Original Research

miRNA-9 inhibits apoptosis and promotes proliferation in angiotensin II-induced human umbilical vein endothelial cells by targeting MDGA2

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Hypertension is a universal risk factor for a variety of cardiovascular diseases. Investigation of the mechanism for hypertension will benefit around 40% of the world's adult population. MicroRNA is crucial for the initiation and progression of cardiovascular diseases. In this study, angiotensin II-treated human umbilical vein endothelial cells were used as a model to imitate the pathological changes in endothelial cells under hypertensive conditions. We demonstrated that microRNA-9 (miR-9) suppressed angiotensin II-induced apoptosis and enhanced proliferation in human umbilical vein endothelial cells. Direct interaction between miR-9 and mitochondria associated membrane domain containing glycosylphosphatidylinositol anchor 2 (MDGA2) was determined. Moreover, miR-9 suppressed MDGA2 levels by binding to the 3' UTR site of the MDGA2 gene. This negative regulation of MDGA2 by miR-9 significantly increased proliferation and decreased apoptosis. Re-introduction of MDGA2 in the miR-9 overexpressed human umbilical vein endothelial cells and normalized proliferation, apoptosis, and the cell cycle. In summary, the present study demonstrated miR-9 inhibited expression of MDGA2 leading to the inhibition of apoptosis and promotion of proliferation in angiotensin II-treated human umbilical vein endothelial cells.

Keywords

miRNA-9; MDGA2; human umbilical vein endothelial cells; proliferation

1. Introduction

Hypertension is a major risk factor for the development of stroke, coronary artery diseases, heart failure, and renal disease (Stocker et al., 2017). Damage and dysfunction of endothelial cells have been found critical in the initiation of hypertension and other cardiovascular diseases (Brandes, 2014). Damage of endothelial cells is proportionally correlated with the severity of hypertension (Brandes, 2014). Previous studies have demonstrated that endothelial cell injury results in elevated vascular permeability, vasoconstriction, and increased blood pressure (Dzau, 1990). These changes further disrupted the vascular endothelial cell function

(Dzau, 1990). The vascular endothelium is important for the investigation of mechanisms for hypertensive genesis and progression.

MiRNAs are a family of small noncoding RNAs composed of 22-26 nucleotides. They can suppress post-transcriptional expression of genes by targeting the 3'-untranslated regions of mRNA. MiRNAs have been involved in a variety of physiological and pathological processes such as cell differentiation and proliferation, inflammation, and carcinogenesis (Rupaimoole and Slack, 2017). It has been widely acknowledged that miRNAs are also associated with cardiovascular diseases (Marques and Charchar, 2015). For example, downregulation of miRNA-133 (miR-133) has been observed to lead to significant cardiac hypertrophy (Carè et al., 2007). Cardiac-specific miR-208 derived from the intron of the αMHC gene was *de rigueur* for cardiomyocyte hypertrophy and fibrosis in response to stress and hypothyroidism (Williams et al., 2009). MiR-145 is abundantly expressed in the pulmonary smooth muscle cells and knock-out or inhibition of miR-145 showed significant protection from pulmonary arterial hypertension (Caruso et al., 2012). Elevation of miR-145 in the smooth muscle cells from the pulmonary artery has been observed in patients diagnosed with pulmonary arterial hypertension (Caruso et al., 2012). Mature miR-155, found down-regulated in pregnant women with severe pre-eclampsia, reduced angiotensin II-induced activation of ERK1/2 signaling (Cheng et al., 2011). Identification of novel miRNA targets in the pathogenesis of hypertension would significantly contribute to the development of new therapeutic approaches.

The sequence of miR-9 is conserved from flies to humans, however, the expression pattern differs (Yuva-Aydemir et al., 2011). It was found that the expression level of miR-9 was down-regulated in the peripheral blood mononuclear cells extracted from hypertensive patients and positively correlated with the pulse pressure (mean, 24 h) and left ventricular mass index (Kontaraki et al., 2014). These findings indicated that miR-9 may be associated with target-organ damage in hypertensive patients. MiR-9 greatly influenced migration and angiogenesis of endothelial cells (Zhuang et al., 2012) and transcription of vascular endothelial growth factors (Ma et al., 2010). However, the influence of miR-9 on the
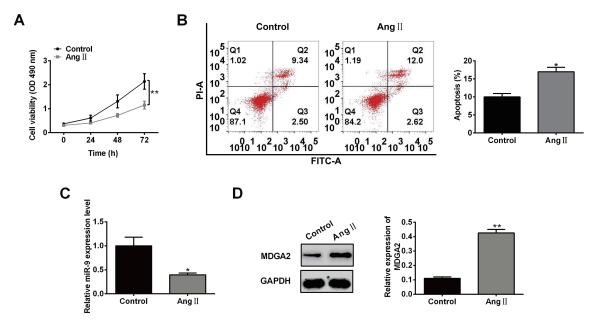
Figure 1. Angiotensin II-induced HUVEC apoptosis is associated with decreased miR-9 and increased MDGA2. HUVECs were treated with angiotensin II for indicated timeframes and were subjected to MTT assay (A) and flow cytometry analysis (B). (C) miR-9 level was determined in the HUVECs treated with angiotensin II for 72 hrs. (D) mRNA and protein levels of MDGA2 were measured in the control and in the angiotensin II-treated HUVECs. ** indicated $P < 0.01$.

endothelial cells in the context of hypertension remains uninvestigated. MAM (mitochondria associated membrane) domain containing glycosylphosphatidylinositol anchor 2 (MDGA2) was initially found to be widely expressed in the central neurons and regarded critical to the development of central diseases (Connor et al., 2016; Joset et al., 2011). Recent studies showed MDGA2 inhibited cell proliferation and induced cell apoptosis, the function of which resembled tumor suppression. Its hypermethylation was proposed to be an independent prognostic marker for gastric cancer (Wang et al., 2016). The role of MDGA2 in hypertension has not been thoroughly studied.

In the current study, the human umbilical vein endothelial cells (HUVECs) sharing similarities to the vascular endothelial cells were used along with angiotensin II to generate a hypertension-like endothelial cell model. The influence of miR-9 on proliferation and apoptosis of HUVECs and interaction with MDGA2 were investigated to demonstrate the miR-9 mediated molecular mechanisms for endothelial dysfunction over hypertension.

2. Material and methods

2.1 Cell culture

HUVECs were obtained (Lonza SanDiego, CA), cultured in M199 medium, supplemented with fetal bovine serum (FBS, 20%), given penicillin and streptomycin (2 mM), amphotericin B (2 mM), endothelial cell growth supplement (40 μg/mL), and heparin (Sigma, St. Louis. MO, 100μg/mL). Cells were cultured at 37°C within a humidified atmosphere containing 5% CO_2.

HUVEC cells were treated with angiotensin II for 12 hrs., 24 hrs., 48 hrs. and 72 hrs. for cell viability assay. Cells treated with angiotensin II for 72 hrs. were used for real-time PCR and western blot analysis.

2.2 Transfection

According to the manufacturer's protocol, transfections were performed using the Lipofectamine 3000 (Thermo Fisher Scientific) for overexpression (plasmid) or the RNAiMax for miRNA mimics and inhibitors. Growth medium was replaced with reduced-serum, antibiotic-free opti-MEM medium while transfection and refreshed with regular complete M199 medium 12 hrs. posterior to the transfection.

Empty pCDNA3.1 vector was used as the negative control, and pCDNA3.1 was used as the vector to construct full length MDGA2 overexpression plasmids. MiR-9 mimics inhibitors, siRNA targeting MDGA2, and corresponding controls were purchased from Sigma Aldrich.

2.3 MTT assay

Cells treated with angiotensin II were transferred into fresh medium containing 10 μL sterile MTT dye (5mg/mL). The solution was aspirated after 4 hrs. of incubation and DMSO (150 μL) was added. The absorbance of the resulting solution was read at a wavelength of 490 nm with a spectrophotometer (Shimadzu, Japan).

2.4 RNA extraction, DNA synthesis, and quantitative reverse transcript PCR (qRT-PCR)

Cells were washed with cold PBS prior to total RNA isolation using the Easypure RNA extraction kit (Transgen Biotech, Beijing, China) according to the manufacturer's protocol. Approximately 1 μg of total RNA was used to synthesize cDNA with the first-strand cDNA synthesis kit (Transgen Biotech, Beijing, China). Quantitative PCR was performed on Bio-Rad C1000 thermal cycler (Bio-Rad, Hercules, CA): 95°C for 5 min, 40 cycles of 95°C for 20 sec, 60°C for 30 sec, then 72°C for 3 min. The primers used: MDGA-2 forward GATCCTGATGTCTCTCCGGG and reverse
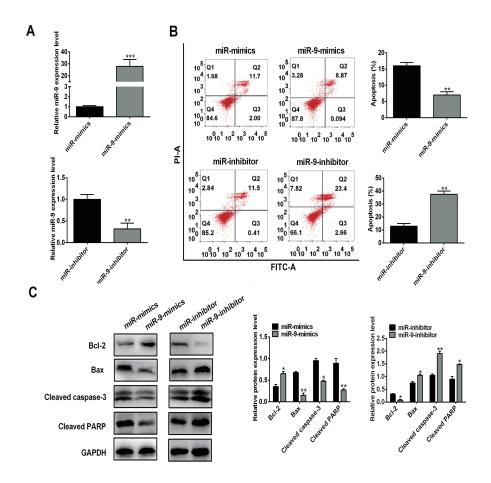
Figure 2. miR-9 negatively regulates angiotensin II-induced HUVEC apoptosis. (A) HUVECs were either transfected with overexpressed vector or inhibitor, and the level of miR-9 was measured. (B) Percentage of apoptosis of the modified cell lines was examined by flow cytometry analysis. (C) Expression levels of proteins relevant to apoptosis were measured in the aforementioned cell lines. * indicated $P < 0.05$, and **$P < 0.01$, ***$P < 0.001$.

TGGAACTGTGCTGCTGGATA, GAPDH forward CCTGCAC-CACCAACTGCTTA and reverse GGCCATCCACAGTCTTCT-GAG.

For miRNA expression, qScript microRNA cDNA Synthesis Kit (Quantabio, Beverly, MA) was used to synthesize cDNA according to the manufacturer's protocol. Quantitative PCR was performed under the following conditions: 95°C for 2 min, 40 cycles of 95°C for 5 sec and 60°C for 30 sec. miRNA qRT-PCR was performed using the miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) and miScript Primer Assay on miR-9 (Thermo Fisher, Waltham, MA). U6 small RNA Assay (Thermo Fisher, Waltham, MA) was used as an internal control for normalization.

2.5 Flow cytometry analysis of apoptosis

Cells were washed with cold PBS and stained with Annexin V conjugated with PE (Thermo Fisher, Waltham, MA, 1:20 diluted) at room temperature for 20 min. The stained cells were subjected to flow cytometry analysis after incubation.

2.6 Flow cytometry analysis of cell cycle

Cells were washed with cold PBS and fixed with cold 75% ethanol for 30 min. After three washes, cells were treated with ribonuclease A at 37°C for 20 min (Aidlab Biotechnologies, Beijing, China) and were stained with propidium iodide (50 μg/mL) at

4°C overnight (Thermo Fisher, Waltham, MA). The stained cells were analyzed using flow cytometry (BD, Franklin Lakes, NJ) with excitation at a wavelength of 488 nm.

2.7 Western blot

Cells were lysed with the RIPA buffer and proteinase inhibitor cocktail (Sigma, St. Louis, MO). Total protein was quantified by Bradford assay (Bio-Rad, Hercules, CA) with 20 μg/gel proteins for electrophoresis. The protein was transferred onto PVDF membrane and incubated with primary antibody and then the secondary after wash. The antibodies used included: anti-MDGA2, anti-BCL-2, anti-Bax (Thermo Fisher, Waltham, MA), anti-cleaved caspase-3, anti-cleaved PARP, anti-cyclin D1, anti-CDK4, anti-PCNA (Abcam, Cambridge, UK). Secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX).

2.8 Construction and luciferase assay

The 3'-UTR of Mdga2 gene was amplified and cloned into a pMIR-report vector (Thermo Fisher Scientific, Waltham, MA) located downstream of the luciferase gene. Constructed luciferase pMIR-report and internal control vectors were co-transfected at a ratio of 50:1 into HUVEC cells. The luciferase activity assay was performed 48 hrs. posterior to the transfection using the Dual-Glo luciferase assay system (Promega, Madison, WI).
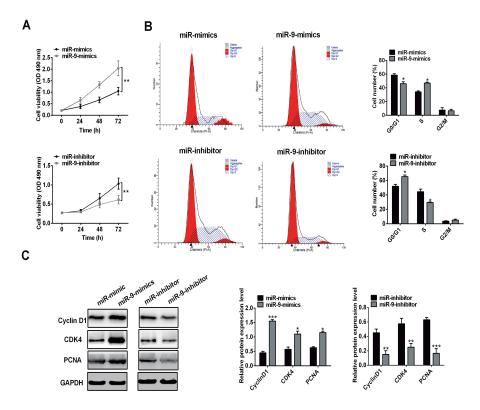
Figure 3. miR-9 positively regulates angiotensin II-induced HUVEC proliferation. (A) Cell proliferation assay was performed on the HUVECs with either miR-9 overexpressed (miR-9 mimics) or inhibited (miR-9 inhibitor). (B) Cell population within different cell cycle phases was measured with the flow cytometry. (C) Expression levels of proteins regulating the G0/G1 to S transition were measured in the aforementioned cell lines. * indicated $P < 0.05$, and **$P < 0.01$, ***$P < 0.001$.
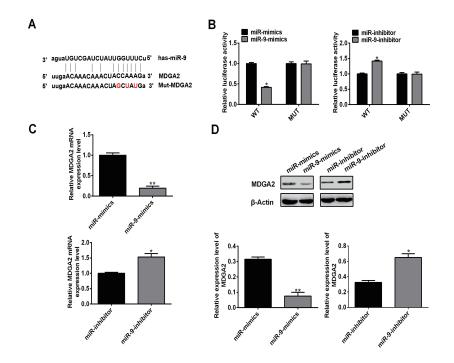

Figure 4. miR-9 directly suppressed MDGA2. (A) Predicted interaction between miR-9 and MDGA2. (B) HUVECs either with miR-9 overexpressed (miR-9 mimics) or miR-9 inhibited (miR-9 inhibitor) were transfected with luciferase vectors containing wild type 3' UTR sequence of MDGA2 or MDGA2 3' UTR sequence with mutated binding site of miR-9. Luciferase activity was measured and normalized in the transfected HUVECs. (C) mRNA and (D) protein levels of MDGA2 were measured in the miR-9 overexpressed/inhibited HUVECs. * indicated $P < 0.05$, and **$P < 0.01$.
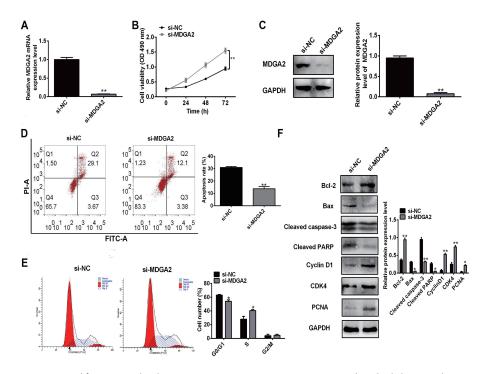
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Figure 5. MDGA2 suppresses proliferation and enhances apoptosis. (A) MDGA2 gene was knocked down in the HUVECs. (B) MTT assay was performed to evaluate the cell proliferation of the HUVECs transfected with control siRNA or siRNA targeting MDGA2. (C) western blot analysis of the HUVEC cells with control siRNA or reduced MDGA2. (D) Apoptotic rates were determined by flow cytometry with Annexin V staining. (E) Cell cycle was determined by flow cytometry with propidium iodide staining. (F) Western blot analysis of apoptosis-related and cell cycle-related proteins in the HUVECs with control siRNA or reduced MDGA2. * indicated $P < 0.05$, and **$P < 0.01$.

2.9 Statistical analysis

The statistical analyses were performed using the GraphPad© Prism. The data were analyzed by either unpaired t-test or two-way ANOVA analysis followed by multiple comparisons. For the MTT assay, two-way ANOVA was used to demonstrate the significance comparing the group parameters.

3. Results

3.1 Angiotensin II induces HUVEC apoptosis and decreases miR-9 expression

To confirm that angiotensin II induced human endothelial cell apoptosis (Dimmeler et al., 1997), the cell viability and cell apoptosis were investigated in the HUVEC + angiotensin II model. We observed a decreased (Fig. 1A) and increased apoptosis (Fig. 1B) in angiotensin II-treated HUVEC cells compared with the control group. In these angiotensin II-treated cells, a significant reduction in the expression level of miR-9 was observed (Fig. 1C), accompanied with significant increase in MDGA2 at both protein and mRNA levels (Fig. 1D).

3.2 miR-9 plays a negative role in angiotensin II-induced HUVEC apoptosis

To further investigate the role of miR-9 in the angiotensin II-treated HUVEC cells, miR-9 was overexpressed or inhibited to observe the corresponding changes in the cell model. qRT-PCR was used to confirm the overexpression and inhibition of miR-9 (Fig. 2A). Decrease and increase in cell apoptosis was observed in the miR-9-overexpressed and miR-9 inhibited cells, respectively (Fig. 2B). Furthermore, the expression levels of multiple proteins involved in the classic apoptosis pathway were evaluated and were

found significantly reduced in the miR-9-overexpressed cells: Bax, cleaved caspase-3, cleaved PARP as well as BCL-2, an inhibitor of pro-apoptotic proteins (Fig. 2C). The opposite was observed in the miR-9-inhibited cells (Fig. 2C).

3.3 miR-9 plays a positive role in angiotensin II-induced HUVEC proliferation

The influence of miR-9 on proliferation was investigated using the aforementioned miR-9 overexpressed and miR-9 inhibited cells. Results of MTT assay showed increase in proliferation in the miR-9 overexpressed cells, but decrease in the miR-9 inhibited cells (Fig. 3A). MiR-9 overexpression resulted in a significantly reduced number of cells in the G0/G1, but increased cell population in the S phase (Fig. 3B). The opposite was observed in the miR-inhibited cells (Fig. 3B). Results of western blot showed the expression levels of several proteins relevant to the cell cycle (cyclin D1, CDK4 and PCNA) increased in the miR-9-overexpressed cells (Fig. 3C), but decreased in the miR-9 inhibited cells (Fig. 3C),. This suggests that miR-9 induced expression of cyclin D1, CDK4 and PCNA lead to the enhanced cell population in the S phase of the cell cycle.

3.4 miR-9 directly interacts with MDGA2 to inhibit its expression

The major function of miRNAs is to regulate the expression of target genes. The target binding gene of miR-9 was predicted using the open resource website targetscan.org. A binding site at 3' UTR region of MDGA2 was found to be a potential target of miR-9 (Fig. 4A). In order to investigate if miR-9 was able to directly regulate the expression of MDGA2, luciferase assay was
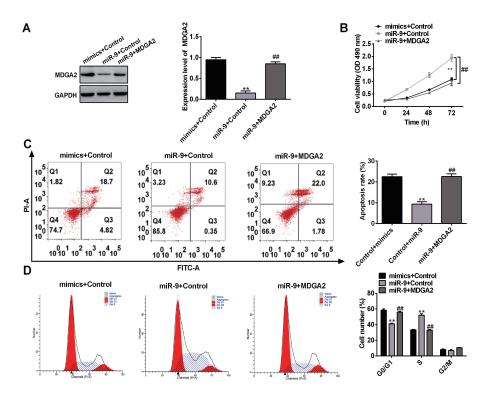
Figure 6. miR-9-suppressed apoptosis is dependent on MDGA2. (A) western blot analysis of MDGA2 level in HUVECs transfected with: control miR-9 mimics and control MDGA2 expressing vector, miR-9 miRNA and control MDGA2-expressing vector, miR-9 and MDGA2 expressing vector, respectively. (B) MTT assay was performed to measure the proliferation of the three cell lines. (C) Apoptotic rate was determined by flow cytometry analysis of Annexin V staining. (D) Cell cycle was analyzed by flow cytometry with prodidium iodide staining. ** indicated $P < 0.01$, ##$P < 0.01$.

performed. The results showed that the relative luciferase activity significantly decreased in the miR-9 overexpressed HUVEC cells, while the mutation at the miR-9 potential binding site in the 3'UTR region of MDGA2 nullified this effect (Fig. 4B). The luciferase activity increased in the miR-9 inhibited HUVEC cells, while mutation of miR-9 binding site nullified the miR-9 regulatory effect (Fig. 4B).

Given the fact that miR-9 could regulate the 3' UTR region of MDGA2, it was speculated that the changes in expression level of miR-9 in the HUVEC should influence the expression level of MDGA2. MDGA2 at mRNA and protein levels were then measured. The results showed a negative correlation between the expression level of miR-9 and MDGA2 in the HUVEC cells, as shown at mRNA level in Fig. 4C and at protein level in Fig. 4D. The above observations suggested that miR-9 directly bound to the 3' UTR site of MDGA2 negatively regulated the expression level of MDGA2.

3.5 Knockdown of MDGA2 significantly impacts angiotensin II-induced apoptosis and proliferation

With the above conclusion in hand, it was further speculated that MDGA2 may participate in the regulation of apoptosis and proliferation. Transfection of siRNA mix was confirmed to effectively lower the expression levels of MDGA2 mRNA (Fig. 5A) and protein (Fig. 5C). Increased proliferation (Fig. 5B) and decreased apoptosis (Fig. 5D) upon angiotensin II treatment were observed in the HUVEC cells. Cell cycle analysis showed elevation in the

cell population in the S phase and decreased in the G0/G1 phase (Fig. 5E). Expression levels of proteins (i.e. Bax, cleaved caspase-3, cleaved PARP) in the classic apoptosis pathway were found significantly reduced, apart from the increased expression of BCL-2 (Fig. 5F). Significant increase in the expression of cyclin D1, CDK4, and PCNA was also observed, which promotes the transition of cells from the G0/G1 phase to the S phase (Fig. 5F).

3.6 Impacts of miR-9 on angiotensin II-induced HUVEC apoptosis and proliferation are MDGA2 dependent

In order to demonstrate the effects of MDGA2 and miR-9 combined on HUVEC cell proliferation and apoptosis, a rescue experiment was performed. MDGA2 was overexpressed in the miR-9 overexpressed HUVEC cells, and Fig. 6A confirmed the successful expression of the exogenous MDGA2 protein. Proliferation, apoptosis, and cell cycle assays were performed on the modified cells treated with angiotensin II. The results of the proliferation assay showed increased proliferation by miR-9. Overexpression of MDGA2 in the miR-9 overexpressed cells led to significant decrease in proliferation (Fig. 6B). Annexin V staining showed significant elevation in the apoptosis of the HUVEC cells with both miR-9 and MDGA2 overexpressed compared to cells with only miR-9 overexpressed (Fig. 6C). Cell cycle analysis demonstrated that overexpression of MDGA2 in the miR-9 overexpressed cells resulted in the similar cell population in the G0/G1 and S phases to those in the HUVEC cells expressing miR-9 mimics and MDGA2 control (Fig. 6D).
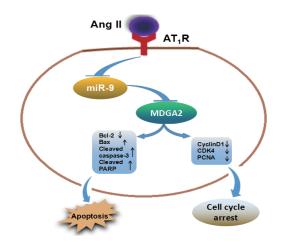
Figure 7. Schematic figures of miR-9/MDGA2 in cell apoptosis and cell cycle arrest. The binding of angiotensin II and its receptor AT1R led to suppression of miR-9. miR-9 negatively regulated the expression of MDGA2, further regulating the expression of apoptosis-related genes and the cell cycle arrest-related genes. Upregulation of miR-9 could thus aid the human endothelial cells to survive the angiotensin II induced abnormalities.

4. Discussion

Hypertension is a condition when effectively treated, may significantly reduce cardiovascular diseases. A number of mechanisms for hypertension have been demonstrated including the involvement of both peripheral and central renin angiotensin systems (Cabandugama et al., 2017), dysfunction of endothelial cells (Brandes, 2014), overactivation of sympathetic systems (Mancia and Grassi, 2014), intestinal gut dysbiosis (Yang et al., 2015), and the complex interactions among organs (Yang and Zubcevic, 2017). The present study focused on the endothelial cells, which are the direct communicator to blood pressure. Previous studies found that damage to endothelial cells significantly influenced the vascular system physiology (Brandes, 2014). The HUVEC cells were used with the pre-treatment of angiotensin II, a potent vasoconstrictor that induced pathological changes (Dimmeler et al., 1997).

The importance of microRNAs in cardiovascular diseases is rising (Marques and Charchar, 2015). *In vivo* studies showed that angiotensin II induced changes in the microRNA profile during hypertension (Eskildsen et al., 2013). The miR-22 inhibitor significantly reduced blood pressure (Friese et al., 2013), indicating that microRNA could be targeted for hypertension treatment. miR-9 was examined in the angiotensin II-treated HUVEC cells due to significant down-regulation of miR-9 in the peripheral blood mononuclear cells that were collected from hypertensive patients (Kontaraki et al., 2014). Moreover, expression level of miR-9 was closely associated with mean pulse pressure and left ventricular mass index (Kontaraki et al., 2014). MiR-9 was significantly reduced in the hypertension-like endothelial cell model, which was characterized by an increase in apoptosis and decrease in proliferation. Subsequent experiments with overexpression or inhibition of miR-9 in the cell model confirmed miR-9 acted as an apoptosis suppressor and a proliferation inducer.

An open resource target scan was used to predict the target un-der the direct regulation of miR-9; MDGA2 was found and then confirmed to be negatively regulated by miR-9 in the miR-9 overexpression or inhibition cell lines. MDGA2 is mainly expressed in the neurons and plays an important role in autism (Connor et al., 2016; Joset et al., 2011). Additionally, it suppresses excitatory synapse development, and its mutation leads to elevated excitation and hyperconnectivity among neurons (Connor et al., 2016). Until recently, MDGA2 was proposed to be a tumor suppressor in gastric cancer (Wang et al., 2016); however, little is known about MDGA2 in the endothelial cells. It was hypothesized that MDGA2 may function as a negative regulator in hypertension-like endothelial cells. The results showed that knocking down MDGA2 increased proliferation and reduced apoptosis, which indicates a similar role of MDGA2 in gastric cancer cells and endothelial cells. An MDGA2-expressing vector was transfected into the miR-9 overexpressed cells to rescue the inhibition of MDGA2. It was observed that expression level of MDGA2 was normalized along with miR-9-induced increased proliferation, decreased apoptosis, and an altered cell cycle. Therefore, it was concluded that miR-9-induced changes through the regulation of MDGA2.

The role of miRNAs in the regulation of cardiovascular diseases has attracted broad research interest (Marques and Charchar, 2015; Kontaraki et al., 2014). Racial differences of miRNA expression have also been demonstrated in the case of hypertension (Dluzen et al., 2016). Modulation of miRNA to regulate protein expression is a potential approach to treat cardiovascular disease. The present study utilized HUVECs as a model to demonstrate the effect of miR-9. The study provided promising results but with limitations such as the lack of tissue-specific targeting (Marques and Charchar, 2015). Due to the ubiquitous expression of miRNAs *in vivo*, side effects of miRNA therapy are difficult to predict. The delivery and side effects of miR-9 were thus required to be further evaluated *in vivo*. Moreover, the potential mechanisms (i.e. defects in miRNA processing, DNA methylation, etc) relevant to miRNA regulation are also of interest to be investigated.

5. Conclusion

In summary, our results showed that angiotensin II signaling suppresses the expression of miR-9, which influences the proliferation and apoptosis of hypertension-like endothelial cell. This observation plays a role in endothelial cells that are related to the mechanisms for hypertension. Moreover, this effect of miR-9 was manifested through its suppression of MDGA2, and its downstream proteins relevant to cell apoptosis and cell cycle arrest (Fig. 7). This was the first work to show the suppression of MDGA2 in the endothelial cells in the context of hypertension suggesting a potential therapeutic target for the treatment of hypertension.

Conflict of Interest

The authors wish to confirm that there are no known conflicts of interest associated with this publication.

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