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Increased expression of microRNA-221 inhibits PAK1 in endothelial progenitor cells and impairs its function via c-Raf/MEK/ERK pathway

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ABSTRACT

Coronary artery disease (CAD) is associated with high mortality and occurs via endothelial injury. Endothelial progenitor cells (EPCs) restore the integrity of the endothelium and protect it from atherosclerosis. In this study, we compared the expression of microRNAs (miRNAs) in EPCs in atherosclerosis patients and normal controls. We found that miR-221 expression was significantly up-regulated in patients compared with controls. We predicted and identified p21/Cdc42/Rac1-activated kinase 1 (PAK1) as a novel target of miR-221 in EPCs. We also demonstrated that miR-221 targeted a putative binding site in the 3′UTR of PAK1, and absence of this site was inversely associated with miR-221 expression in EPCs. We confirmed this relationship using a luciferase reporter assay. Furthermore, overexpression of miR-221 in EPCs significantly decreased EPC proliferation, in accordance with the inhibitory effects induced by decreased PAK1. Overall, these findings demonstrate that miR-221 affects the MEK/ERK pathway by targeting PAK1 to inhibit the proliferation of EPCs.

1. Introduction

The incidence of coronary artery disease (CAD) is increasing and there is an urgent need to elucidate the mechanisms responsible for this disease and to develop new therapies. Circulating endothelial progenitor cells (EPCs) are stem cells that circulate from the bone marrow to peripheral vessels and maintain physiological, as well as pathological neovascularization [1]. EPCs differentiate into mature endothelial cells in response to endothelial cell injury via a self-healing process, while transplanted EPCs can also restore the integrity of damaged endothelium [2,3]. A previous study found that some microRNAs (miRNAs) were dysregulated in EPCs of atherosclerosis patients [4]. In addition EPC differentiation was partly inhibited by miRNA-107 via hypoxia-inducible factor-1b [5]. Although EPCs can promote cell recovery to some extent, a fuller understanding of the mechanisms involved in endothelial injury at the molecular level will provide more therapeutic options for CAD patients.

The newly identified, small non-coding miRNAs have been reported to be involved in various physiological and pathological processes, including cell proliferation. Although this provides cues for elucidating the mechanisms of diseases such as cancer, the details remain unclear. miR-221 has been widely reported to be up-regulated in many cell types, including EPCs in patients with CAD [4], glioma cells [6–8], glioblastoma cells [9–11], and prostate carcinoma cells [12]. Thus, miR-221 may potentially regulate the proliferation of different cell types depending on the cellular context and target genes regulated.

P21-activated kinases (PAKs) are a family of serine/threonine protein kinases that have been shown to be important regulators involved in cancer cell signaling networks [13–15] and cardiac physiology [16]. The six mammalian PAKs are categorized into two subgroups: group I (PAK1, 2, and 3) and group II (PAK4, 5, and 6) [17]. Previous studies showed that PAK1 was regulated by several miRNAs in different cellular processes [18–23]. However, no miRNAs have yet been reported to target PAK1 in EPCs.

In this study, we examined miR-221 expression in EPCs of atherosclerosis patients, and investigated the regulatory relationship between miR-221 and PAK1. The results of this study will further our understanding of the role of miRNAs in EPC proliferation in CAD.

2. Materials and methods

2.1. Patient recruitment

All patients were from the Tenth People's Hospital (Shanghai) and blood samples were collected before treatment. The control

Abbreviations: CAD, coronary artery disease; EPCs, endothelial progenitor cells; miR-221, microRNA-221; PAK1, p21/Cdc42/Rac1-activated kinase 1; UTR, untranslated regions.

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group comprised healthy volunteers. All protocols were approved by the Research Ethics Board of the Tenth People's Hospital. Informed consent for study participation was acquired from all patients and volunteers. Patients with multiple cardiac risk factors and stable CAD were recruited in this study.

2.2. Cell culture and reagents

All procedures were approved by the Institutional Animal Care Committees of Shanghai Tenth's hospital. Eight-week-old male Lewis rats were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China). The rats were killed by injection of an overdose of sodium pentobarbital. The bone marrow cavities were flushed twice with 0.01 mol/l precooled phosphate-buffered saline (PBS). The cell pellets were washed twice with PBS and resuspended in M199 medium (Gibco, America). Bone marrow mononuclear cells (BMMCs) were separated from the cell suspension by centrifugation through a Ficoll-isopaque (Sigma, America) density gradient. To obtain EPCs, the BMMCs were allowed to adhere to 6-well plates in M199 medium for 1 h at 37 °C in a 5% CO_2 incubator. Non-adherent cells were then collected and cultured in M199 medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), 10 ng/ml vascular endothelial growth factor (PeproTech, America) and 2 ng/ml basic fibroblast growth factor (PeproTech) at 37 °C in a 5% CO_2 incubator. After 3 h, non-adherent cells were removed and adherent cells were collected and cultured for 7 days.

2.3. Immunofluorescence and flow cytometric analysis

Blood was obtained from atherosclerosis patients and controls. EPCs were isolated using a Becton–Dickinson FACS Aria cytometer (BD Biosciences, America).

2.4. Western blotting analysis

EPCs were obtained and treated with 300 μl lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (Sigma) and 1× proteasome inhibitor (Sigma). Twenty micrograms of proteins were loaded onto 10% SDS–polyacrylamide gels and transferred onto PVDF membranes (Millipore, Germany). Western blotting was performed using primary antibodies to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:500 dilution in 5% milk) and PAK1 (Abgent, America) and secondary goat anti-rabbit antibodies (1:4000 dilution, PerkinElmer, America). Autoradiographs were scanned and analyzed semiquantitatively.

2.5. RNA extraction and real-time quantitative PCR

Total RNA was extracted from EPCs using Trizol reagent according to the manufacturer's instructions (Qiagen, Germany). RNA was reversely transcribed to cDNA using PrimeScript^{R}RT Master Mix Perfect Real Time (Takara, Japanese) and amplified using a PCR kit (Qiagen). Reverse transcription primers were used as described previously [24]. Expression levels of miRNA-221 were measured using TaqMan real-time PCR and reactions were performed using a Roche Light Cycler 480 system (Roche Diagnostics GmbH, Mannheim, Germany). All reactions were performed in triplicate. U6 RNA was used as an endogenous control.

2.6. Plasmid constructs and generation of stable cell lines

All constructs were validated by sequencing. To obtain stable expression of miR-221 in EPC cells, we used human genomic DNA to amplify human pri-miRNA with the following primers: miR-221 forward 5′-CAGCATACATGATTCCTTGTGA-3′, reverse 5′-CTTTGGTGTTTGAGATGTTTGG-3′. The PCR products were then inserted into a pCDH-CMV-MCS-EF1-GFP lentiviral vector. pri-miRNA-221 plasmid DNA and the transfection complex DNAs were transfected into EPCs using Lipofectamine 2000 (Invitrogen, America) according to the manufacturer's recommendations. pri-miRNA-control plasmid DNA was used as a negative control.

A fragment of 3′UTR of PAK1 containing the putative miR-221 binding site was amplified from human cDNA and cloned into psi-CHECK-2 (Promega, America) at BamHI and DpnII cutting sites. Some nucleotides were mutated by PCR at the putative target sites and the mutated sequence was 5′-GAAAUGGUCUUAGCUUAGAUGCA-3′. Both the wild-type and mutant 3′UTRs were located just downstream of the luciferase gene sequence. The constructed vectors were named PAK1-WT and PAK1-MUT.

2.7. Cell transfection and cell proliferation assay

Cells were grown to 30–40% confluency and transfected with control siRNA (cat. sc-37007 Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or human PAK1 siRNA (sequence cat. PAK1: sc-29700, Santa Cruz, Biotechnology, Inc.) using Lipofectamine 2000. Unless otherwise indicated, 50 nM of RNA duplex and 200 nM of miRNA inhibitor were used for each transfection.

In vitro growth of EPCs was measured using the cell proliferation assay WST-1 (Roche). A total of 4000 cells with 100 μl culture medium were seeded into each well of 96-well plates and transfected with siRNA-control or siRNA-PAK1 at a final concentration of 50 nM, and then further incubated at 37 °C for 0, 3, 6, and 9 days, respectively. Following the addition of 10 μl WST-1 reagent to each well at 2 h, the OD 450 nm value in each well was determined using a microplate reader.

2.8. Luciferase reporter assay

EPCs were seeded in 96-well clusters and co-transfected with 50 nM lenti-miR-221 and 10 ng of firefly luciferase reporter comprising wild-type or mutant 3′-UTR of the target gene PAK1. Luciferase activity was detected 48 h after transfection using a dual-luciferase reporter assay system (Promega) and normalized to *Renilla* activity.

2.9. Statistical analysis

All data from three independent experiments were expressed as mean ± SEM and processed using SPSS 16.0 statistical software. Differences among groups in terms of cell proliferation were analyzed using Student's *t*-tests. A p value of <0.05 was considered to be statistically significant.

3. Results

3.1. Identification of EPCs

We collected blood from atherosclerosis patients and the corresponding control group and analyzed the miRNA expression profiles. EPCs were separated by flow cytometry and showed similar morphologies in both groups (Fig. 1A). The purities of the cells assessed by three EPC-specific markers CD34, CD133 and KDR were up to 85%, 92% and 94%, respectively. The purity increased to 98%, after flow cytometry (Fig. 1B). Purified EPCs were subjected to RT-PCR. We compared the miR-221 levels detected by RT-PCR in the normal and atherosclerosis groups. Expression levels of microRNA-221 were significantly up-regulated in atherosclerosis patients (Fig. 1C).

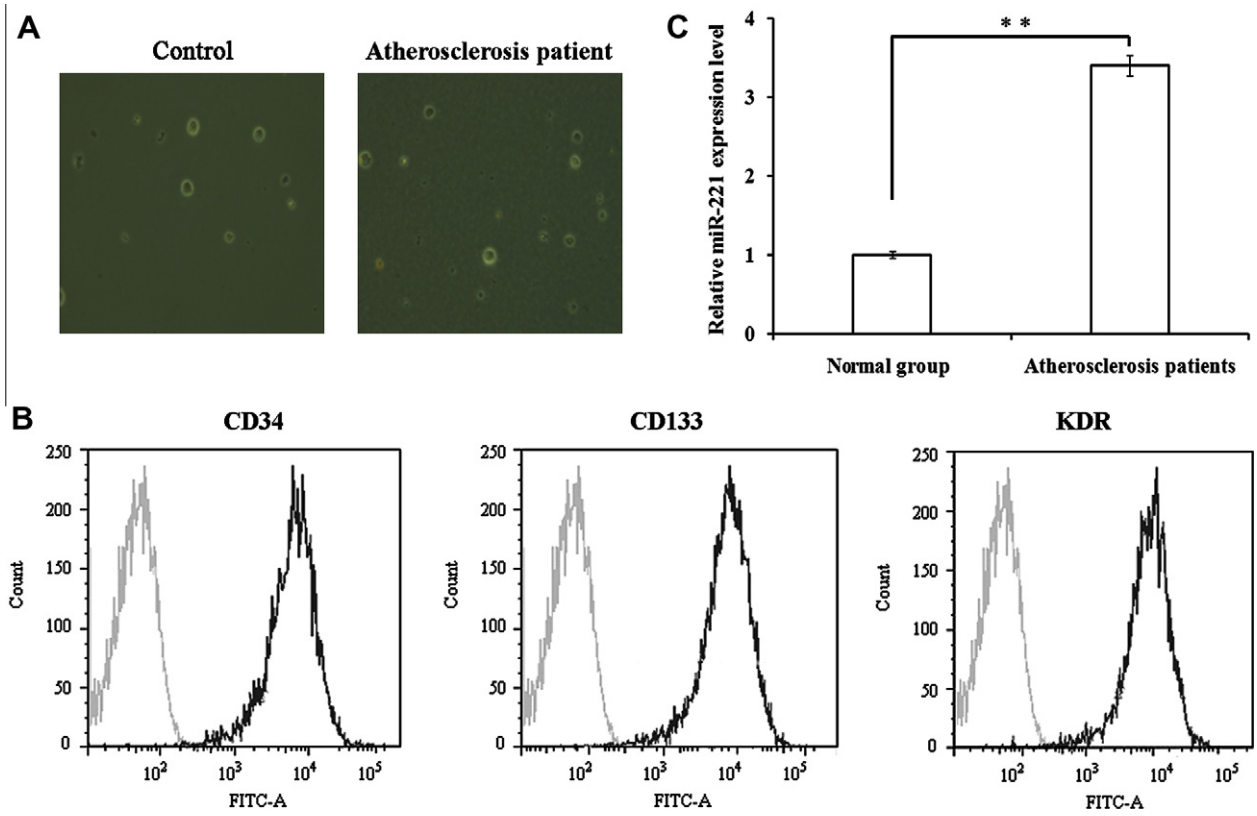

Fig. 1. Identification of EPCs and quantification of miR-221 expression levels in atherosclerosis patients and healthy controls. (A) Morphology of EPCs under 20× phase-contrast microscope after flow cytometry. (B) Flow cytometry data showing numbers of $CD34^+$, $CD133^+$, and KDR^+ EPCs. (C) RT-PCR quantification of miR-221 from total RNA extracted of EPCs from atherosclerosis patients and normal controls.

3.2. miR-221 expression and EPC proliferation under hypoxic conditions

Before examining miR-221 in EPC from atherosclerosis patients, we mimicked the atherosclerotic microenvironment by culturing rat EPCs under hypoxic conditions, and quantified the expression levels of miR-221. miR-221 levels were significantly increased in hypoxic EPCs (Fig. 2A and B). The cell proliferation ability of the EPCs was also reduced under hypoxia (Fig. 2C), suggesting a possible relationship between up-regulated miRNA-221 and EPC proliferation.

3.3. miRNA-221 directly targets PAK1

We performed computational target prediction to identify functionally relevant targets of miR-221 in EPCs of atherosclerosis patients. We hypothesized that PAK1, which possesses a putative binding site for miR-221, might be a target gene of miR-221. To confirm this, we cloned the 3′UTR of PAK1 mRNA (PAK1-WT-UTR) into psi-CHECK-2 downstream of the reporter gene. A mutant version (PAK1-MUT-UTR) with a mutated binding site was also constructed (Fig. 3A). The vectors pCDH-miR-221 or pCDH-miR-control and PAK1-WT-UTR or PAK1-MUT-UTR were cotransfected into EPC cells. Overexpression of miR-221 significantly reduced luciferase activity (Fig. 3B), whereas luciferase activity was unaffected by transfection with PAK1 3′UTR with four mutated nucleotides in the miR-221 seed binding site. miR-221-mediated repression of luciferase activity was abolished by the mutant putative binding site. We constructed stable EPCs that overexpressed miR-221. To verify the results of the gain-of-function study, we detected the expression levels of miR-221 in stable cell lines infected with a lentivirus carrying miR-221 (miR-221) or the control (miR-control). Levels of miR-221 expression were significantly increased in the miR-221 group (Fig. 3C), demonstrating that the stable cell lines successfully expressed miR-221. Moreover, the enhanced miR-221 expression in EPCs significantly repressed PAK1 protein expression, compared with the miR-control (Fig. 3 D and E). Overexpression of both miR-221 and siRNA-PAK1 affected the viability of EPCs (Fig. 3F). These initial experiments suggest that miR-221 may negatively regulate the expression of PAK1 by directly targeting the 3′UTR of its mRNA, and thus influencing EPC viability.

3.4. miRNA-221 potentially inhibits EPC proliferation via c-Raf/MEK/ERK pathway

Cell proliferation in response to mitogenic stimulation involves phosphorylated ERK1/ERK2. We investigated the role of the Raf/MEK/ERK pathway in miR-221 anti-cell proliferation by targeting PAK1 and found that specific transient miR-221 expression reduced phosphorylated pRAF, pMEK, and pERK1/2 activity in accordance with previous reports [25] (Fig. 4). Taken together, these findings indicate that miR-221 inhibits the PAK4/Raf/MEK/ERK pathway to suppress EPC proliferation.

4. Discussion

Although miRNA dysregulation has been observed in various pathological processes, the molecular mechanisms underlying miRNA modulation of these pathological processes remain unclear. Numerous important discoveries have recently demonstrated the involvement of miR-221 in many cell types, including EPCs in atherosclerosis patients, glioma cells [6–8], glioblastoma cells [9–11] ,

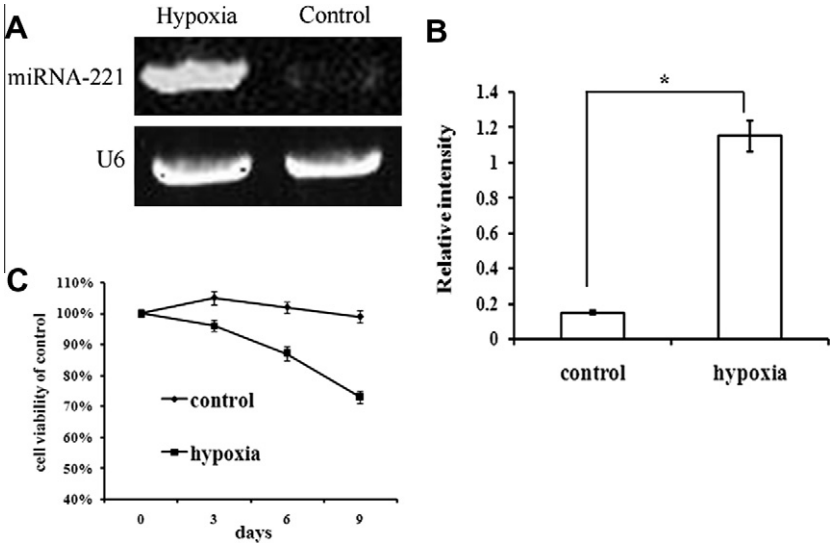

Fig. 2. miR-221 levels and cell viability under hypoxic and normal conditions. (A) RT-PCR of miR-221 from total RNA extracts from EPCs under hypoxic and normal conditions. U6 levels were used as an internal control. (B) Quantification of (A). Error bars = SEM. miRr-221 was decreased under hypoxic conditions (*$p < 0.05$). (C) Cell numbers at 0, 3, 6 and 9 days after seeding in 6-well plates, normalized to the cell number at day 0.

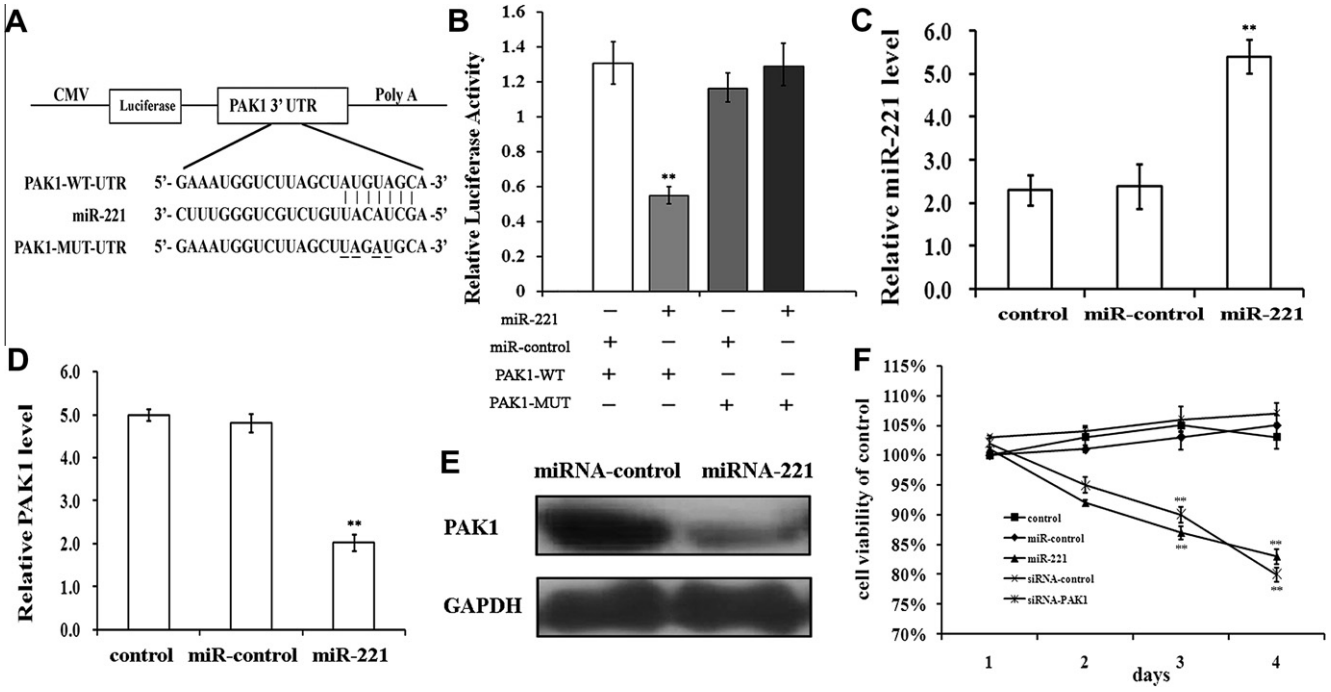

Fig. 3. miR-221 binds directly to the PAK1 3′UTR region. (A) Construct sequence alignment of the miR-221 seed region and the predicted miR-221 binding site in PAK1 3′UTR (WT), and the construct obtained by site-directed mutagenesis of four base pairs of the miR-221 seed-sequence (MUT). (B) Repression of luciferase activity by PAK1 3′UTR was dependent on miR-221 in EPCs. The mutated PAK1 3′UTR abolished miR-221-mediated repression of luciferase activity (*$p < 0.01$). (C) miR-221 level was increased by stable transfection with plasmids containing the miR-221 sequence, compared with non-transfected control EPCs and miR-control-transfected EPCs. (D) PAK1 mRNA levels were decreased in miR-221-stably transfected cells, compared with non-transfected control EPCs and miR-control-transfected EPCs (*$p < 0.01$). (E) Compared to miR-control-transfected EPCs, endogenous PAK1 protein levels were inhibited by miR-221 overexpression. (F) Cell viability was decreased in both miR-221- and siRNA-PAK1-transfected EPCs after different time points (1, 2, 3 and 4 days). Error bars = SEM.

and prostate carcinoma cells [12], indicating a key role for miR-221 in carcinogenesis and cancer development. The results of the current study also suggest that miR-221 plays a critical role in EPCs in atherosclerosis patients.

We elucidated the potential mechanisms of miR-221 in endothelial injury. Expression levels of miR-221 were commonly up-regulated in EPCs from atherosclerosis patients compared with normal EPCs. This was in accordance with the results of other studies indicating that miR-221 was up-regulated in various unhealthy cell lines. We also performed functional assays in rat EPCs cultured under hypoxic conditions to mimic the atherosclerotic microenvironment. We found that miR-221 inhibited cell proliferation in stable EPC cell lines overexpressing miR-221. These results imply that miR-221 may play a fundamental role in inhibiting endothelial cell recovery.

PAK1 is part of a subfamily of serine/threonine kinases involved in vascular integrity and plays a fundamental role in several pathways. Down-regulation of the PAK1 gene in zebrafish endothelial

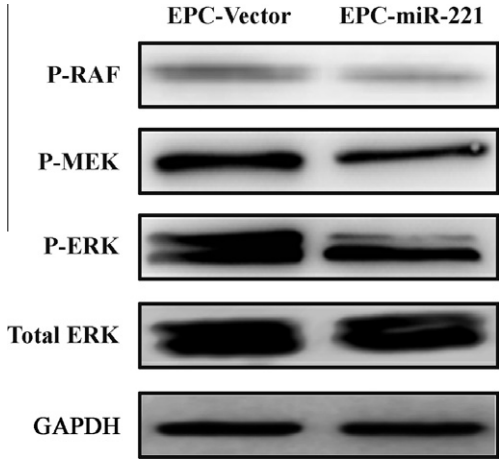

Fig. 4. Raf/MEK/ERK pathway was inhibited by overexpression of miR-221.Total cellular proteins were extracted and subjected to Western blot analysis. Stable overexpression of miR-221 decreased the protein levels of phosphorylated (p)RAF, pMEK and pERK. Total ERK was unaffected. GAPDH was used as an internal control.

cells was shown to rescue cranial hemorrhage [21]. The mechanisms of PAK1, which involve the MEK1/ERK1/2 signaling pathways, are linked to cell proliferation. We addressed the molecular mechanisms involved in miR-221-mediated effects by focusing on PAK1, which was predicted to be a target of miR-221 by informatics screening. miR-221 bound directly to the specific complementary site in the PAK1 3′-UTR of PAK1, and overexpression of miR-221 in EPCs dramatically decreased the mRNA and protein expression levels of PAK1. Knockdown of PAK1 by siRNA suppressed cellular growth, similar to the results of the gain-of-function study by miR-221 overexpression. These findings thus revealed that the inhibitory effect of PAK1 was similar to that of overexpression of miR-221. This is the first study to show that PAK1 is negatively regulated by miR-221 at the post-transcriptional level, by binding to a specific target site within the PAK1 3′UTR. These findings suggest that this might represent a potential molecular mechanism by which miR-221 participates in endothelial cell injury.

In summary, this study demonstrated that miR-221 is dysregulated in EPCs of atherosclerosis patients. We demonstrated that miR-221 plays an important role in inhibiting cell proliferation by directly targeting PAK1, a newly identified target gene. These results suggest that miR-221 overexpression and inhibition of the PAK1/MAPK signaling pathway may provide targets for strategic therapeutic applications in atherosclerosis patients.

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