs [Yasumoto et al., 2001]. JNK activation is involved in SMC proliferation and intimal hyperplasia induced by balloon injury, and transfer of dominant negative c-Jun inhibits SMC proliferation and neointimal thickening [Yasumoto et al., 2001]. However, JNK/c-Jun have a pro-apoptotic role in vascular endothelial cells [Murakami et al., 2005], and over-expression of c-Jun induce apoptosis [Yasumoto et al., 2001]. In current study, we showed that PDCD4 was a pro-apoptotic gene in AFs and MFs. PDCD4 siRNA prompted proliferation, and meanwhile significantly increased JNK/c-Jun activity, which revealed that JNK/c-Jun acted as a positive regulator of cell growth in AFs and MFs. It is also suggested that miR-21- or PDCD4 siRNA- mediated proliferation is partly attributable to JNK/c-Jun activation.

In conclusion, we reveal that miR-21 has an important role in AFs and MFs by directly modulating their proliferation and apoptosis, and further affecting vascular remodeling. The miR-21/PDCD4/JNK/c-Jun may be an important signal transduction pathway in regulating their proliferation and apoptosis. These findings provide novel insights into the mechanisms and treatment of vascular remodeling.

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