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Original Article

MicroRNA 181b promotes vascular smooth muscle cells proliferation through activation of PI3K and MAPK pathways

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Abstract: Vascular smooth muscle cells (VSMCs) hyperplasia is a common feature of pathologic cardiovascular event such as restenosis and atherosclerosis. The role and mechanisms of microRNAs (miRs) in VSMCs proliferation are poorly understood. Here, we report that miR-181b promotes VSMCs proliferation and migration. In an animal model, miR-181b was significantly increased in the rat carotid artery after balloon catheter injury. Delivery of miR-181b inhibitor to injured artery exhibited a marked inhibition of neointimal hyperplasia. Transfection of miR-181b with "mimics" to A10 cells accelerated cell proliferation, which was accompanied by an increase of cell migration. The induction of A10 cells proliferation by miR-181b appeared to be involved in activation of S and G2/M checkpoint, concomitant with decreases in cell-cycle inhibitors p21 and p27, and increases in cell-cycle activators CDK4 and cyclinD1. In contract, miR-181b inhibition attenuated A10 cells proliferation, inhibited cell migration and arrested cell cycle transition. Moreover, forced miR-181b expression elevated the phosphorylation levels of Akt and Erk1/2, whereas inhibition of miR-181b produced the opposite effects. Additionally, inhibition of PI3K and MAPK signaling pathways with specific inhibitors, but not inhibition of JNK pathway, significantly abolished the effects of miR-181b in promoting cell proliferation. These findings demonstrate that miR-181b enhances the proliferation and migration of VSMCs through activation of PI3K and MAPK pathways.

Keywords: Neointimal formation, vascular smooth muscle cells, proliferation, miRNA-181b

Introduction

The characteristics of restenosis include the thickening of intima resulting from the proliferation and migration of vascular smooth muscle cells (VSMCs) [1]. VSMCs are the major component of the vasculature, and play a critical role in maintaining vascular tone and blood pressure [2]. Under normal conditions, VSMCs in mature animals are mainly retained in a non-proliferative state, and their principal functions are differentiation and contraction [3, 4]. However, after vessel injuries, the damaged endothelial cells and inflammatory cells invade the sub-endothelial layer and release cytokines such as interleukin-1, platelet-derived growth factor, endothelin and angiotensin II [3, 5]. Subsequently, the complex interaction between VSMCs and these cytokines results in proliferation and migration of VSMCs, which plays crucial roles in the pathogenesis of restenosis and atherosclerosis [1, 6]. Thus, the balance between differentiation and proliferation of VSMCs is important for maintaining vascular homeostasis. Although some signaling pathways associated with the proliferation of VSMCs have been identified, the detailed molecular mechanisms to modulate these alterations required further investigation.

MicroRNAs (miRs) are a class of highly conserved, single stranded, non-coding RNAs with small lengths of 18-25 nucleotides that regulate the target genes expression at post-transcriptional level via binding to complementary sequences in their 3'-untranslated regions (3'-UTR) [7, 8]. miRs have gradually emerged as an essential regulator of VSMCs proliferation and have been found to be involved in various aspects of cardiovascular diseases. For example, miR-21 [9], miR-143 [10], miR-145 [11], miR-221 and miR-222 [12] have been suggest-

ed to play pivotal roles in VSMCs proliferation and restenosis. Recent studies have demonstrated that miR-181b is involved in proliferation of various cells, such as astrocytoma [13], ovarian cancer cells [14] and metanephric mesenchymal cells [15]. However, the functional role of miR-181b in VSMCs remains unknown.

In the present study, we first found that miR-181b is significantly increased after balloon-injury to the carotid artery. Indeed, miR-181b promotes cell proliferation and migration in vitro. Taken together, our results reveal that miR-181b may be a potential therapeutic target to inhibit VSMCs proliferation and restenosis.

Materials and methods

Cell culture

The smooth muscle cell line A10 (CRL-1476), derived from the thoracic aorta of rat embryo, was obtained from ATCC (VA, USA) and was maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) supplemented with 10% fetal calf serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen) at 37°C in 5% CO_2 atmosphere. In this study, A10 cells were arrested by replacing the medium with 0.1% FBS/DMEM for 24 h before corresponding treatments. LY294002 (20 μM), PD98059 (10 μM) and SP600125 (10 μM) (Sigma St.Louis, USA) were used to inhibit phosphorylation of PI3K/AKT, ERK/MAPK and JNK pathway in miR-181b inhibitor-transfected cells, respectively.

miRNA transfection

The oligonucleotides miR-181b mimics (181b-m), mimics negative control (NC-m), miR-181b inhibitor (181b-i) and inhibitor negative control (NC-i) were obtained from GenePharma (Shanghai, China). A 10 cells were transfected with miRNAs at final concentration of 50 nM using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 6 h incubation in a CO_2 incubator at 37°C, the medium was changed, and the cells were allowed to grow for various times as indicated.

Balloon angioplasty and miR-181b inhibition in vivo

All animal protocols were confirmed with the relevant guidelines and regulations approved by the Institutional Animal Care and Use Committee of China Medical University. 250-300 g male Sprague-Dawley rats were purchased from the Medical Experimental Animal Center of China Medical University and the common carotid artery balloon angioplasty was performed as previously described [16]. Briefly, under anesthesia with Ketamine (100 mg/kg, i.p.) and Xylazin (5 mg/kg, i.p.), a longitudinal midline incision on the common external and internal carotid arteries was performed. The common and internal carotid arteries were ligated temporarily using vessel clips. A 2F Fogarty balloon catheter (Edwards Lifesciences, CA, USA) that was introduced via the external carotid artery and advanced to the common carotid artery. Inflation and retraction of the balloon catheter were repeated four times. Subsequently, miR-181b inhibitor and inhibitor negative control (50 μg/rat) were delivered to the injured artery and incubated for 20 min. Then the external carotid artery was tied off and rats were allowed to recover from anesthesia. The animals were sacrificed and the carotid arteries were collected for various times as indicated.

Quantitative real-time PCR analysis

Total RNA was extracted from A10 cells or carotid artery using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcriptions were performed by using SuperScript III First-Strand Synthesis system (Invitrogen). The levels of mature miRs were quantified using Taqman miRNA probes (Applied Biosystems, CA, USA) with a Fast RT-PCR system (ABI 7300, Applied Biosystems). The sequence-specific primers for quantitative RT-PCR of has-miR-181a (PN4373117), has-miR-181b (PN4373116), has-miR-181c (PN4373115), has-miR-181d (PN4373180) and U6 (PN4373381) were obtained from Qiagen (CA, USA). The U6 small nuclear RNA was selected as a control.

Morphometry

At the time of euthanasia, the injured segment of the common carotid artery was dissected from the surrounding tissue, fixed in 10% formalin, and embedded longitudinally in paraffin. Samples were cut into 4 μm longitudinal sections to assess intimal lesion size by hematoxylin-eosin (HE) staining. With an analysis pro-

gram (ImageJ, Version 1.41, NIH, Maryland, USA), the external plastic layer (EPL) area, the internal plastic layer (IPL) area, and the luminal area were measured. The intimal/medial ratio was calculated:

Intimal thickness = (IPL area/π)$^{1/2}$-(luminal area/π)$^{1/2}$

Medial thickness = (EPL area/π)$^{1/2}$-(IPL area/π)$^{1/2}$.

Western blot

A10 cell extracts were subjected to western blot analysis as described previously [13]. Briefly, after determination of the protein concentration using a BCA kit (Beyotime Institute of Biotechnology, Jiangsu, China), equal amount of protein was size-fractionated electrophoretically using 8%-12% polyacrylamide gelelectrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). Following incubation with 5% non-fat milk for 1 h, the membranes were incubated with following target primary antibodies at 4°C overnight: Mouse-anti-JNK (sc-6254), mouse-anti-JNK (sc-7345), rabbit-anti-p21 polyclonal antibody (sc-397), mouse-p27 monoclonal antibody (sc-393380), rabbit-anti-CDK4 polyclonal antibody (sc-260), mouse-cyclin D1 monoclonal antibody (sc-246) and mouse-anti-GAPDH monoclonal antibody (sc-166574) (1:1,000; Santa Cruz Biotechnology, CA, USA); rabbit anti-p-AKT (Thr308) polyclonal antibody (#9611), rabbit-anti-p-ERK1/2 (Thr202/Tyr204) monoclonal antibody (#4370), rabbit-anti-AKT polyclonal antibody and rabbit-anti-p-ERK monoclonal antibody (#4348) (1:500; Cell Signaling Technology, CA, USA). After incubation with the appropriate secondary peroxidase-conjugated antibodies including HRP-conjugated anti-rabbit or anti-mouse (diluted 1:1000; Beyotime) for 1 hour, the antigen-antibody complex was developed by a chemiluminescence system (Cell Signaling Technology) and the absorbance of the protein bands was analyzed by ImageJ software.

Proliferation assay

In vitro A10 cells proliferation was determined by cell counting and 5-bromo-2'-deoxyuridine (BrdU) assay as previously described [17]. Cells in the logarithmic phase of growth were seeded in 96-well at 2×10^3/well density and cultured for 24, 48 and 72 h. Cell number was examined using Cell Counting Kit-8 (Dojindo Molecular Technologies, MD, Japan) according to the manufacturer's instructions. BrdU assay was measured by using BrdU (Sigma) uptake. The optical density (OD) was determined at a wavelength of 570 nm with a microplate reader (Bio-Rad 550, CA, USA).

Cell cycle analysis

A10 cells were collected with 0.25 g/L trypsin and centrifuged. The cell pellet was re-suspended in 10 mL phosphate-buffered saline (PBS) and 1 mL 70% ethanol was added. After incubation at -20°C for 15 min, the cells were centrifuged and washed with cold PBS. Then cells were resuspended in PBS with 20 mg/mL PI and incubated for 30 min at room temperature. The DNA sample of each sample was measured by FACScalibur flow cytometry (BD Biosciences, CA, USA). The data analysis was performed using Cell Quest (BD Biosciences) software. The results were presented as percentages of the total cell count in different phases of the cell cycle, namely the G0/G1 phase, S phase and G2/M phase.

Wound healing assay

Wound healing assay was performed as previously described [13]. In brief, cells were cultured to 95% confluence in 6-well plates. Cell monolayers were scraped with a sterile 100 μl Pipettes tip across the diameter to form wound gaps. The distance between the wound edges was measured immediately and again 48 h later. The images of wound area were obtained by a microscope (IX71, Olympus). The total distance migrated by wounded A10 cells was evaluated using ImageJ software and was presented as a percentage of the initial wound distance.

Chemoinvasion assay

The cells invasion was performed using BD Matrigel invasion chambers (BD Biosciences) following the manufacturer's protocol. Cells were suspended in serum-free DMEM medium at density of 2×10^4 cells/well and seeded in the upper chamber with a Matrigel-coated filter. The lower chambers of the apparatus contained DMEM medium supplemented 10% FBS

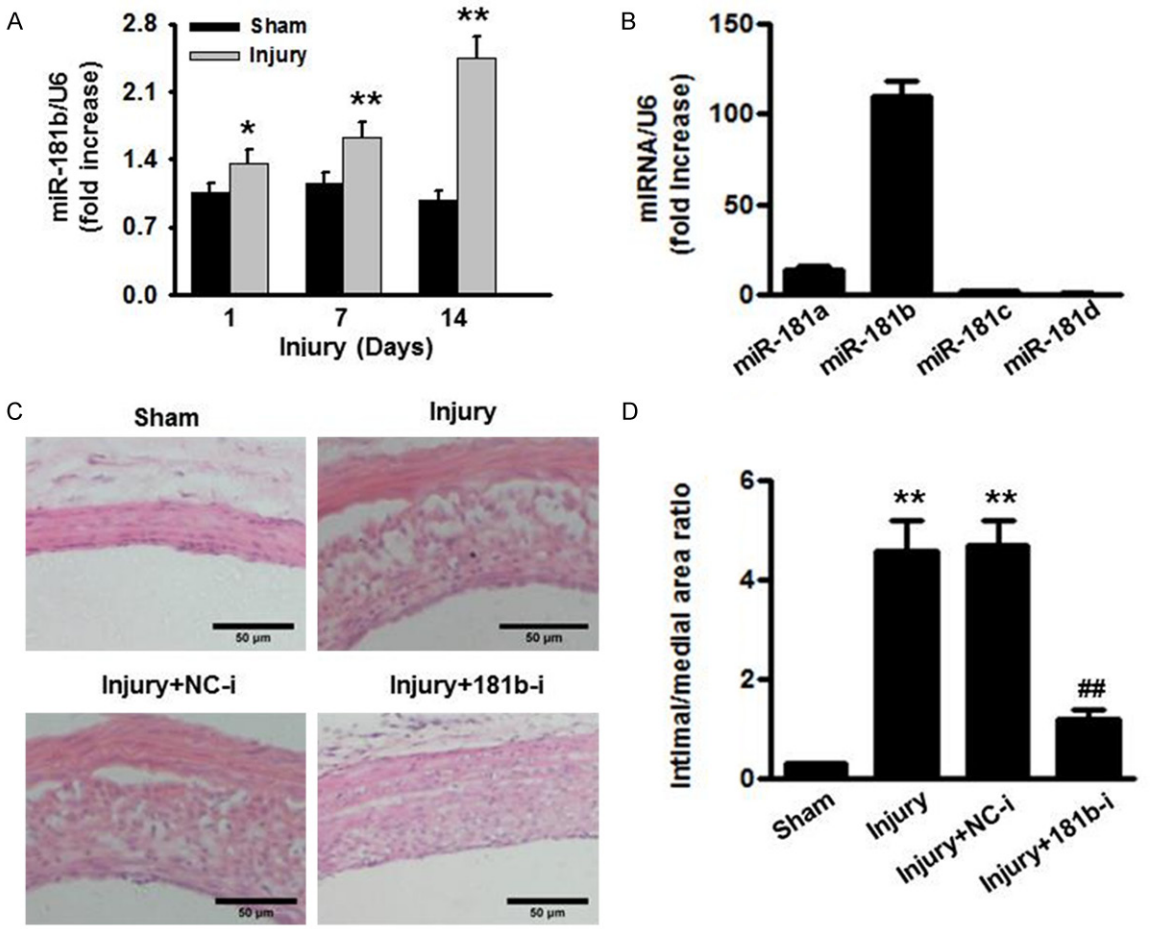

Figure 1. MiR-181b is involved in neointimal formation in response to injured carotid artery. A. MiR-181b expression in injured carotid artery and the corresponding sham group was measured at 1-, 7- and 14-day after operation by quantitative real-time PCR. *P<0.05, **P<0.01 vs. corresponding sham groups, n = 10 in each group. B. Quantitative real-time PCR analysis of miR-181a, miR-181b, miR-181c and miRNA-181d mRNA expression in carotid artery. C. 14 days after injury, HE staining was performed to observe the intima and media of carotid artery. D. Intimal/medial area ratio after balloon injury was quantified. **P<0.01 vs. sham group; ##P<0.01 vs. injury group, n = 6 in each group.

as a chemoattractant. After 72 h incubation at 37°C, the cells on the upper membrane surfaces were removed by using a cotton swabs and the cells on lower surface were fixed with methanol and stained with 0.1% crystal violet. The images of the migrating cells were observed under a microscope (IX71, Olympus, MD, Japan) The number of migrated cells was quantified by counting cells using ImageJ software.

Statistical analysis

All data were expressed as mean ± SEM at from least three independent experiments. 1-way ANOVA using the Bonferroni multiple comparison post hoc test with a 95% confidence interval was employed in SPSS 16.0 system (SPSS Inc., IL, USA). Relationships were considered statistically significant when $P<0.05$.

Results

Inhibition of miR-181b represses neointimal formation after common carotid balloon catheter injury

To investigate the role of miR-181b in participating vascular remodeling, expression of miR-181b was detected in the carotid artery after a balloon angioplasty. Quantitative real-time PCR revealed that at 1, 7 and 14 days after balloon injury, miR-181b expression was significantly increased as compared with those in sham

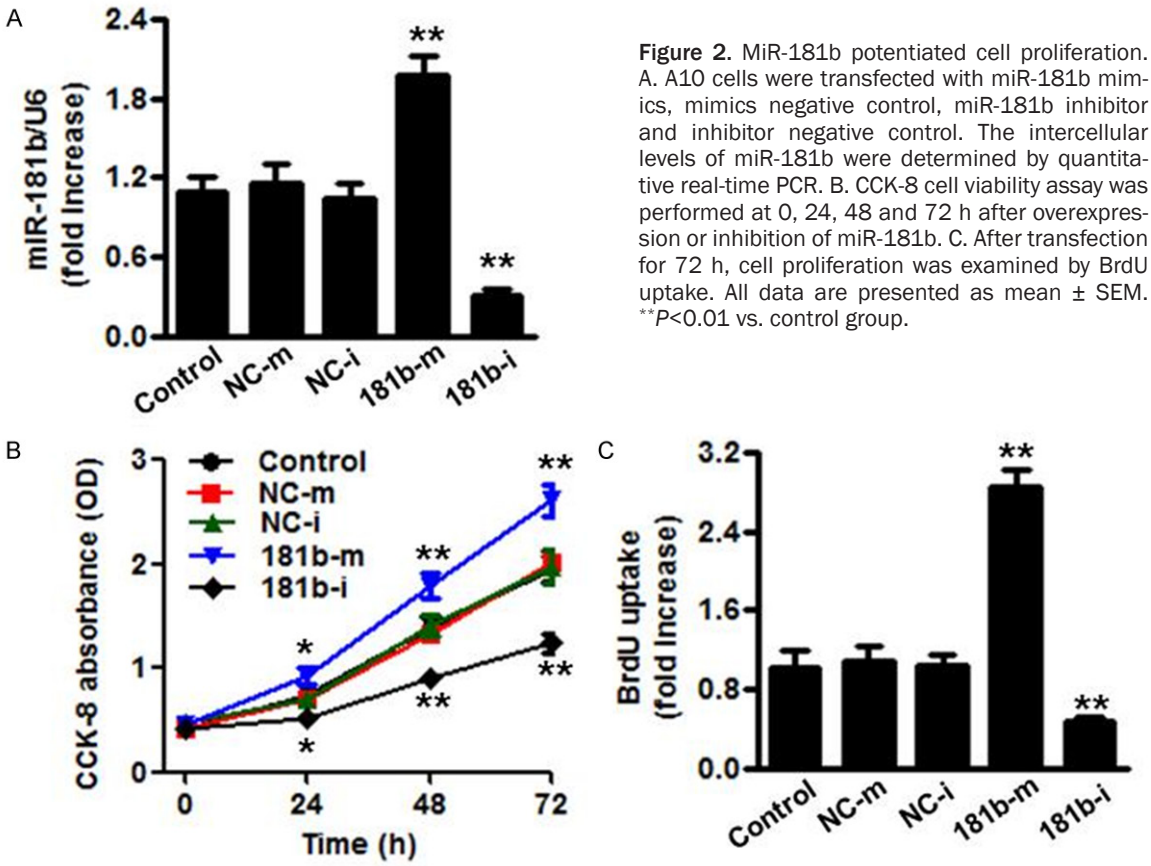

Figure 2. MiR-181b potentiated cell proliferation. A. A10 cells were transfected with miR-181b mimics, mimics negative control, miR-181b inhibitor and inhibitor negative control. The intercellular levels of miR-181b were determined by quantitative real-time PCR. B. CCK-8 cell viability assay was performed at 0, 24, 48 and 72 h after overexpression or inhibition of miR-181b. C. After transfection for 72 h, cell proliferation was examined by BrdU uptake. All data are presented as mean ± SEM. $^{**}P<0.01$ vs. control group.

group (n = 10 in each group) (**Figure 1A**). MiR-181 family comprises 4 miRs: miR-181a, miR-181b, miR-181c and miR-181d. Therefore, we next analyzed the expression pattern of miR-181 family in carotid artery. As showed in **Figure 1B**, the level of miR-181b was most abundant in carotid artery, as evidenced by 8-fold higher than miR-181a and 74-fold higher than miR-181c, respectively. Moreover, the expression of miR-181d was too faint to be detected. These data indicate miR-181b is the dominant member of miR-181 family expressed in carotid artery and may play a functional role in the regulation of neointimal hyperplasia.

To verify this assumption, miR-181b inhibitor or inhibitor negative control was delivered upon carotid artery injury. 2 weeks after carotid artery balloon injury, HE staining showed that carotid artery intima was remarkably thickened, neointimal protruding into the lumen. There were no differences in the neointimal thickness between inhibitor negative control group and carotid artery injury alone. Notably, administration of miR-181b inhibitor significantly decreased the intimal hyperplasia, as demonstrated by an obvious decrease in intima/media area ratio (**Figure 1C** and **1D**).

MiR-181b promotes A10 cells proliferation

To examine whether miR-181b affects the survival of VSMCs, A10 cells, a rat VSMCs line, were transfected with miR-181b mimics, mimics negative control, miR-181b inhibitor or inhibitor negative control and analyzed for cell viability. The expression of miR-181b was markedly increased after transfection with miR-181b mimics, whereas miR-181b inhibitor significantly decreased miR-181b expression in vitro (**Figure 2A**). The CCK-8 assay showed that overexpression of miR-181b significantly increased cell number even on the first day of transfection. The cell number was gradually increased as the treatment interval increased. In contrast, in cells transfected with miR-181b inhibitor, cell number was obviously decreased. The relative cell proliferation rate of the miR-181b-transfected cells at 72 h was 63.3%, and the relative cell proliferation rate of the miR-

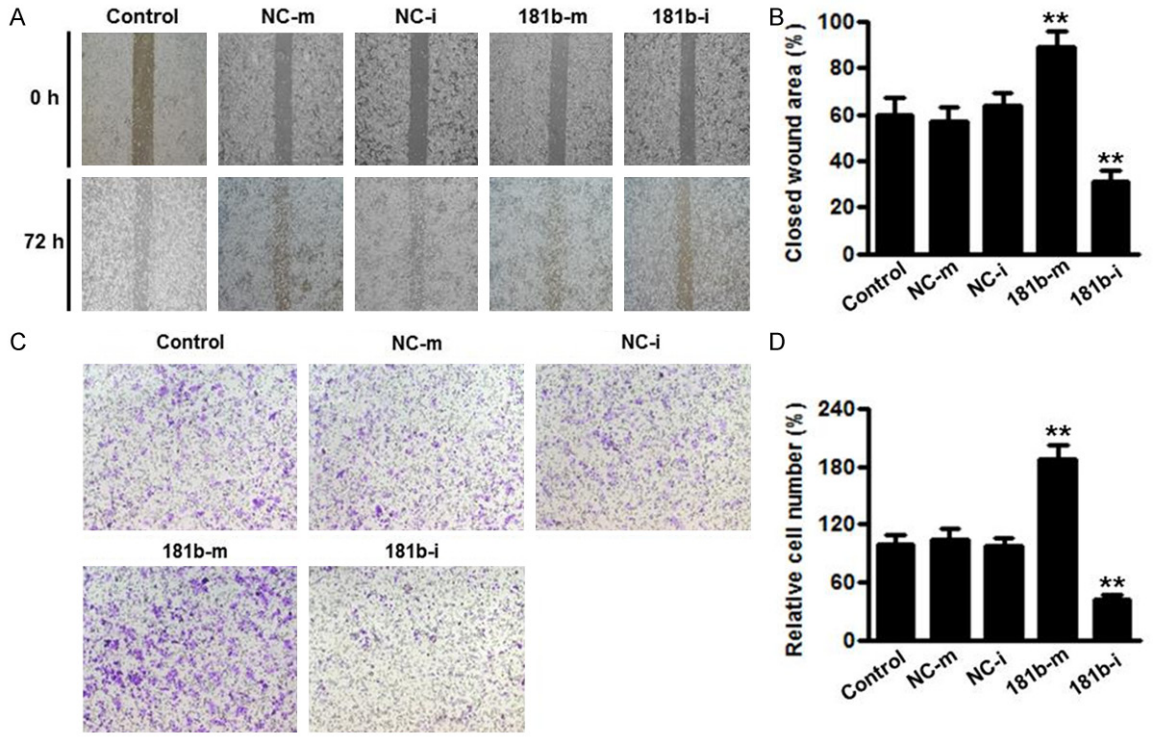

Figure 3. The role of miR-181b in cell migration and invasion in A10 cells. (A) Overexpression or knockdown of miR-181b by transfection with miR-181b mimics or miR-181b inhibitor for 72 h, respectively. Wound healing assay was performed. The representative images from four independent experiments are shown. (B) Quantification results of the closed wound area. (C) and (D) A10 cells migration was examined by Transwell analysis after treatment mentioned in (A). The images shown are representative images from four independent experiments (C), and a statistical analysis was performed (D). All data are presented as mean ± SEM. $^{**}P<0.01$ vs. control group.

181b inhibitor-transfected cells at 72 h was 133.8% (**Figure 2B**). BrdU uptake is another important parameter for determining cell proliferation. In agreement with CCK-8 results, miR-181b mimics significantly increased BrdU uptake at 72 h, whereas knockdown of miR-181b produced the opposite effects (**Figure 2C**). Mimics negative control or inhibitor negative control transfection did not significantly alter the rate of cell proliferation.

MiR-181b accelerates cell migration and invasion

VSMCs play a critical role in neointimal hyperplasia. Thus, we further investigated whether miR-181b is associated with the migration ability of VSMCs. The migratory and invasive behavior of A10 cells was analyzed. Transient transfection of miR-181b mimics potentiated the migration capacity of A10 cells, whereas inhibition of miR-181b attenuated wound closure, which represented as lowing of cell motility (**Figure 3A** and **3B**). A Transwell invasion assay, an alternative assay for determining cell movement, revealed that knockdown of miR-181b reduced the invasive ability of A10 cells. However, miR-181b mimics-transfected A10 cells exhibited a higher mobility in Transwell assays (**Figure 3C** and **3D**). These data suggest that miR-181b was positively correlated with the mobility of A10 cells.

Knockdown of miR-181b inhibits cell cycle progression in A10 cells

Flow cytometric cell cycle analysis was performed using propidium iodide (PI) staining to assess the effect of miR-181b on cell cycle progression (**Figure 4A**). The results showed that, in A10 cells overexpressing miR-181b, 42.7% of cells were in the G0/G1 phase, 45.3% of cells were in the S phase, and 8.9% of cells were in the G2/M phase, as compared to 65.6%, 30.3% and 6.9% of control cells, respectively, indicating miR-181b promotes cell cycle progression

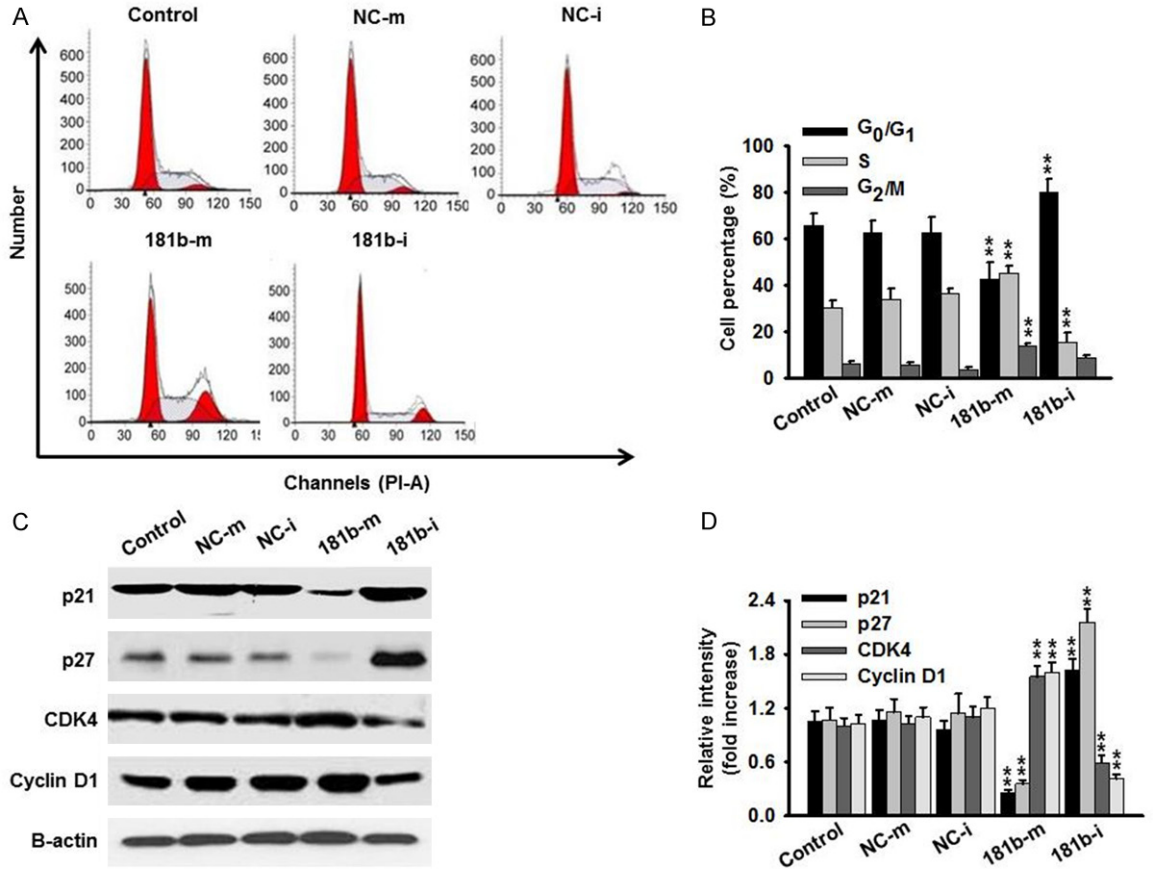

Figure 4. MiR-181b promotes cell cycle transition in A10 cells. A10 cells were transfected with miR-181b mimics, mimics negative control, miR-181b inhibitor or inhibitor negative control. A. Cell cycle profiles were determined by flow cytometry (stained with PI). B. The percentages of cells in the G0/G1, G1/S and G2/M phases of the cell cycle were analyzed. C. Representative western blots of p21, p27, CDK4 and cyclin D1 in A10 cells. D. Densitometric analysis of p21, p27, CDK4 and cyclin D1 expression. All data are presented as mean ± SEM. $^{**}P<0.01$ vs. control group.

and enhances cells' entrance into S phase. On the contrary, inhibition of miR-181b significantly suppressed cell cycle transition (**Figure 4B**). To further examine the molecular mechanism by which miR-181b enhances cell cycle transition, we analyzed the expression of p21, p27, CDK4 and cyclin D1 in A10 cells. As shown in **Figure 4C** and **4D**, overexpressing miR-181b markedly decreased p21 and p27 expression, while CDK4 and cyclin D1 expression were elevated. However, inverse results were observed in group of miR-181b inhibitor (**Figure 4C** and **4D**).

MiR-181b increases A10 cell proliferation via PI3K and MAPK signaling pathway

It has been documented that PI3K, MAPK and JNK pathways may be responsible for transduction of signaling to trigger cell proliferation [18]. To determine the underlying molecular mechanisms, the phosphorylation of AKT, ERK and JNK was measured. Western blot showed that transient transfection of miR-181b mimics significantly elevated AKT and ERK phosphorylation, whereas inhibition of miR-181b attenuated the phosphorylation of AKT and ERK obviously. Of note, neither miR-181b mimics nor miR-181b inhibitor produced significant effects on JNK phosphorylation, indicating JNK pathway may be not involved in miR-181b-mediated cell proliferation (**Figure 5A** and **5B**). In order to verify this assumption, A10 cells were pretreated with LY294002 (20 μM), a specific inhibitor to PI3K, or PD98059 (10 μM), a specific inhibitor to MAPK, or SP600125 (10 μM), a specific inhibitor to MAPK, and then examined their effects on miR-181b-induced cell proliferation. Our results showed that both LY294002 and PD98059 remarkably attenuated the stim-

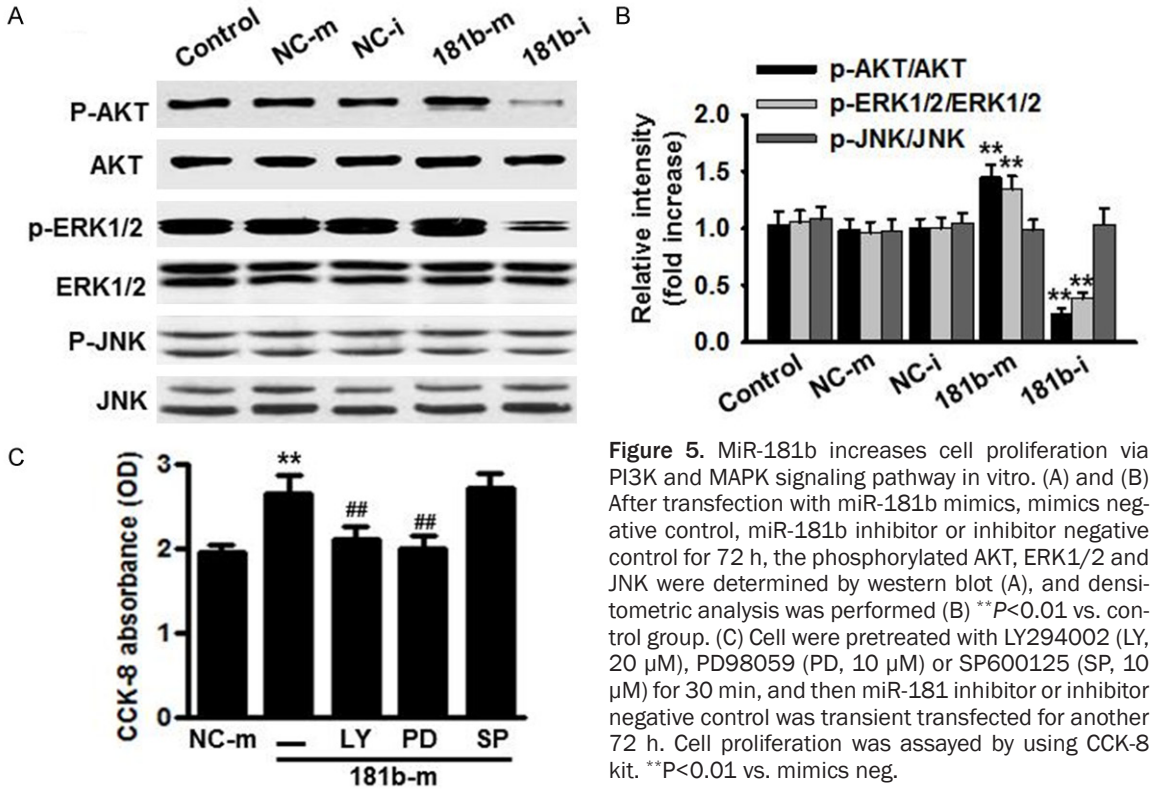

Figure 5. MiR-181b increases cell proliferation via PI3K and MAPK signaling pathway in vitro. (A) and (B) After transfection with miR-181b mimics, mimics negative control, miR-181b inhibitor or inhibitor negative control for 72 h, the phosphorylated AKT, ERK1/2 and JNK were determined by western blot (A), and densitometric analysis was performed (B) $^{**}P<0.01$ vs. control group. (C) Cell were pretreated with LY294002 (LY, 20 μM), PD98059 (PD, 10 μM) or SP600125 (SP, 10 μM) for 30 min, and then miR-181 inhibitor or inhibitor negative control was transient transfected for another 72 h. Cell proliferation was assayed by using CCK-8 kit. $^{**}P<0.01$ vs. mimics neg.

ulatory effect of miR-181b on cell proliferation. However, no significant differences were observed after pretreatment with SB600125 (**Figure 5C**). Together, these data suggest that miR-181b potentiated A10 cell proliferation via activation of PI3K and MAPK pathway, but not JNK pathway.

Discussion

With an increasing number of target genes of miRs have been identified by experimental assays and clinical studies, miRs are emerging as new regulators of VSMCs proliferation. Depletion of miR-21 results in decreased cell proliferation and increased cell apoptosis by targeting PTEN and Bcl-2 [9]. The levels of miR-221 and miR-222 are specifically up-regulated in VSMCs in the injured vascular walls, resulting in VSMCs proliferation and migration by targeting p27 and p57, respectively [12]. Moreover, the expression of miR-145 is selectively reduced in response to PDGF-bb, and this reduction is positively correlated with VSMCs proliferation and neointimal formation [11]. MiR-181b belongs to the miR-181 family, which includes 4 mature miRs: miR-181a, miR181b, miR-181c and miR-181d. They are encoded by 6 primary miRs sequences located on 3 different chromosomes [19]. Although recent study has demonstrated that miR-181b is able to negative regulate the expression of pro-inflammatory molecules and thus protects against vascular inflammation [19], the functional role of miR-181b in VSMCs and cardiovascular system is not fully understood. In the present study, our results are the first to our knowledge to demonstrate that the expression of miR-181b is up-regulated in injured rat carotid artery. Moreover, we found miR-181b was highly expressed in carotid artery based on quantitative real-time PCR, but the endogenous expression of miR-181a, miR-181c and miR-181b was very faint, indicative of a critical role of miR-181b in neointimal formation. Indeed, inhibition of miR-181b by administration of miR-181 inhibitor significantly blocked neointimal hyperplasia in balloon injury models. Furthermore, we found that miR-181b positively regulated proliferation in A10 cells as well as cell migration and invasion. Expectedly, knockdown of miR-181b exhibited the opposite effects. Of note, different functional role of miR-181b in proliferation have been reported. For example,

miR-181b promotes cell growth in cervical cancer cells [20] and ovarian cancer cells [14], whereas decreases cell survival rate in astrocytoma [13] and metanephric mesenchymal cells [15]. Therefore, cell type-specific response should be noted.

Next we examined the effects of miR-181b on cell-cycle distribution to investigate the mechanisms by which miR-181b modulates the proliferation of VSMCs. Our results showed that overexpression of miR-181b promoted cell cycle progression via activation of S and G2/M checkpoint. In contrary, inhibition of miR-181b inhibited cell cycle transition. The cyclin-CDK complexes, such as cyclin D1 and CDK4, have been found to be an essential regulator of cell cycle progression by promoting the transition from G1 to S phase [21]. On the other hand, CDK inhibitors (CDKIs) including p21 and p27, are widely reported to restrict transition in the cell cycle by suppressing several cyclin-CDK complexes [22]. In this study, we found up-regulation of miR-181b obviously decreased p21 and p27 expression, whereas the expression of CDK4 and cyclin D1 was significantly elevated. In addition, inverse results were observed in miR-181b inhibitor-transfected cells. Together, our results demonstrate that miR-181b promotes cell proliferation by regulating expression of cell cycle regulator, in turn results in facilitating cell cycle progression.

PI3K, MAPK and JNK pathways are thought to be responsible for signal transduction to trigger cell proliferation [18]. We therefore hypothesized that these signaling pathways might be involved in the stimulatory effects of miR-181b on VSMCs proliferation. In the current study, we found that overexpression of miR-181b could activate PI3K and MAPK pathways, as evidenced by increased AKT and ERK1/2 phosphorylation. However, no significant effects were found on the phosphorylation of JNK. To further determine the possible involvement of these pathways, we inhibited the target pathways by specific inhibitors. As expected, LY294002 and PD98095 almost abolished the effects of miR-181b in increasing cell number, whereas SP600125 produced no effects. The results indicate PI3K and MAPK are the critical upstream signaling molecules responsible for miR-181b in the regulation of VSMCs proliferation.

In summary, we initially demonstrate that miR-181b expression is markedly increased during the development of neointimal formation. MiR-181b overexpression activates PI3K and MAPK signaling pathways, which underlies, at least in part, the effects of miR-181b in promoting VSMCs proliferation.

Disclosure of conflict of interest

None.

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