

# Silencing miR-16 Expression Promotes Angiotensin II Stimulated Vascular Smooth Muscle Cell Growth

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## Abstract

miRNAs are a class of non-coding endogenous small RNAs that control gene expression at the posttranscriptional level and involved in cell proliferation, migration and differentiation. Dysregulation of miRNA expression is involved in a variety of human diseases including cardiovascular diseases. miRNAs have been shown to regulate vascular smooth muscle cell (VSMC) function and play vital roles in hypertension, restenosis and atherosclerosis. Here we reported that miR-16 as one of miRNAs in the miR-15 family was highly expressed in vascular smooth muscle cells (VSMCs) and involved in angiotensin II (Ang II) mediated VSMC signaling pathways. Ang II downregulated miR-16 expression in VSMCs. Lentiviral vector mediated miR-16 knockdown promoted Ang II-induced cell proliferation and migration. Moreover, silencing miR-16 enhanced Ang II induced cell cycle associated gene expression and promoted Ang II-activated cell proliferative pathways ERK1/2 and p38. Our finding demonstrated for the first time that miR-16 was a potential therapeutic target by participating in the Ang II-associated multiple signaling pathways in cardiovascular diseases.

**Keywords:** Vascular smooth muscle; Cell growth; miRNA

## Introduction

microRNAs (miRNAs) are noncoding endogenous small RNAs with a length of 18 to 25 nucleotides (nt) that negatively regulate gene expression at the posttranscriptional level by either degrading the target transcript through perfect complementary match or blocking protein translation by binding the 3' untranslated region (UTR) through imperfect base-pairing [1,2]. Accumulating evidence reveals that miRNAs play important roles in a variety of human diseases, such as cancer, diabetes, viral infection, neurodegenerative diseases, and cardiovascular diseases [3-16]. Ang II is a multifunctional peptide that promotes VSMC growth and involved in hypertension, restenosis and atherosclerosis [17-19]. miRNAs have been shown to target AngII signaling pathways in VSMCs. miR-155, miR-365 and miR-761 inhibited VSMC proliferation induced by Ang II [20-22]. miR-483-3p regulated four different components of renin-angiotensin system (RAS) in VSMCs including angiotensinogen, angiotensin converting enzyme 1 (ACE-1), ACE-2 and AT<sub>1</sub>R [23]. Ang II also promoted VSMC proliferation by upregulating miR-130a [24]. Those studies indicated that miRNAs are important regulators in Ang II-mediated signaling pathways in VSMCs.

miR-16 is one of miRNAs in the miR-15 family and matured from miR-15a/16-1 and miR-15b/16-2 clusters. The miR-15a/16-1 cluster locates in chromosome 13 in humans and 14 in mice and not annotated in the rat genome database. We previously identified the miR-15a/16-1 cluster in rats, locating in chromosome 15, which is highly conserved among different mammalian species [25]. The miR-15b/16-2 cluster locates in chromosome 3 in humans, 2 in rats and 3 in mice and is also highly conserved. The sequence difference between the miR-15a/16-1 and miR-15b/16-2 clusters is that miR-15a has 4-nt differences with miR-15b in the mature sequence. miR-16-1 and miR-16-2 share exactly the same mature sequences, suggesting that both of them may have the same target genes.

The role of miR-15 family in cancers have been extensively investigated and it is well-known that miR-15 family functions as tumor suppressor by targeting several different genes such as cyclin D3 (CCND3), cyclin E1 (CCNE1) and CDK6, thus triggers an accumulation of cells in the G0/G1 phase to block cell cycle progression and inhibits

cell proliferation [26,27]. miR-15a was recently found to play a role in ischemia-induced cerebral vascular endothelial injury by targeting Bcl-2 [28]. Moreover, miR-16 regulated angiogenic signaling in endothelial cells by targeting VEGFR and FGFR [29]. However, it is completely unknown how miR-16 contributes to the Ang II-stimulated cell growth and regulates signaling pathways in VSMCs. In this study, we found that miR-16 was highly expressed in VSMCs and the expression level of miR-16 was comparable to that of miR-143 and miR-145, two VSMC specific miRNAs. Using lentiviral vector, we silenced the expression of miR-16 in VSMCs and determined the functions of miR-16 in VSMCs by studying VSMC growth and found that silencing miR-16 in VSMCs promoted Ang II-induced cell proliferation, migration by promoting ERK1/2 and p38 cellular survival pathways.

## Materials and Methods

### Cell culture

Mouse VSMC line was purchased from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Hyclone; Logan, UT), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA). HEK293 FT cells were cultured in DMEM media with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% glutamine, 1% nonessential amino acid and geneticin with a final concentration of 1 µg/ml.

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## Cell proliferation assay

To examine the cell proliferation, VSMCs transduced with scramble control and miR-16 knockdown (KD) lentiviral vectors were labeled 24 h after following transduction using BrdU according to manufacturer's instructions in triplicate (Roche Diagnostics, Indianapolis, IN). Cell proliferation was calculated using the ratio of BrdU positive cells to cell nuclei stained with propidium-iodide (PI) or DAPI. Cell proliferation was also examined with MTT assay as we published previously [30].

## Cell migration assay

$5 \times 10^5$  cells VSMCs transduced with miR-16KD and control lentiviral vectors, plated in triplicate in a 6-well plate and grown overnight. A scratch was created using a sterile 20  $\mu$ l pipette-tip and washed 3 times with PBS. Fresh growth medium was added for additional 24 h. Migration rate was calculated using to (area of the wound area at 0 h- the wound area at 24 h)/the wound area at 0 h. Cell migration was also examined using trans well plates as we published previously [30].

## Lentiviral vector construction and virus production

The lentiviral miR-16KD and control vector were constructed by inserting antisense-miR-16 or luciferase sequences into a lentiviral shRNA vector pLenti-U6-pgk-puro (UTHSC Viral Vector Core), respectively. Lentivirus was packaged in 293FT cells and purified through ultracentrifugation as described previously [31].

## Detection of miRNA using real-time RT-PCR

Total RNA was extracted from VSMCs transduced with miR-16KD and control lentiviral vectors. PolyA tailing real-time RT-PCR was performed as described previously [32]. Forward primers for individual miRNAs are listed in Table 1. The SYBR Green-based real-time PCR was performed using a LightCycler 4800 real-time PCR instrument (Roche Applied Science; Indianapolis, IN). Melting curve analysis was performed to examine the PCR product specificity. The relative expression was normalized to U6 small nuclear RNA by the  $\Delta_2$ Ct method, and expressed as mean  $\pm$  SD.

## Western blot

VSMCs transduced with miR-16KD and control lentiviral vectors were collected in RIPA buffer (Thermo Scientific; Rockford, IL) containing 1% Halt Proteinase inhibitor Cocktail (Thermo Scientific; Rockford, IL). An equal amount of protein (40  $\mu$ g/lane) was loaded on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. The membrane was blocked with 5% non-fat milk for 1 h and incubated with primary antibodies against  $\beta$ -actin, GAPDH (Sigma; St. Louis, MO), phospho-ERK, phospho-p38, Total ERK1/2 and p38 (Cell Signaling; Danvers, MA).

miRNAs	Primer sequences
miR-15a	5' TAGCAGCACATAATGGTTTGTG
miR-15b	5' TAGCAGCACATCATGGTTTACA
miR-16	5' TAGCAGCACGTAATATTGGCG
miR-143	5'GGTGCACTGCTGCATCTCTGG
miR-145	5'GTCCAGTTTTCCAGGAATCCCT
miR-21	5' TAGCTTATCAGACTGATGTTGA
miR-124	5' CGTGTTACAGCGGACCTTGAT

Table 1: Primers used for amplifying miRNAs by PCR.

## Statistical analysis

Data shown represent the mean  $\pm$  standard deviation (SD) from at least three different experiments. The differences were analyzed using Student's *t*-test. P values < 0.05 were considered significant.

## Results

### miR-16 is highly expressed in VSMCs and Ang II downregulated miR-16 expression

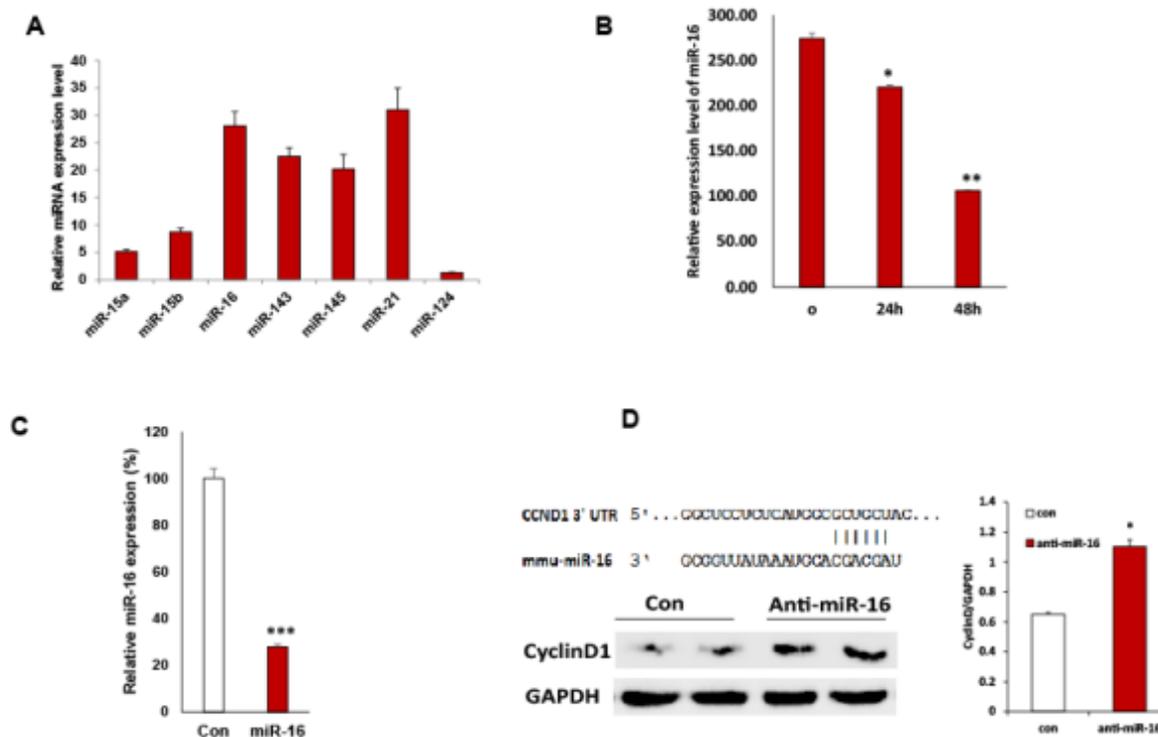
To determine the endogenous expression of miR-15 family in VSMCs, we performed polyA tailing real-time RT-PCR and examined the expression levels of individual miRNA in this family, including miR-15a, 15b, miR-16 and compared with VSMC specific miR-143 and 145. The expression of miR-15a and miR-15b was relatively lower than miR-16. However, miR-16 was highly expressed and comparable to miR-145, miR-143 and miR-21 in VSMCs (Figure 1A). To determine how Ang II regulated miR-16 expression, we treated VSMCs with 100 nM Ang II for 24 and 48 h, miR-16 expression was reduced over 20% at 24 h and over 70% at 48 h as determined by poly A tailing real-time RT-PCR (Figure 1B). Since miR-16 was highly expressed in VSMCs, we knocked down miR-16 expression using lentiviral vector. Following virus transduction and puromycin selection, miR-16 expression was reduced over 70% compared to control cells transduced with empty vector (Figure 1C). miR-16 targets cell cycle associated gene cyclin D1 and binding site was indicated in Figure 1D as published previously [33]. We also determined the expression of miR-16 target gene cyclin D1, which was upregulated in miR-16 knockdown cells compared to control (Figure 1D).

### Silencing miR-16 expression promoted Ang II-stimulated VSMC cell proliferation

To determine the role of miR-16 on VSMC proliferation, we labeled cells transduced with lentiviral miR-16 knockdown (KD), overexpression and control vectors using BrdU. Cell proliferation was examined by counting BrdU positive cells compared with cell nuclei stained with PI or DAPI. Knockdown of miR-16 significantly increased VSMC proliferation while overexpression of miR-16 reduced it compared to controls (Figures 2A and 2B). In addition, to examine whether miR-16 is involved in Ang II induced cell proliferation, miR-16KD and control VSMCs were treated with 50 and 100 nM Ang II for 24 and 48 h, cell proliferation was measured using MTT assay. Knockdown of miR-16 significantly promoted Ang II-stimulated cell proliferation at 24 and 48 h compared to control cells in both 50 and 100 nM doses. Ang II also significantly promoted cell proliferation in miR-16KD and control cells treated with 50 and 100 nM compared to 0 nM at 24 or 48 h (Figure 2B).

### Silencing miR-16 expression promoted Ang II-induced VSMC cell migration

VSMC migration is a key event during vessel injury or atherosclerosis and VSMCs migrated into neointima from media area of vessels. Inhibition of VSMC migration is a therapeutic strategy in proliferative vascular diseases. To examine whether miR-16 plays a role in VSMC migration, we first determined cell migration using a wound healing assay. Knockdown of miR-16 significantly inhibited cell migration (Figure 3A). We also verified cell migration using trans well plates and similarly knockdown of miR-16 inhibited VSMC migration (Figure 3B). To examine whether miR-16 is involved in Ang II-induced



**Figure 1:** miR-16 expression in VSMCs and Ang II downregulates miR-16 expression in VSMCs. **A.** miR-16 expression level in VSMCs detected by poly tailing realtime RT-PCR. **B.** miR-16 expression in Ang II treated wildtype VSMCs and normalized to non-coding RNA U6 (\*p<0.05;\*\*p<0.01). **C.** miR-16 expression in miR-16 knockdown (KD) and control VSMCs. **D.** miR-16 target gene cyclinD1 expression in miR-16KD and control cells was detected by Western blot (\*p<0.05).

cell migration, we treated miR-16KD and control cells using 100 nM Ang II or vehicle and then examined cell migration using transwell plate. Knockdown of miR-16 significantly promoted cell migration induced by Ang II compared to control cells, although Ang II induced cell migration in both miR-16KD and control cells (Figure 3C).

### Silencing miR-16 enhanced Ang II induced VSMC proliferative signaling

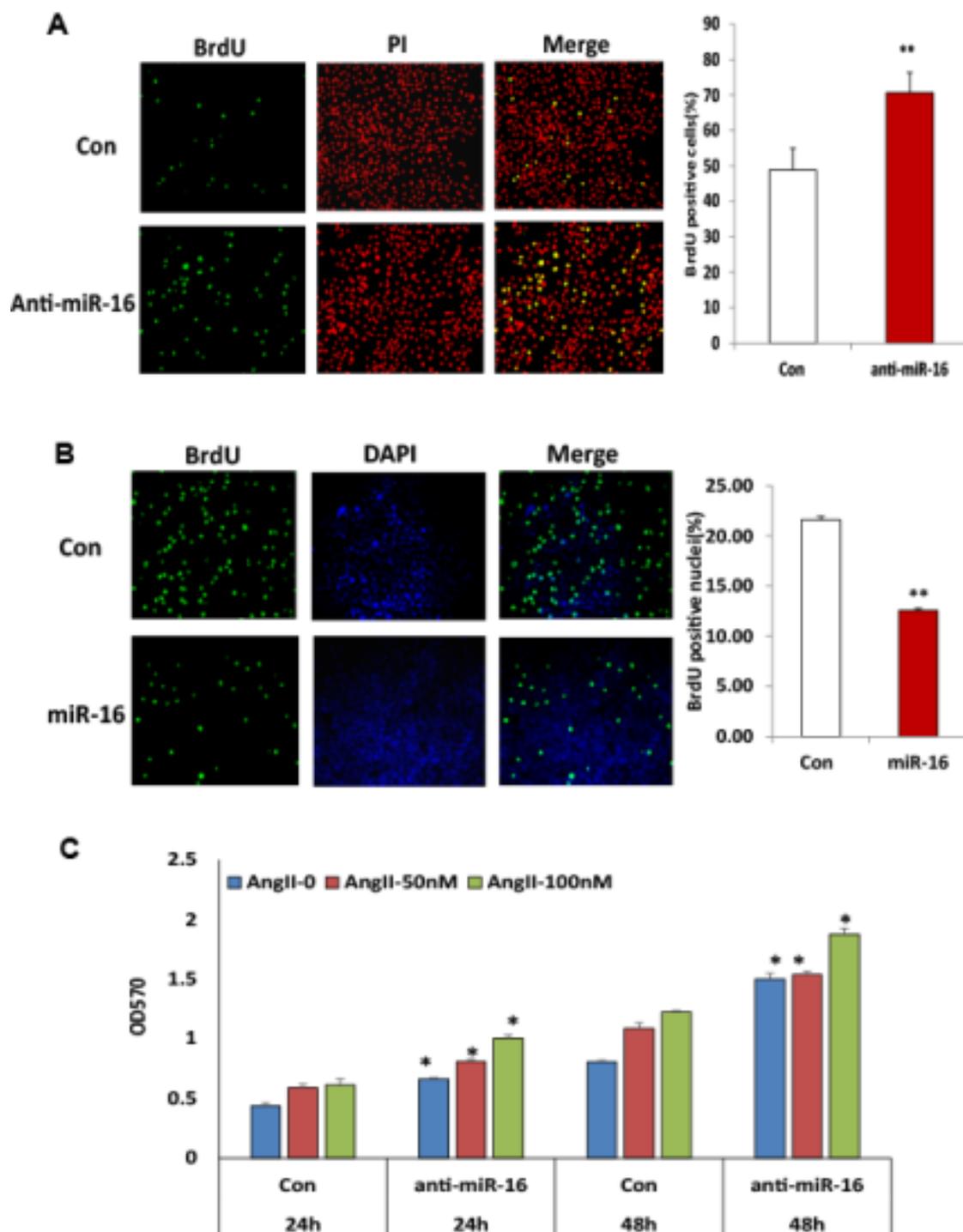
miRNAs function by inhibiting target gene expression at the posttranscriptional level. miR-16 targets cell cycle protein cyclin D1 in VSMCs. To determine whether miR-16 is involved in Ang II regulated cell cycle pathways, we treated miR-16KD and control cells with Ang II and then examined cell cycle genes CDK6 and p21 expression using Western blot. Knockdown of miR-16 significantly promoted Ang II induced CDK6 but inhibited p21 expression (Figure 4A). In addition, we examined two VSMC cell survival pathways ERK1/2 and p38 to determine whether miR-16 was involved in Ang II regulated cell survival signaling in VSMCs. miR-16KD and control cells were treated with 100 ng Ang II for 5 and 10 min following serum starvation. Knockdown of miR-16 expression promoted Ang II-induced activation of ERK1/2 and p38 (Figure 4B). Our results demonstrated that knockdown of miR-16 enhanced Ang II-induced cell survival signaling pathways.

### Discussion

In this study, for the first time we showed that miR-16 was highly expressed in VSMCs with a comparable level to miR-21, miR-145

and miR-143. miR-16 can be expressed from miR-16-1 and miR-16-2 two different clusters, which may lead to a relatively high expression level compared to other family members in the miR-15 family such as miR-15a and miR-15b. Ang II mediated signaling pathways in VSMCs are very important and dysregulation is associated with various cardiovascular diseases including hypertension, restenosis and atherosclerosis. We have identified that miR-16 was involved in Ang II mediated signaling pathway in VSMCs. Ang II downregulated miR-16 expression in VSMCs. It was reported previously that Ang II regulated multiple miRNA expression in VSMCs including up or downregulated miRNAs, for example, miR-132 was upregulated by Ang II and miR-15b, one of miRNAs in miR-15 family was downregulated by Ang II in VSMCs [34]. Long non-coding RNAs (lnc RNAs) were also involved in Ang II signaling pathways in VSMCs that lnc-Ang362 was regulated by Ang II and functioned as a host for miR-221 and miR-222 to promote VSMC proliferation [35]. Those studies including ours demonstrated that Ang II functions in VSMCs not only by regulating coding genes, but also non-coding RNAs including miRNAs and lncRNAs.

Knockdown of miR-16 led to the inhibition of Ang II-induced cell proliferation and migration in VSMCs. miRNAs function by targeting multiple genes and downstream pathways. miR-16 targeted cell cycle associated genes such as cyclin D1, D2 and cyclin E1 in cardiomyocytes and promoted cardiomyocyte hypertrophy [33]. miR-16 also targeted other genes such as FGFR2 and VEGFR2 in vascular endothelial cells, thus disrupted angiogenesis [29]. We have shown that miR-16 targeted cyclin D1 in VSMCs and knockdown of miR-16 led to upregulation of cyclin D1 expression, indicating that knockdown of miR-16 enhanced

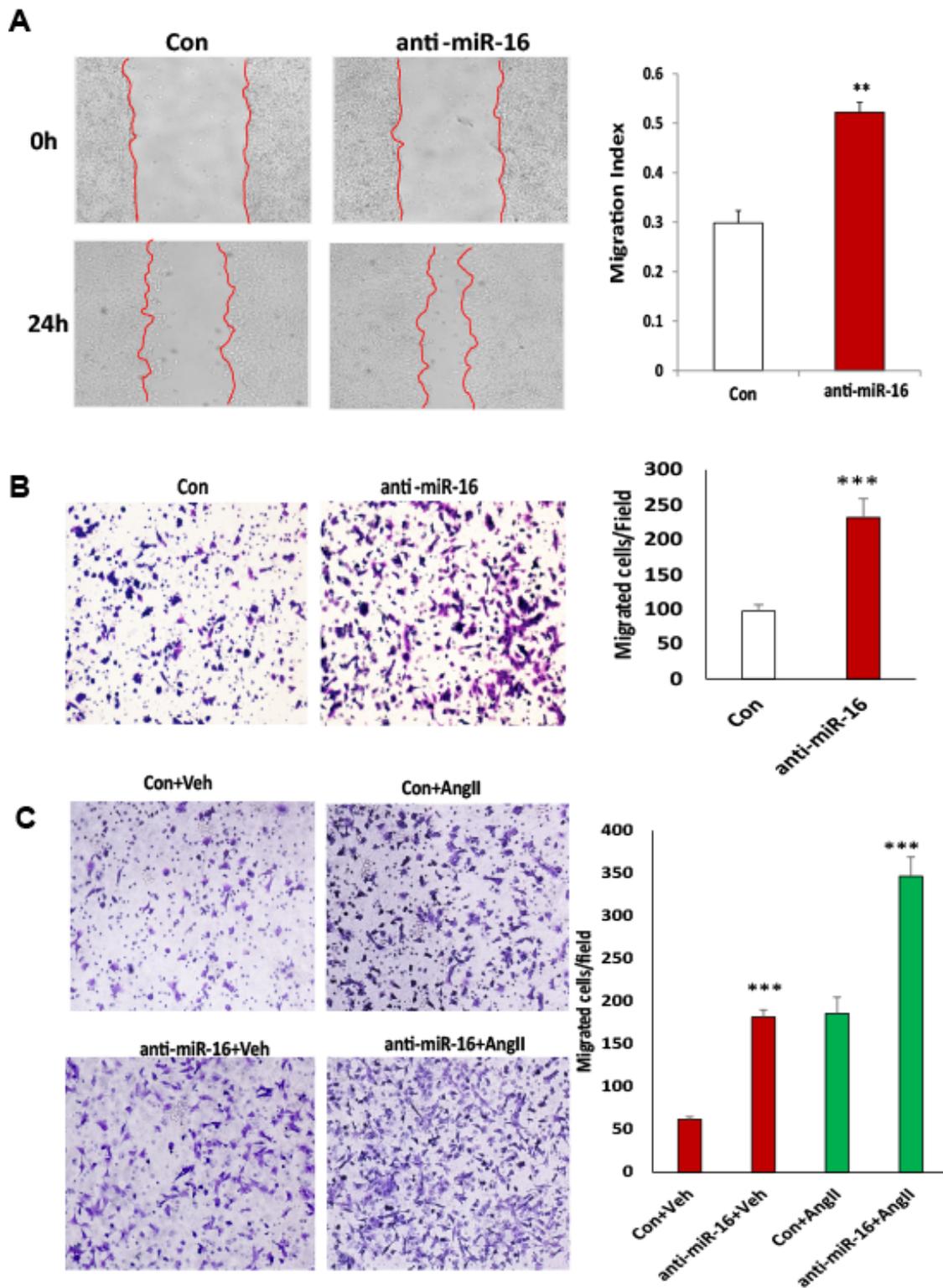


**Figure 2:** Knockdown of miR-16 promoted Ang II-induced cell proliferation.

A. Cell proliferation in miR-16KD cells was measured by BrdU labelling. Cell nuclei were stained with PI (\*\*p<0.01).

B. Cell proliferation in miR-16 overexpression was measured by BrdU labelling; Cell nuclei were stained with DAPI (\*\*p<0.01).

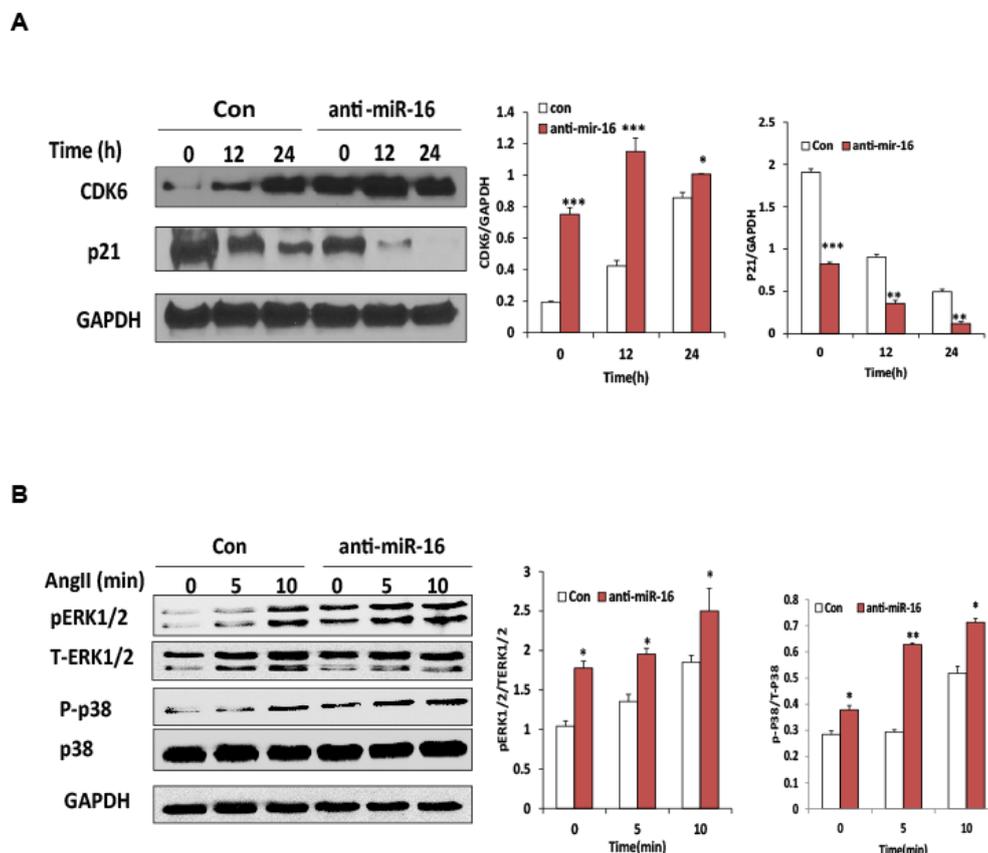
C. Cell proliferation in VSMCs transduced with lentiviral anti-miR-16 and control (Con) vectors were treated with 50 and 100 nM Ang II for 24 and 48 h and measured with MTT assay (\*p<0.05;\*\*p<0.01).



**Figure 3:** Knockdown of miR-16 promoted Ang II-induced cell migration.

A, B. Cell migration in VSMCs transduced with lentiviral anti-miR-16 and control vectors was determined using a wound healing assay and transwell plates (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

C. Ang II induced cell migration in VSMCs transduced with lentiviral anti-miR-16 and control vectors was examined using transwell plates (\*\*\* $p < 0.001$ ).



**Figure 4:** Knockdown of miR-16 enhanced Ang II activated cell proliferative pathways. A. Cell cycle proteins in VSMCs transduced with lentiviral anti-miR-16 and control vectors were examined using Western blot following Ang II treatment for 12 and 24 h. B. Two cellular survival pathways ERK1/2 and p38 in VSMCs transduced with lentiviral anti-miR-16 and control vectors were examined by Western blot following Ang II treatment (\* $p < 0.05$ ; \*\* $p < 0.01$ ).

Ang II stimulated cell growth. Although miR-16 may target other multiple genes in VSMCs, it is essential to further identify those target genes to determine how miR-16 is involved in Ang II mediated multiple functions using gain and loss of functions. There are also other components in RAS system and it is not clear whether miR-16 directly or indirectly regulates angiotensinogen, ACE-1, ACE-2, AT<sub>1</sub>R and AT<sub>2</sub>R. Further studies are needed to understand how miR-16 regulates Ang II mediated functions through those components.

It is well known that Ang II promotes VSMC growth by activating cellular survival pathways. We showed that knockdown of miR-16 enhanced the cellular proliferative signaling pathways ERK1/2 and p38, suggesting that miR-16 functions by antagonizing Ang II signaling in VSMCs. In our recent studies, we generated transgenic rats expressing miR-15b/16-2 cluster and found that overexpression of miR-15b/16-2 attenuates Ang II induced hypertension and also resulted in reduced vascular reactivity induced by vasoconstrictor phenylephrine and endothelial-1. Both ERK1/2 and p38 pathways were attenuated in the thoracic aorta of 15b/16-2 in transgenic rats (Data not shown). Our data demonstrated that knockdown of miR-16 enhanced the VSMC proliferative signaling induced by Ang II.

## Conclusion

In summary, our study demonstrated that miR-16 was a key regulator of VSMC growth and migration and involved in Ang II mediated cell

signaling in VSMCs. Overexpression of miR-16 is a novel approach in treating VSMC vascular diseases by inhibiting VSMC proliferation.

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