

## Original Article

# MiRNA-22 attenuates angiotensin II-induced cerebrovascular smooth muscle cells proliferation by targeting PDPK1

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**Abstract:** Cerebrovascular smooth muscle cells hyperplasia is the major contributor of cerebrovascular remodeling and stroke. However, the mechanisms have not been defined. MicroRNAs modulate various cellular processes by regulating their target genes expressions. In this study, we found that Angiotensin II (AngII) increased cell viability and decreased miR-22 expression in rat basilar artery smooth muscle cells (BASMCs). Moreover, the continuous increase in cell viability was negatively correlated with changes in miR-22 expression after AngII treatment. Up-regulation of miR-22 with miR-22 precursor decreased cell viability induced by AngII, while the down-regulation of miR-22 with antisense had the opposite effect. In addition, miR-22 overexpression inhibited AngII-induced cell cycle transition by inducing cell cycle arrest at G<sub>1</sub>/S point. However, inhibition of miR-22 further enhanced the effect of AngII on cell cycle. Luciferase reporter assay and western blotting showed that miR-22 negatively regulated the expression of 3-phosphoinositide-dependent protein kinase 1 (PDPK1) by directly targeting the 3'-UTR of PDPK1. Furthermore, the results revealed that miR-22 attenuated AngII-induced BASMCs proliferation by inhibiting AKT and mTOR phosphorylation. Taken together, these data demonstrate that miR-22 exhibits anti-proliferative effects in BASMCs by regulating PDPK1 expression and its downstream AKT/mTOR signaling. Our findings suggest that up-regulation of miR-22 may be a novel strategy for the treatment of cerebrovascular remodeling and stroke.

**Keywords:** Cerebrovascular smooth muscle cells, proliferation, miR-22, PDPK1, AKT/mTOR signaling

## Introduction

In cerebral arteries, chronic hypertension results in vascular remodeling, which is one of the important risk factors of stroke [1]. Remodeled cerebral arteries with thickened walls and narrowed lumens can be observed in various animal hypertension models such as 1k1c Goldblatt rats, deoxycorticosterone acetate salt rats, stroke-prone spontaneously hypertensive rat, and angiotensin II (AngII)-infused rats [2-4]. One prominent feature of remodeled arteries and stroke is cerebrovascular smooth muscle cells (SMCs) hyperplasia [4]. However, the underlying mechanisms are poorly understood.

3-phosphoinositide-dependent protein kinase 1 (PDPK1) is a Ser/Thr kinase and a key activator of a number of protein kinases that play a critical role in regulating cell proliferation, such as AKT, protein kinase C and S6 kinase [5, 6].

Although PDPK1 was initially shown to be an important oncogene in multiple types of cancer, it is worth noting that PDPK1 was also involved in cerebrovascular remodeling [4, 7, 8]. AKT is a well characterized cellular substrate for PDPK1 [5]. Binding of the products of phosphoinositide 3-kinase (PI3K), PtdIns-3,4-P<sub>2</sub> or PtdIns-3,4,5-P<sub>3</sub>, to interact with the pleckstrin homology domains of AKT and PDPK1, these proteins are recruited to plasma membrane and consequently lead to AKT activation [4]. Additionally, PDPK1-mediated activation of AKT results in phosphorylation of the downstream kinase mTOR [8]. The PDPK1/AKT/mTOR kinase signaling pathway has been reported to promote cerebrovascular SMCs proliferation, vascular remodeling, and hypertension development [4, 7].

MicroRNAs (miRNAs) are small non-coding RNAs of approximately 17-25 nucleotides, which lead to degrade their cognate target

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genes by binding the 3'-untranslated regions (3'-UTRs) [9, 10]. A steadily growing number of studies have demonstrated that miRNAs play significant roles in diverse cellular processes, including apoptosis, proliferation, metabolism and differentiation [11]. Among miRNAs, miR-22 is a 22-nucleotide non-coding RNA and originally identified as a tumor suppressor in Hela cells [10]. Further, its expression can be detected in various cancer tissues [12]. Up-regulation of miR-22 is known to suppress lung, ovarian and colon cancer progression [13-15]. Although the role of miR-22 in cancer has been extremely studied, its functions in cardiovascular system have not been addressed. This study aims to investigate the role of miR-22 in cerebrovascular SMCs proliferation and explore the potential targets of this miRNA. Our results demonstrate that miR-22 plays an anti-proliferative role in rat basilar artery smooth muscle cells (BASMCS) by targeting PDPK1, indicating miR-22 may be a potential therapeutic target for the treatment of cerebrovascular remodeling and stroke.

### Materials and methods

#### Cell culture

BASMCS were isolated and cultured as described previously [4, 16]. Male Sprague-Dawley (SD) rats were obtained from Experimental Animal Central of Chinese Academy of Medical Science (Shanghai, China). All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Cangzhou Central Hospital. After being anesthetized with pentobarbital sodium, cerebral basilar arteries were dissected rapidly and cut into 1-mm strips and then were incubated in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin (all from Gibco, Grand Island, NY, USA) in a humidified incubator at 37°C with 5% CO<sub>2</sub>. After 5-7 days, the smooth muscle cells began to migrate from the pieces and were used at passages between 8 and 14. In this study, cell growth was arrested by replacing the DMEM/F-12 without FBS for 48 h before treatments.

#### RNA isolation and miR-22 detection

Total RNA was isolated from BASMCS using the mirVana™ miRNA Isolation Kit (Ambio, Austin, TX, USA). Reverse transcription was performed

with the miRNA Reverse Transcription Kit (Applied Biosystems, Forster City, CA, USA) and the PCR amplification was carried out with the miRCURY LNA™ Universal RT microRNA PCR (Exiqon A/S, Vedbaek, Denmark) by using an ABI PRISM 7000 instrument (Applied Biosystems). Measurements were normalized to U6 ( $\Delta C_t$ ) value and data of miR-22 expression was determined by using 2<sup>- $\Delta\Delta C_t$</sup>  method. The primer of detection of miR-22 and U6 were obtained from RiboBio Co., Ltd (Guangzhou, China).

#### *In vitro* miR-22 overexpression or inhibition

BASMCS were transfected miR-22 precursor (pri-miR-22), miR-22 antisense (anti-miR-22), or their negative controls (GenePharma, Shanghai, China) at a final concentration of 20 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 h for transfection, cells were collected for further analysis.

#### Cell viability and proliferation assay

Viability of BASMCS was determined by using CCK-8 assay as previously described [4]. Briefly, the cells (2×10<sup>3</sup> cells per well) were seeded in 96-well plates, and then were transfected with pri-miR-22, anti-miR-22, or their negative controls for 48 h in prior to Angiotensin II (AngII, Sigma, Louis, MO, USA) treatment for another 48 h. Afterward, CCK-8 reagent was added to each well at final concentration of 500 µg/ml for 2 h at 37°C. The absorbance was read at 540 nm with a microplate reader (SpectraMax M5 Molecular Device, Sunnyvale, CA, USA).

At the same time, we also examine the BrdU incorporation to measure the proliferation of BASMCS. After treatment as mentioned above, cells were incubated with 50 mM BrdU (Sigma) for 4 h at 37°C and then fixed with 4% paraformaldehyde. After permeabilized with 0.1% Triton X-100 for 5 min, cells incubated of anti-BrdU monoclonal antibody (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight, following treatment with biotinylated goat anti-mouse IgG antibody (1:100, Cell Signaling Technology, Beverly, MA, USA) for 60 min Phosphate buffer saline (PBS) was used as a negative control by replacing the primary antibody. The BrdU incorporation was expressed as the percentage of total cells.

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## *Cell cycle analysis*

Distribution of BASMCs in cell cycle was determined by flow cytometry as previously described [9]. In brief, cells were harvested and incubated with 50 µg/ml propidium iodide (PI, Sigma) dissolved in PBS for 30 min at 37°C. The stained cells were analyzed by using a Becton Dickinson (San Jose, CA, USA) FACScan flow cytometer. The percentages of cells in different phases of the cell cycle were quantified with the ModFit software program (Verity Software House, Topsham, VT, USA).

## *Luciferase experiment*

Luciferase reporter assay was performed to predict the direct binding of miR-22 to the target gene PDPK1. The rat 3'-UTR of the PDPK1 gene containing miR-22 binding sites was generated from BASMCs genomic DNA and then inserted into the pMIR-Report™ vector (RiboBio Co., Guangzhou, China). Mutation in the miR-128 binding-site of PDPK1 was directly synthesized and inserted into the equivalent reporter vector. HEK293T cells were seeded in 24-well plates with the intensity of  $0.5 \times 10^4$  per cell. The following day, the cells were co-transfected with 3'-UTR of PDPK1 (with either wild-type or mutant miR-128 binding sites), and pri-miR-22 or anti-miR-22 for 48 h by using Lipofectamine 2000. Afterward, the luciferase activity was assessed by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

## *Western blotting*

Western blotting analysis was performed as previously described [1]. Briefly, BASMCs were washed with ice-cold PBS for 3 times and then lysed in RIPA buffer containing protease inhibitor (Merck, Darmstadt, Germany). Equal amount of proteins quantified by Enhanced BCA Protein Assay Kit (Beyotime) were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride membrane (PVDF, Millipore). The membranes were blocked with 10% non-fat milk in TBST (20 mM Tris-Cl, 0.15 M NaCl, 0.05% Tween-20, pH 7.5) at room temperature for 1 h and incubated with antibodies against PDPK1, GAPDH (1:1000, Santa Cruz Biotechnology Inc.), p-AKT, AKT, p-mTOR or mTOR (1:1000, Cell Signaling Technology) overnight at 4°C. After incubation with secondary

antibody (donkey anti-goat or goat anti-rabbit IgG conjugated with the horseradish peroxidase, 1:4000, Cell Signaling Technology) for 1 h, the labeled proteins were visualized with the Enhanced Chemiluminescence Kit (GE Healthcare, Little Chalfont, UK) and quantified using densitometry software (Image J Version 1.44, NIH, Bethesda, Maryland, USA).

## *Statistical analyses*

All data were expressed as mean ± SEM, and n value indicated the number of independent experiments. The one-way analysis of variance (ANOVA) and *post hoc* Tukey's test were used to analyze the differences of multiple groups. Differences were considered to be statistically significant when  $P < 0.05$ . All statistical analyses were performed using SPSS15.0 statistical software (SPSS Inc., Chicago, IL, USA).

## **Results**

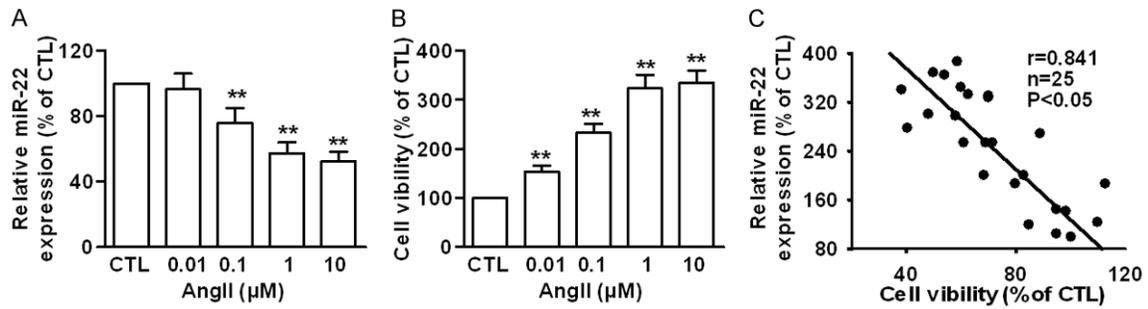
### *The decreased miR-22 expression is negatively correlated with AngII-induced cell viability in BASMCs*

AngII has been suggested to induce BASMCs proliferation and subsequently cerebrovascular remodeling [16, 17]. To unveil the role of miR-22 in participating BASMCs proliferation, we treated cells with various concentrations of AngII (0.01, 0.1, 1 or 10 µM) and then the expression of miR-22 was determined. The results showed that incubation of AngII for 48 h decreased miR-22 expression in a concentration-dependent manner (**Figure 1A**). Moreover, CCK-8 assay demonstrated that viability of BASMCs was gradually increased after AngII treatment (**Figure 1B**). Interestingly, the decreased miR-22 expression was negatively correlated with the increased BASMCs viability induced by AngII (**Figure 1C**), suggesting that the reduced miR-22 may serve as a critical factor in AngII-induced BASMCs proliferation.

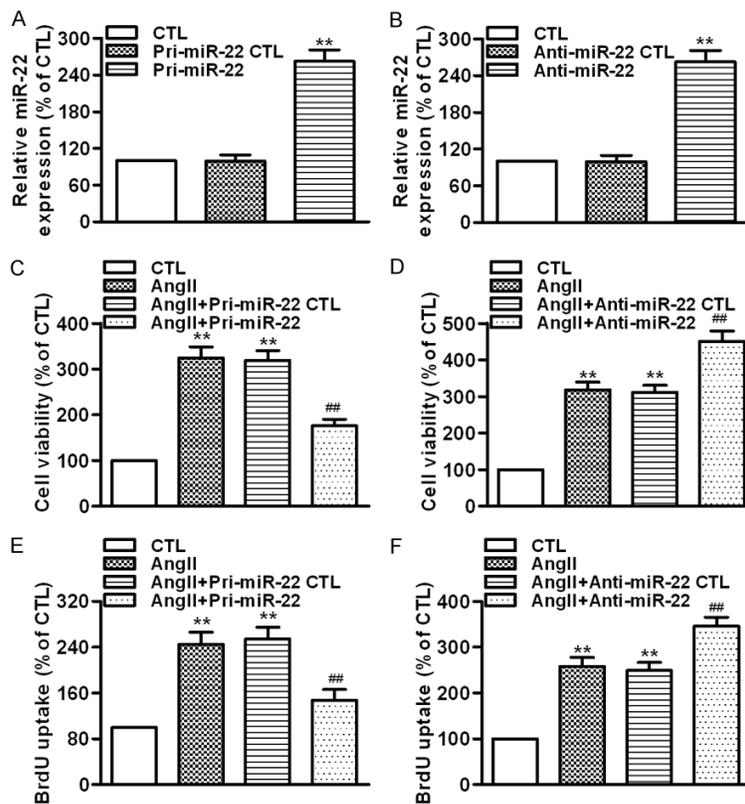
### *Upregulation of miR-22 inhibits AngII-induced BASMCs proliferation*

To confirm whether miR-22 is involved in BASMCs proliferation, cells were transfected with pri-miR-22, anti-miR-22 or their negative control, and then cell viability was determined. Pri-miR-22 significantly increased miR-22 expression more than 2.5-fold, as compared to

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**Figure 1.** MiR-22 expression is negative correlated with AngII-induced cell viability in BASMCs. A. The cells were treated with different concentrations of AngII (0.01, 0.1, 1 or 10  $\mu$ M) for 48 h, the expression of miR-22 was determined by qPCR. B. Cell viability was examined by CCK-8 assay.  $**P<0.01$  vs. control,  $n=6$ . C. The expression of miR-22 was negative correlated with cell viability.



**Figure 2.** Overexpression of miR-22 inhibits AngII-induced the increase of cell viability in BASMCs. (A and B) The cells were transfected with pri-miR-22 (A), anti-miR-22 (B), or their negative control for 48 h, the expression of miR-22 was determined by qPCR.  $**P<0.01$  vs. control,  $n=5$ . (C and D) After treatment mentioned above, cells were incubated with AngII (1  $\mu$ M) for another 48 h. Effects of miR-22 overexpression (C) or inhibition (D) on cell viability was analyzed by CCK-8 assay. (E and F) Cell proliferation was measured by BrdU assay in BASMCs treated with pri-miR-22 (E), anti-miR-22 (F).  $**P<0.01$  vs. control;  $##P<0.01$  vs. AngII,  $n=6$ .

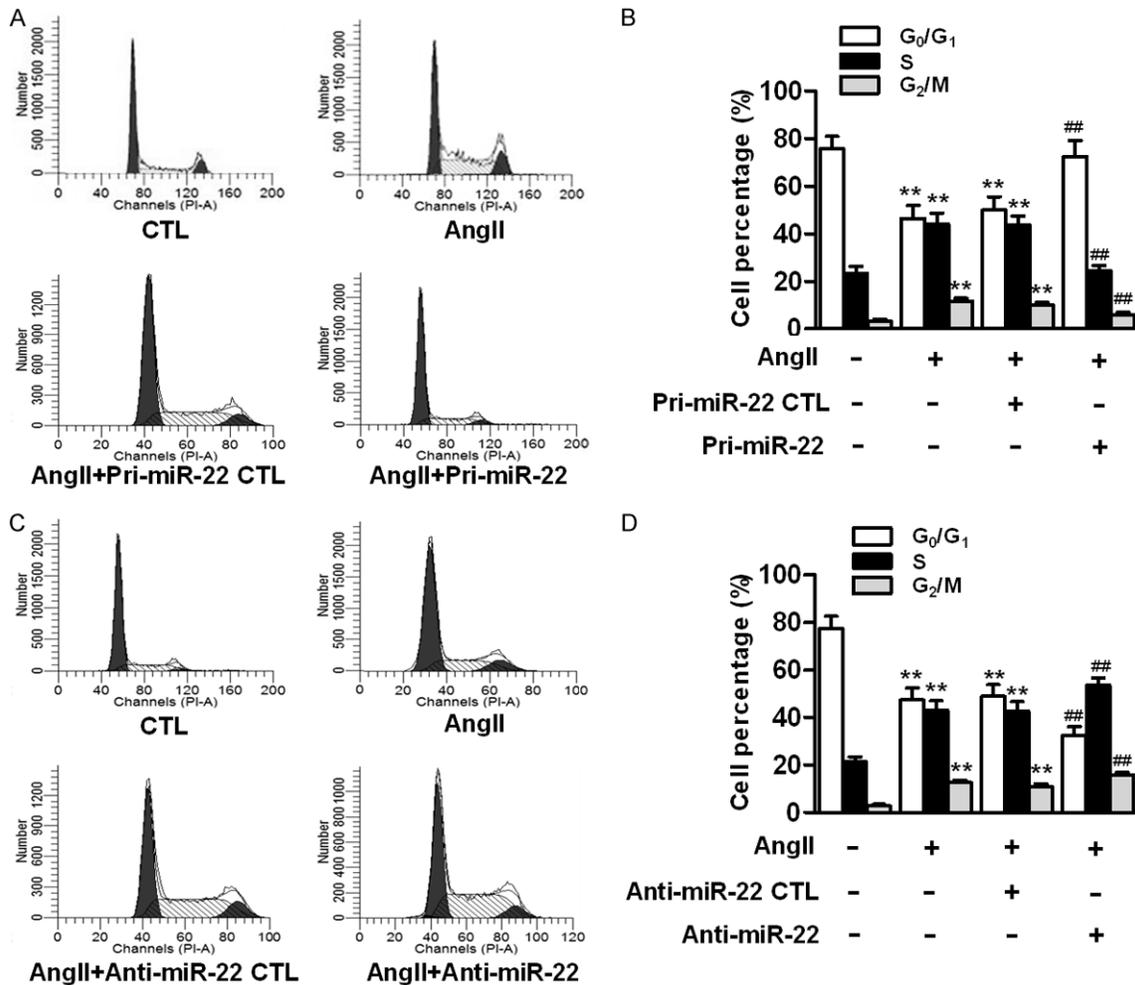
pri-miR-22 negative control (Figure 2A). At the same time, the endogenous expression of miR-22 was remarkably decreased after anti-

miR-22 transfection, while anti-miR-22 negative control had no effect (Figure 2B). CCK-8 assay showed that AngII treatment resulted in a marked increase of cell viability in BASMCs, which was dramatically inhibited by miR-22 overexpression (Figure 2C). In contrast, in cells transfected with anti-miR-22, AngII-induced the increase of cell viability was further enhanced (Figure 2D). In accordance with CCK-8 results, similar tendency was observed in BrdU uptake assay (Figure 2E and 2F). Pri-miR-22 negative control or anti-miR-22 negative control did not change the cell viability and proliferation induced by AngII.

### *Inhibition of miR-22 enhances AngII-induced cell cycle progression in BASMCs*

We next examined the effect of miR-22 on cell cycle progression by flow cytometry using PI staining. As displayed in Figure 3A and 3B, AngII treatment resulted in a decrease from 78.36% to 48.55% in  $G_0/G_1$  phase, and an increase from 25.48% to 44.57% in S phase, as compared to control, indicating AngII promotes cell cycle progression by increasing cell population in S phase.

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**Figure 3.** Knockdown of miR-22 further enhances AngII-induced cell cycle transition in BASMCs. A. The cells were transfected with pri-miR-22 or pri-miR-22 negative control for 48 h in prior to AngII treatment for another 48 h. Cell cycle profiles were determined by flow cytometry. B. The percentages of cells in the G<sub>0</sub>/G<sub>1</sub>, G<sub>1</sub>/S and G<sub>2</sub>/M phases of the cell cycle were analyzed. C. After transfection with anti-miR-22 or anti-miR-22 negative control, BASMCs were treated with AngII for 48 h. Cell cycle transition was examined by flow cytometry. D. The percentages of cells in different phases of the cell cycle were quantified. \*\*P<0.01 vs. control; ##P<0.01 vs. AngII, n=6.

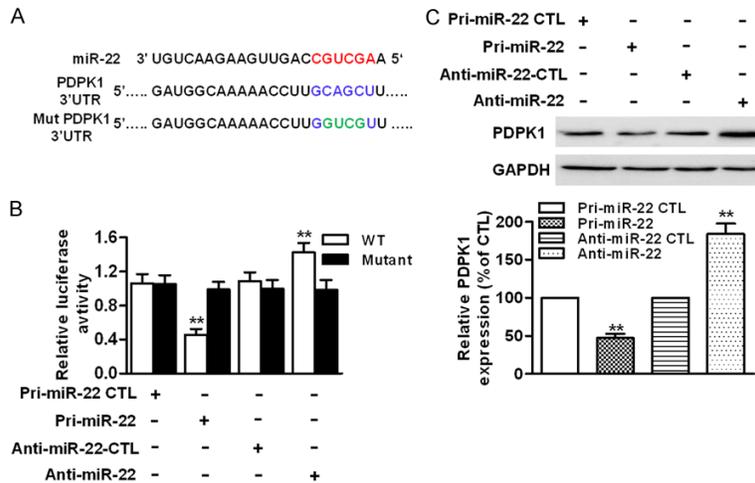
Overexpression of miR-22 significantly blocked the effect of AngII on cell progression, as evidenced by an increase in cell population in G<sub>0</sub>/G<sub>1</sub> phase and a decrease in S phase. However, knockdown of miR-22 dramatically enhanced cell cycle transition induced by AngII (Figure 3C and 3D).

### MiR-22 specifically targets PDPK1

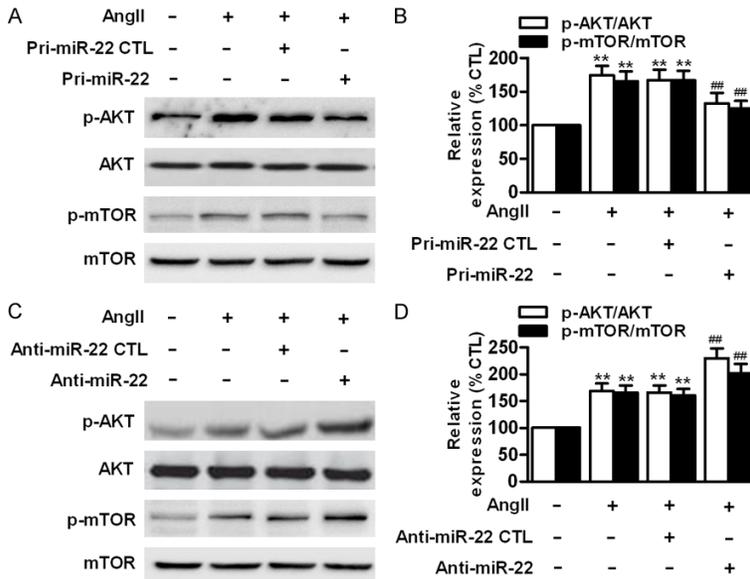
By using Targetscan ([www.Targetscan.org](http://www.Targetscan.org)), we speculated that the target of miR-22 to regulate BASMCs proliferation may be PDPK1 because we found the matching positions for miR-22 within 3'-UTR of the PDPK1 (Figure 4A). Thus, we cloned the 3'-UTR of PDPK1 by PCR

and also constructed the site-mutagenesis to generate luciferase reporter vectors containing wild-type or mutant PDPK1. By co-transfected pri-miR-22, anti-miR-22 or their negative controls with wild-type reporter or mutant one to BASMCs, luciferase assay showed that miR-22 significantly decreased the luciferase activity in wild-type reporter but not in the mutant one. In contrary, inhibition of miR-22 remarkably increased the luciferase activity of wild-type PDPK1, but also had no effects on mutant one (Figure 4B). In agreement with these observations, pri-miR-22 effectively decreased the protein expression of PDPK1, whereas anti-miR-22 increased the expression of PDPK1 in

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**Figure 4.** MiR-22 negatively regulates PDPK1 expression. A. Predicted miR-22 seed matches to the sequence in the 3'-UTR of PDPK1. The complementary sequences are shown in red and blue, and the mutated sequences are shown in green. B. BASMCs were transfected with pri-miR-22, anti-miR-22 or their negative controls for 48 h. The luciferase assay was performed to identify the directly binding of miR-22 to 3'-UTR of PDPK1. C. The protein expression of PDPK1 was examined by western blotting. \*\* $P < 0.01$  vs. corresponding negative control,  $n = 6$ .



**Figure 5.** Effects of miR-22 on AKT/mTOR signaling pathway. A. BASMCs were transfected with pri-miR-22 or pri-miR-22 negative control for 48 h in prior to AngII treatment for another 48 h. AKT and mTOR phosphorylation, and their total protein expressions were detected by western blotting. B. Densitometric analysis was performed. C. After transfection with anti-miR-22 or anti-miR-22 negative control, cells were treated with AngII for 48 h. Representative images of phosphorylated level of AKT and mTOR. D. The densitometric analysis of each band was performed. \*\* $P < 0.01$  vs. control; ## $P < 0.01$  vs. AngII,  $n = 6$ .

BASMCs (Figure 4C). These results indicate that PDPK1 is a direct target of miR-22.

### Effect of miR-22 on AKT/mTOR pathway in BASMCs

It has been suggested that PDPK1 trigger cell proliferation through activation of its downstream signaling AKT/mTOR [4, 8]. Therefore, we measured the effects of miR-22 on the phosphorylation of AKT and mTOR in the presence of AngII. Western blotting revealed that AngII treatment significantly increased the phosphorylation of AKT and mTOR in BASMCs. Transient transfection of pri-miR-22 markedly inhibited the increased phosphorylation of AKT and mTOR, whereas inhibition of miR-22 further enhanced the phosphorylation of AKT and mTOR (Figure 5). These data suggest that miR-22 suppresses AngII-induced BASMCs proliferation via inhibition of AKT/mTOR pathway.

### Discussion

AngII, one of the most potent vasoconstrictor, contributes to hypertensive remodeling of cerebral vessels through increasing SMCs proliferation [16, 17]. Multiple clinical and experimental studies have been demonstrated that AngII concentration in serum is upregulated in hypertension patients or animals [17, 18]. A study in mice with overexpressing the renin-angiotensin system showed that AngII caused hypertrophy and cerebrovascular remodeling [19]. Moreover, brain infarct size and the wall thickness of cerebrovascular have been demonstrated to be reduced after chronic treatment with AngII subtype 1 receptor blocker in spontaneously hypertensive rats [20]. In this study, our findings were consistent

with previous studies, which showed that AngII induced cell viability in BASMCs.

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It has been generally accepted that cerebrovascular SMCs hyperplasia is an important determinant of cerebrovascular remodeling and stroke [1, 4]. Previous studies revealed that miR-22 suppressed multiple cancer cell progression through post-transcriptional regulation of target gene [12, 14], indicating the vital role of miR-22 in regulating cancer cell proliferation. However, whether miR-22 regulates cerebrovascular SMCs proliferation is unknown. To the best of our knowledge, our study first evidenced that miR-22 was expressed in rats BASMCs and decreased after AngII treatment. Interestingly, we also found that the increased cell viability was negatively correlated with a decrease in miR-22 expression.

Hence, we speculated that miR-22 may be involved in the proliferation of cerebrovascular SMCs proliferation, given that miR-22 not only functioned as a tumor suppressor, but also was down-regulated in AngII-treated BASMCs. Both CCK-8 and BrdU results presented here clearly showed that overexpression of miR-22 significantly decreased the proliferation of BASMCs induced by AngII, while opposite effects were observed after miR-22 inhibition. In addition, we further investigated the effects of miR-22 on cell cycle. The results showed that up-regulation of miR-22 increased percentage of cell population in G<sub>1</sub> phase and decreased percentages in S phase, indicating miR-22 exerts its anti-proliferative effects to BASMCs by inducing cell cycle arrest at G<sub>1</sub>/S point. However, down-regulation of miR-22 further promoted AngII-induced cell cycle in BASMCs.

Activation of PDPK1 signaling has been reported to promote cancer progression and is therefore a valid target of anti-cancer therapies [8, 9, 21]. In addition, recently studies suggested that PDPK1 was also a potential therapeutic target for cerebrovascular remodeling and hypertension [7, 8]. Moreover, the secretion or expression of PDPK1 was found to be increased in BASMCs isolated from hypertension rats [4]. Such study, together with our present results indicated that there may be inverse correlation between miR-22 and PDPK1 expression in cerebrovascular SMCs. Although PDPK1 has been demonstrated to be a target of several miRNAs, such as miR-379 [9] and miR-375 [21], no study has suggested a direct relationship between miR-22 and PDPK1. Here, our

data demonstrated that miR-22 suppressed PDPK1 expression by directly binding to the 3'-UTR of PDPK1. It has been documented that PDPK1 is a key component of the PI3K/AKT signaling pathway [4]. Our results showed that the PDPK1 downstream kinases AKT and mTOR phosphorylation were both elevated after AngII treatment in BASMCs, indicating AngII-induced cerebrovascular SMCs hyperplasia may be partially due to the activation of PDPK1/AKT/mTOR signaling pathway. Expectedly, miR-22 up-regulation remarkably attenuated AngII-induced phosphorylation of AKT and mTOR, whereas inhibition of miR-22 was associated with enhanced AKT and mTOR phosphorylation. Therefore, our findings provide a comprehensive understanding of miR-22 in proliferation of cerebrovascular SMCs.

In summary, the present study demonstrates that miR-22 directly targets PDPK1 and subsequently inhibits AngII-induced cerebrovascular SMCs proliferation through AKT/mTOR pathway, indicating overexpression of miR-22 may be a novel approach for the treatment of cerebrovascular remodeling and stroke.

### Disclosure of conflict of interest

None.

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