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LncRNA AK094457 promotes AngII-mediated hypertension and endothelial dysfunction through suppressing of activation of PPARγ

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ABSTRACT

Hypertension is one of the major risk factors for cardiovascular disease worldwide and is striking more young people, which is characterized by impaired vascular endothelial function. To find the functional lncRNAs associated with hypertension, high throughput lncRNA microarray were used to analyze expression profile of the lncRNAs in the aortic vascular endothelial cells (VECs) of spontaneously hypertensive rats (SHRs). The tail vein injection of siRNA was used to study the influence of lncRNA AK094457 inhibition on endothelial function in vivo. In vitro, endothelial function was studied in endothelial cells transfected with lncRNA AK094457-over-expressed vectors and siRNAs. pPPARγ and iNOS protein levels were detected with Western blot. Elisa assay was used to analyze the secretion of AngII, ET-1, ROS and LDH level. The nitrite/nitrate (NO_2^-/NO_3^-) concentration was measured using a colorimetric assay. LncRNA AK094457 was a most upregulated lncRNA in SHRs. It is showed that downregulation of AK094457 significantly reduced rat arterial pressure, increased activation of endothelial PPARγ, and suppressed serum contents of AngII and NO in vivo. Furthermore, results from gain-and-loss of function in primary aortic endothelial cells indicated that AK094457 negatively regulated activation of PPARγ and promoted AngII-mediated endothelial dysfunction, manifested by decreased capacities of cell proliferation and migration, and increased levels of ROS production and LDH release. In conclusion, lncRNA AK094457 is identified as a key regulator in blood pressure and endothelial function, which can increase AngII-induced hypertension and endothelial dysfunction via suppression of PPARγ.

1. Introduction

Hypertension is a common chronic disease and one of the major risk factors for cardiovascular disease worldwide. With economic growth of China, the prevalence of hypertension has increased in the past several decades [1]. At present, it has become a major public health challenge and is striking more young people. Hypertension is influenced by multiple environmental and genetic determinants and is characterized by impaired vascular endothelial function [2]. Disruption of endothelial cell function is characterized by impaired bioavailability of NO and induces vascular disease [3]. Kinds of drugs have been used for the management of blood pressure. However, hypertension can be controlled by drugs, but it's hard to eradicate, because of high incidence of complications such as inflammation and vascular diseases [4,5].

The renin-angiotensin system (RAS) is an important humoral regulation system composed of a series of peptide hormones and enzymes. RAS plays an important role in regulating and maintaining normal blood pressure and the balance of water and electrolyte. It is now revealed that RAS is deeply involved in the pathogenesis of hypertension [6,7]. Angiotensin II (Ang II) is a main active substance in RAS. It not only plays a key role in acute and chronic regulation of systemic arterial blood pressure, but also is an important regulator of cardiovascular function [8,9]. AngII triggers vascular damage via several mechanisms, including oxidative stress, inflammation and apoptosis of endothelial cells and endothelial progenitor cells [10]. Peroxisome proliferator-activated receptor (PPAR)-γ is famous as a master regulator of adipogenesis. Recently, PPAR-γ was reported to play a key role in vascular dysfunction. For example, endothelial PPAR-γ interacted with angiotensin 1–7 responses and activated oxidant-inflammatory signaling and Rho kinase [11]. Activation of brain PPAR-γ was used to reduce central inflammation and brain RAS activity in Ang II-dependent hypertension [12].

Long non-coding RNAs (LncRNAs) are non-protein coding RNAs longer than 200 nucleotides with high abundance in human beings [13]. It functions in many complicated biological events and has attracted increasing attention in recent years. The emerging evidence

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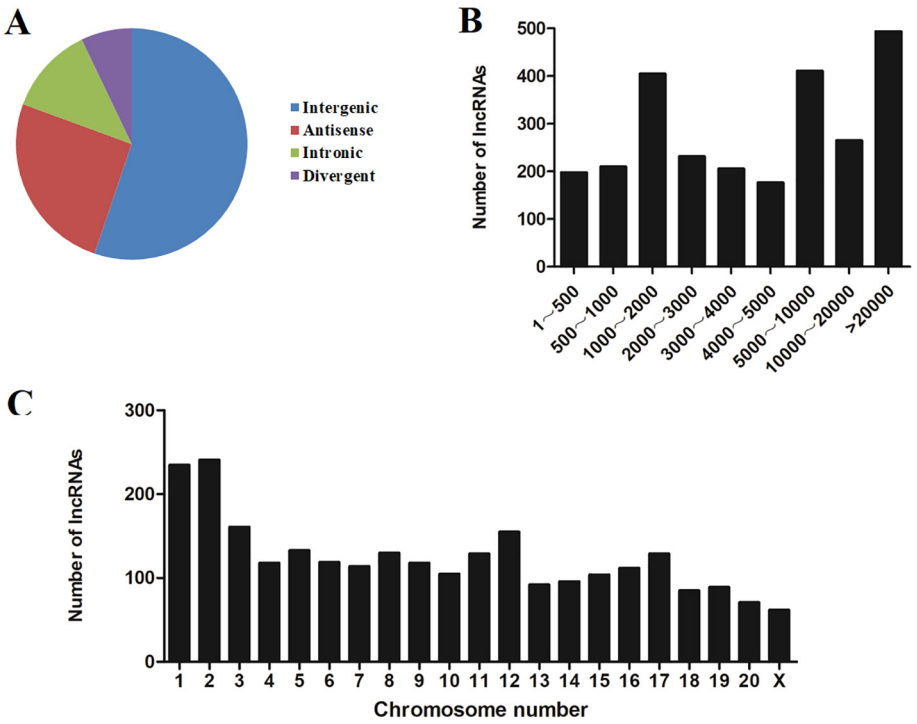

Fig. 1. Microarray expression profile of lncRNAs in the aortic endothelial cells isolated from SHRs. A, Percentage of differentially expressed lncRNAs transcribed from intergenic, antisense, intronic and divergent B, the distribution of differentially expressed lncRNAs with different lengths C, the number of differentially expressed lncRNAs on different chromosomes.

indicates that dysregulation of lncRNAs is associated with development of kinds of human diseases [14]. For example, in Qian's study, loc285194 was reported as a direct target for p53 and inhibited tumor cell growth [15]. Though the significance of lncRNAs is being increasingly recognized in hypertension, it remains nearly completely unexplored. The vascular endothelium is located in the inner layer of blood vessels and regulates vascular wall function [16]. Thus, it has a crucial role in the pathogenesis of hypertension. In this study, we analyzed expression profile of the lncRNAs in the aortic vascular endothelial cells (VECs) isolated from spontaneously hypertensive rats (SHRs). A most upregulated lncRNA AK094457 has been previously reported to be upregulated in hypertensive patients, but it is really unknown about its function in endothelial cells and the development of hypertension [17]. We suggested that lncRNA AK094457 was related to hypertension through suppressing the activation of PPARγ and accelerated Angiotensin II (Ang II) -induced vascular dysfunction.

2. Methods

2.1. Animals

Male SHRs ($n = 20$, weighing 361 ± 22 g) and male normotensive Wistar Kyoto (WKY) rats (not treated, wide type controls, $n = 10$, weighing 326 ± 19 g) were purchased from Charles River (Beijing, China) at an age of 10 weeks. Animals were housed in standard cages in temperature-controlled environment, fed with standard diet and tap water ad libitum and kept on a 12-h light-dark cycle [18]. Tail-cuff sphyg momanometer was used to measure the blood pressure of each animal. The aorta was isolated to the common iliac artery and cut into D-hanks fluid. After removal of the adventitia and fat tissue, the Endothelial cells were cultured as previously reported [19]. The animal experiments were conducted in accordance with the National Instructions of Health Guide for the Care and Use of Laboratory Animals.

2.2. Microarray

SHRs and control rats were used for microarray analysis. RNA was extracted with Trizol (Invitrogen, CA). 5 μg of total RNA was used to synthesize Double-strand cDNA (ds-cDNA) by Invitrogen SuperScript ds-cDNA synthesis kit. ds-cDNA was hybridized using the Human 12 × 135 k Long Non-coding RNA Array (Roche, USA) [12]. The slides were washed in an ozone-free environment and scanned with the Axon GenePix 4000B microarray scanner. The expression data analysis was performed with NimbleScan software (version 2.5). Differentially expressed lncRNAs were identified with the following criteria: a false discovery rate (FDR) -adjusted $P < 0.05$ and a fold change > 1.5. Hierarchical clustering GO analysis and Pathway analysis was performed by KangChen Bio-tech, Shanghai, China.

2.3. RT-qPCR

Total RNA was isolated and reverse transcribed by using Primescript RT master mix (Takara, Biotech, Shiga, Japan). SYBR® Premix Ex Taq™ II kit (Takara, Biotech, Shiga, Japan) was used for real-time PCR. We used GAPDH as a reference gene. $2^{-\Delta\Delta Ct}$ method was performed to analyze the relative expression level.

2.4. Western blot

Sample cells were harvested in RIPA buffer with 1 × Protease Inhibitor Cocktail (Roche Applied Science). Protein concentration was measured by a BCA protein assay kit (Pierce, Rockford, USA). Proteins were separated with SDS-PAGE and transferred onto a nitrocellulose membrane (NC; Millipore, Boston, MA, USA). The membranes were incubated with primary antibodies: anti-PPARγ (ab59256, 1:500, Abcam, USA), anti-AngII (ab18801, 1:500, Abcam), anti-eNOS (ab76198, 1:500, Abcam) and anti-GAPDH (ab8245, 1:1000, Abcam). The horseradish peroxidase conjugated antibodies (1:2, 000; bs-0295-HRP; Bioss, Beijing, China) was used as a secondary antibody. Signals were detected with western chemiluminescent HRP substrate (Millipore, USA). Image J was used for statistical analysis.

2.5. Elisa assay

Elisa assay was performed to determine AngII, Endothelin-1 (ET-1), reactive oxygen species (ROS) and LDH level with Elisa kits (R&D

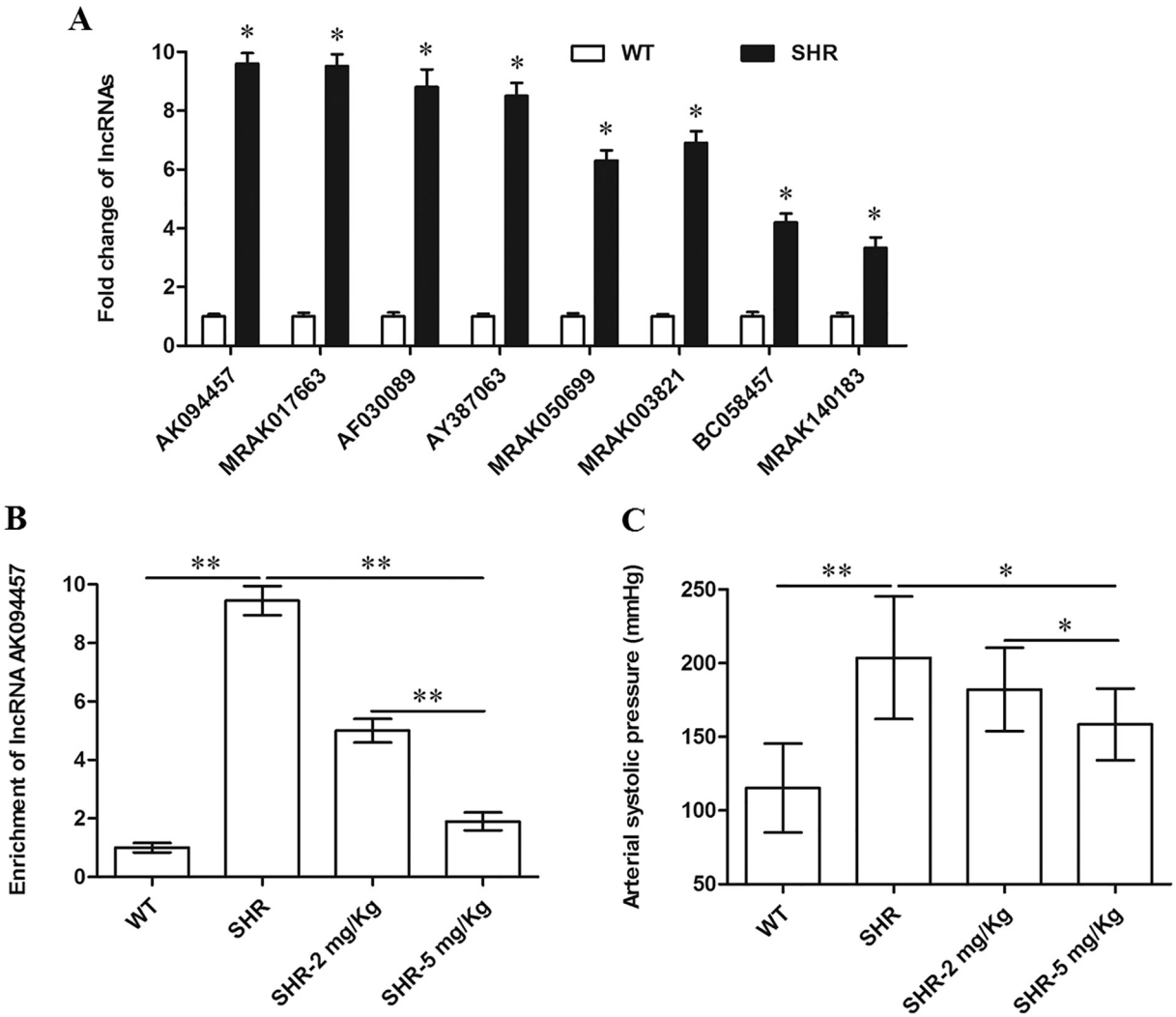

Fig. 2. Expression of lncRNA AK094457 correlates with the arterial systolic pressure. A, the top 10 significant up-regulated lncRNAs (except for them from divergent region) of endothelial cells from SHR were validated with RT-PCR. B, lncRNA AK094457 was detected in SHRs with tail injection of 2 mg/kg or 5 mg/kg siRNAs weekly for a total of 12 weeks and control group. C, systolic pressure was measured in tail injected SHR and control group after training for 7 days. $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 1
The most 10 upregulated lncRNAs in SHRs.

LncRNA name	Target gene	Fold-change	FDR	Class
MRAK140183	PGC1a	6.89	1.50E-08	Intergenic
BC058457	Dctn4	7.23	9.05E-10	Intronic
MRAK003821	Camkk2	7.99	1.76E-12	Intergenic
MRAK050699	Nrp1	8.54	4.51E-06	Intergenic
AY387063	RGD	9.01	1.99E-05	Antisense
XR_009519	Rt1	9.45	3.23E-07	Divergent
AF030089	Dclk1	9.99	1.28E-07	Intronic
MRAK149123	LOC310926	11.30	1.90E-06	Divergent
MRAK017663	RGD	12.01	6.12E-08	Antisense
AK094457	PPARγ	12.55	1.34E-11	Intergenic

Notes: FDR, false discover rate.

Systems, Minneapolis, USA). The absorbance of the samples was red at 450 nm using a reference at 570 nm. The concentrations were calculated through the standard curves.

2.6. Determination of nitrite/nitrate concentrations

Blood samples were collected overnight and prepared after centrifugation. The serum nitrite/nitrate (NO_2^-/NO_3^-) concentration which represent NO production can be measured using a colorimetric assay based on Griess reaction (Cayman Chemical Company, Ann Arbor, Michigan, USA) as previously described [20]. The absorbance of the sample was measured at a wavelength of 540 nm and compared with known concentrations of sodium nitrite.

2.7. Cell culture and transfection

SD rats were killed by decortication and immersed in beaker containing 750 mL/L ethanol for 5 min. The thoracic and abdominal cavity of rats were opened quickly, and the aorta was isolated. The aorta was placed in a 6 cm diameter single-hole culture dish containing PBS (containing 1% blue chain double antibody, the same below) and transferred to a sterile operating table. With the help of ophthalmic corneal scissors, tweezers and iris restorer, peripheral blood vessel fat and connective tissue were gently removed. The external impurities and blood cells were removed by rinsing three times and transferred to a spare single-pore Petri dish containing PBS. The vascular fragment was digested with 2 mL type I collagenase in at 37 °C for 30 min, then discarded, and digested by adding serum containing medium to stop digestion. The obtained cells were inoculated into 6-well plates and incubated at an atmosphere of 5% CO_2 at 37 °C. The medium was

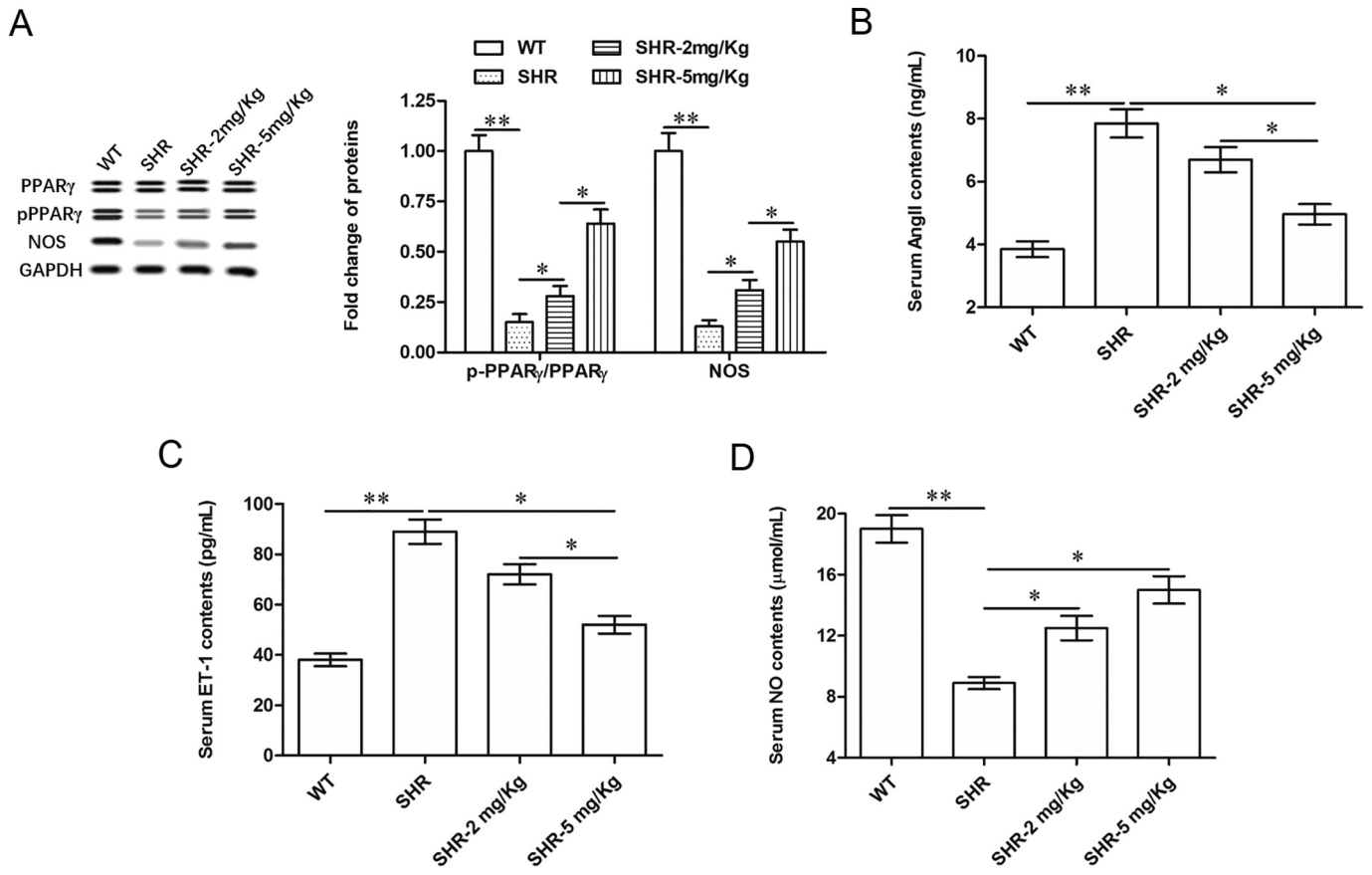

Fig. 3. AK094457 induces vascular dysfunction through inhibiting the activation of PPARγ. A, PPARγ, pPPARγ and iNOS protein level was determined in tail injected SHR and control group by Western blotting. B, C and D, the serum AngII, ET-Iand NO content was measured by ELISA in SHRs with tail injection group and control. $^{*}P < 0.05$; $^{**}P < 0.01$.

changed into fresh after 72 h and changed again after another 48 h. When reaching 80% of confluence, the ells were digested with trypsin and passed on.

The fragments of lncRNA AK094457 were cloned into pcDNA vector in Genewiz Inc. (Suzhou, China). SiRNAs of lncRNA AK094457 was designed and synthesized in GenePharma Inc. (Shanghai, China). For transfection, the 3rd passage of the obtained cells was subgrown and cell transfection was performed when the cells reached 70% of confluence. In a six plate, 1 μg vector or 50 nM siRNAs and their control were transfected into Endothelial cells using 8 μL Lipofectamine 2000 regent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In vivo study, mice were tail injected by different dose of siRNAs weekly for a total of 12 weeks.

2.8. Cell viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Amresco, USA) was used to detect the cell viability [14]. After transfection for 48 h, 1.0×10^4 cells were plated in 96-well plates (Corning, MA) per well and incubated for 12 h. then the cells were cultured in MTT solution (5 mg/ml) for 4 h. The media were discarded and 200 μl DMSO per well. Finally, the absorbance was measured using a microplate reader (Bio-Rad, USA) at 490 nm wavelength.

2.9. Migration assays

Cell migration was performed using 8 μm pores Transwell chambers (Corning, USA). After 48 h of transfection, 10^5 cells were plated in the wells above in 200 μl serum-free medium. 800 μl medium containing 10% serum was added to the bottom wells. After fixed in 4% paraformaldehyde solution, the transwelled cells were then stained with 0.1% crystal violet (Amresco, USA). The cells were observed under a NIKON Eclipse TE300 microscope and calculated from six fields for each well.

2.10. Statistical analysis

A mean ± SD was used to present the data. The data analysis was conducted using GraphPad Prism 6 software (GraphPad Inc., San Diego, USA). The independent student's *t*-test was applied to compare the quantitative parameters between two groups. Analysis of Variance (ANOVA) was used to compare several groups. It is statistically significant when $P < 0.05$.

3. Results

3.1. Microarray expression profile of lncRNAs in the aortic endothelial cells isolated from SHRs

We performed a microarray analysis of lncRNA in the aortic Endothelial cells isolated 3 SHRs and control rats. A total of 2598 differentially expressed lncRNAs were detected. Among them, lncRNAs transcribed from intergenic, antisense, intronic and divergent accounted for 55.2%, 25.4%, 12.3% and 7.1% respectively (Fig. 1A). The distribution of lncRNAs with different lengths was shown in Fig. 1B, whereas the number of them on different chromosomes is shown in Fig. 1C.

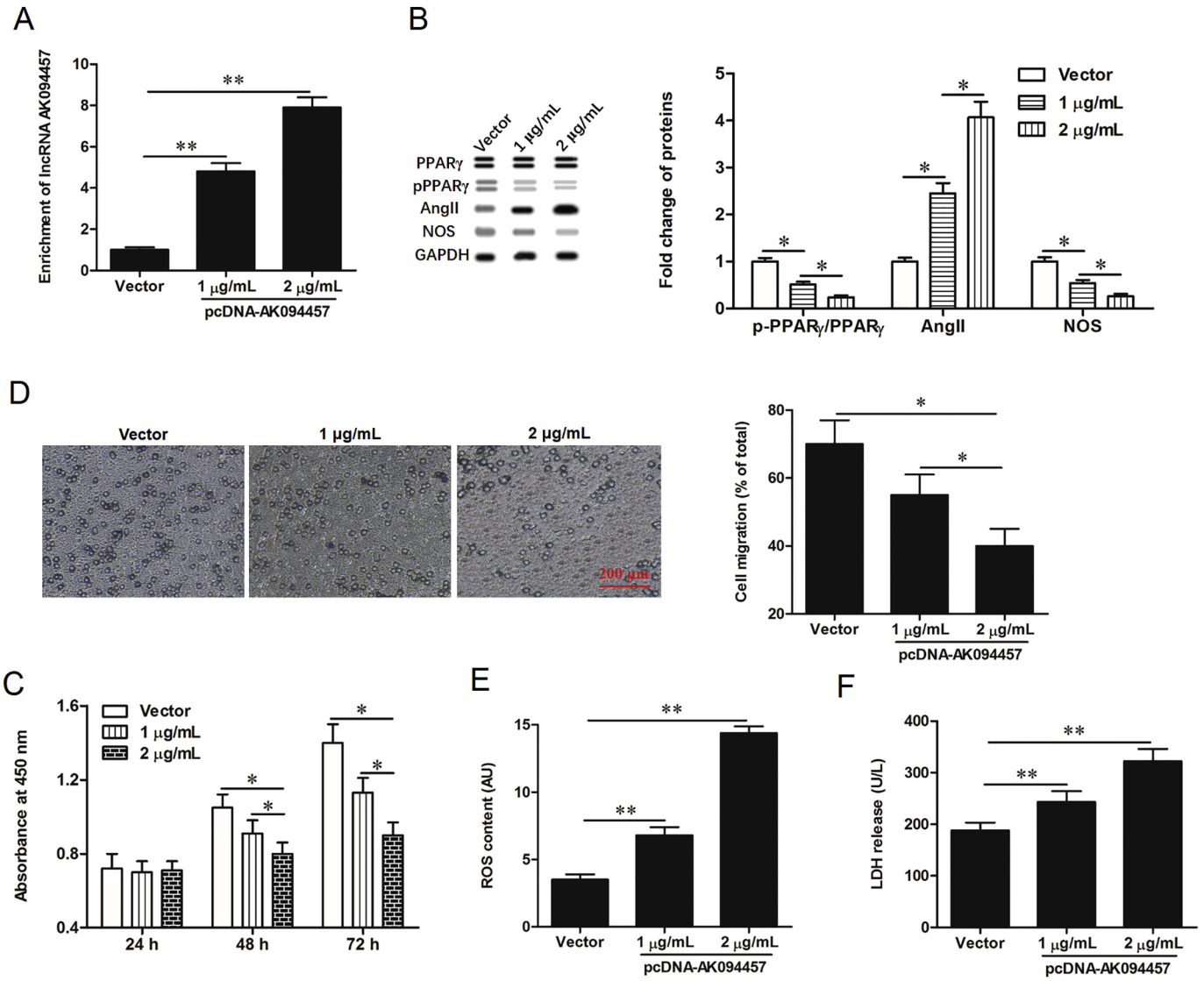

Fig. 4. AK094457 overexpression promotes AngII-induced vascular dysfunction. Primary aortic endothelial cells were isolated and cultured in vitro. The pcDNA-AK094457 expression vector at different concentrations and empty vector were transfected into the endothelial cells. Following transfection for 48 h, A, AK094457 level was detected in endothelial cells transfected with different dose of AK094457 vector and control by qPCR. B, PPARγ, pPPARγ, AngII and iNOS expression protein level was detected by Western blotting in endothelial cells in AK094457 overexpressed group and control. C and D, cell viability and migration ability were analyzed by CCK-8 and Transwell cell migration assays in different transfection group. E and F, ROS content and LDH release was determined in vector transfection group and control by corresponding detection kits. The magnification of the image is 100. $*P < 0.05$; $**P < 0.01$.

3.2. Expression of lncRNA AK094457 correlates with the arterial systolic pressure

To validate the results of microarray analysis, RT-PCR was performed to detect the top 10 significant up-regulated lncRNAs, two of them not detected for they are from divergent region (Fig. 2A). 10 lncRNAs with the highest up regulation in SHRs were listed in Table 1. In terms of the fold change in expression level, lncRNA AK094457 is the most significant. lncRNA AK094457 had been reported to be related to antihypertension [8]. We found that AK094457 was significantly increased in SHR, whereas it was downregulated after tail injection of 2 mg/kg or 5 mg/kg siRNAs weekly for a total of 12 weeks (Fig. 2B). We also found that systolic pressure was raised in SHR but fallen in the groups of AK094457 inhibited (Fig. 2C).

3.3. AK094457 induces vascular dysfunction through inhibiting the activation of PPARγ

PPARγ expression did not changed as AK094457 level was altered in SHR. Expression of p-PPARγ and iNOS were decreased in SHR but they picked up in groups tail injected with 2 mg/kg or 5 mg/kg siRNA (Fig. 3A). In contrast, serum AngII and ET-1 contents were upregulated in SHR but suppressed when AK094457 was inhibited (Fig. 3B and C). Similarly, secretion of NO is consistent with the iNOS level (Fig. 3D).

3.4. AK094457 overexpression promotes AngII-induced vascular dysfunction

The aortic endothelial cells were isolated from SHRs and transfected with different dose of AK094457 vector. Compare to control, AK094457 level was upregulated in different dose of vector transfection group (Fig. 4A). PPARγ protein level was not changed in any vector transfection group. AK094457 overexpression significantly inhibited

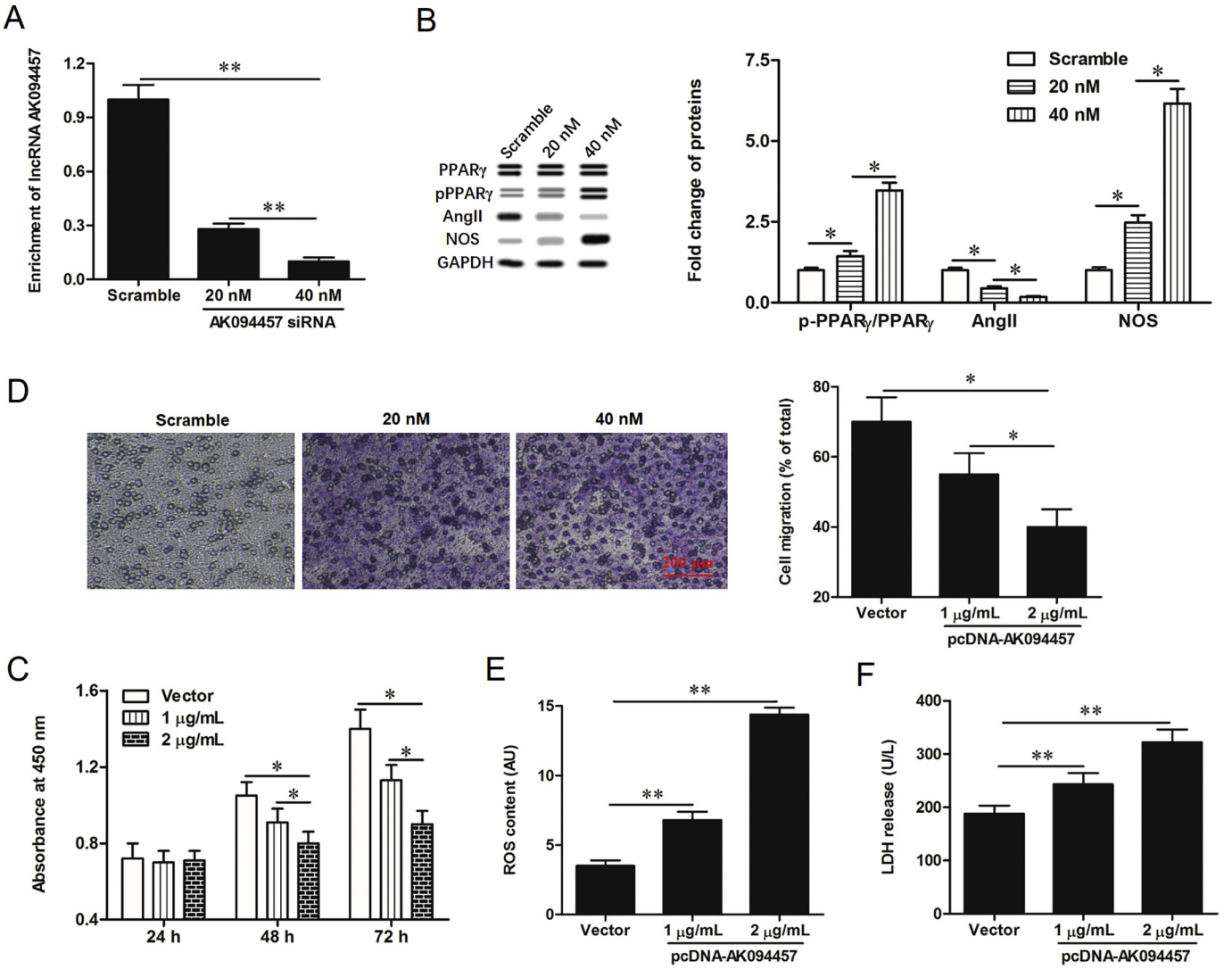

Fig. 5. AK094457 inhibitors relieve AngII-induced vascular dysfunction. The AK094457 siRNA at different concentrations and empty vector were transfected into the endothelial cells. Following transfection for 48 h, A, AK094457 level was detected by qPCR. B, PPARγ, pPPARγ, AngII and iNOS expression were detected by Western blotting. C and D, cell viability and migration ability were analyzed. E and F, ROS content and LDH release were determined. The magnification of the image is 100. $^{*}P < 0.05$; $^{**}P < 0.01$.

pPPARγ and iNOS expression but promoted AngII level (Fig. 4B). After transfection for 48 and 72 h, the overexpression of AK094457 obviously suppressed cell viability and migration ability (Fig. 4C and D). Simultaneously, ROS content and LDH release was also increased in vector transfection group (Fig. 4E and F).

3.5. AK094457 inhibitors relieve AngII-induced vascular dysfunction

In the aortic endothelial cells, AK094457 level was significantly decreased after transfected with different dose of siRNA (Fig. 5A). We found that AK094457 depletion significantly promoted pPPARγ and iNOS expression but inhibited AngII level, although PPARγ protein level was not changed (Fig. 5B). After transfection for 48 and 72 h, the suppression of AK094457 obviously increased cell viability and migration ability (Fig. 5C and D). ROS content and LDH release was also decreased when AK094457 was inhibited (Fig. 5E and F).

3.6. AK094457 suppressed PPARγ activation by deactivating AMPK

PPARγ could be activated by several kinase signaling pathways, among which the adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) was a most common one in endoderm-derived cells, such as adipocytes and endothelial cells. Here, we found that AK094457 overexpression suppressed, while AK094457 knockdown increased the activation of AMPK (Fig. 6A and B). Inhibition of AMPK or PPARγ by their specific inhibitors (Compound C and T0070907 respectively) could antagonize the activation of PPARγ and rescue the upregulation of AngII (Fig. 6B), but had no effect on expression of AK094457 (Fig. 6A). Inconsistent with the change of the protein levels, inhibition of AMPK or PPARγ by their specific inhibitors could antagonize the increase in cell proliferation and migration (Fig. 6C and D) and rescue the decrease in production of ROS and LDH (Fig. 6E and F).

4. Discussion

The vascular endothelium is located in the inner layer of blood vessels and regulates vascular wall function, which has a crucial role in the process of the regulation of blood pressure. Hypertension is commonly related to impaired vascular endothelial function. It is increasingly found that lncRNAs played an important role in many diseases. The alteration of lncRNAs expression profile and the mechanism involved in the regulation of hypertension remain unclear. In this study,

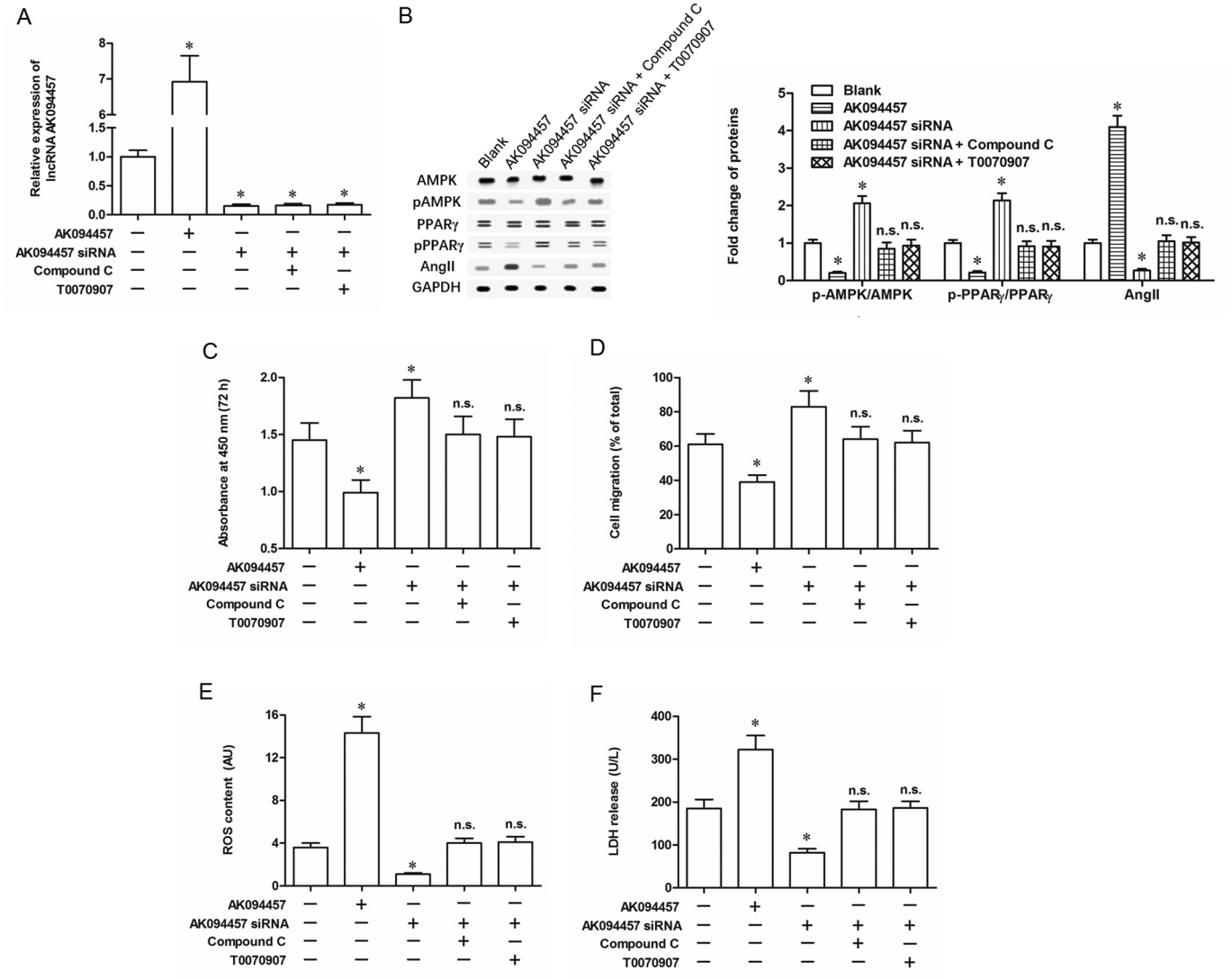

Fig. 6. Inhibition of AMPK or PPARγ by their specific inhibitors could rescue AK094457-siRNA-induced endothelial cell dysfunction. Primary aortic endothelial cells were isolated and cultured in vitro. The pcDNA-AK094457 expression vector (2 μg/mL), AK094457-siRNA (40 nM), AK094457-siRNA (40 nM) plus Compound C (50 nM), and AK094457-siRNA (40 nM) plus T0070907 (1 nM) were respectively used to incubated with the endothelial cells. Following treatment for 48 h, A, AK094457 level was detected by qPCR. B, AMPK, p-AMPK PPARγ, p-PPARγ, and AngII expressions were detected by Western blotting. C and D, cell viability and migration ability were analyzed. E and F, ROS content and LDH release were determined. $^*P < 0.05$, n.s. represents non-significant.

our data on Microarray revealed multiple lncRNAs dysregulated in SHRs. Signaling pathway and molecular function analyses revealed that, among the top 10 upregulated lncRNAs, AK094457 was mainly associated with kinase signaling pathway, endothelial function, cell proliferation, apoptosis and migration, while other lncRNAs were associated with angiogenesis and tumorigenesis signaling pathways and cell differentiation. We also found that AK094457 is upregulated in the aortic endothelial cells isolated from SHRs.

AK094457 has been previously reported to be upregulated in hypertensive patients, but it is really unknown its function in Endothelial cells and the development of hypertension [17]. We found that PPARγ is regulated by lncRNA AK094457 in endothelial cells of SHR. The phosphorylated PPARγ was significantly decreased in SHR and lncRNA AK094457 overexpressed endothelial cells. Peroxisome proliferator activated receptors (PPAR) are a family of ligand-activated transcription factors. PPARγ is mainly expressed in adipose tissue and cells of the monocyte lineage, which influence cellular energy homeostasis and insulin activity [21]. The PPARs are phosphoproteins and their transcriptional activity is activated by phosphorylation [22]. Through our results, systolic pressure was raised in SHR but fallen in AK094457 inhibited groups. The p-PPARγ was inhibited by upregulation of lncRNA AK094457 in SHR. ROS and LDH level was downregulated in lncRNA AK094457 overexpressed endothelial cells but upregulated when treated with siRNAs. These results imply lncRNA AK094457 suppresses the activation of PPARγ which may accelerate the generation of reactive ROS, inflammation, atherosclerosis and vascular remodeling. The increased serum LDH may be related to tissue injury, inflammation and hypoxia [23]. LDH converts pyruvate to lactate, when oxygen is not available. In hypertensive patients, the increased level of lncRNA AK094457 may inhibit PPARγ activity which is involved in the progress of hypertension.

In the mechanism exploration, we found that lncRNA-AK094457 could inhibit activation of AMPK that could not only trigger PPARγ phosphorylation and endothelial function, but also play important roles in regulating cell behaviors including cell proliferation, apoptosis, migration and differentiation [24,25]. Inhibition of AMPK or PPARγ by their specific inhibitors could rescue AK094457-siRNA-induced endothelial cell dysfunction. In fact, many lncRNAs have been involved in regulation of kinase pathways including AMPK. For example, lncRNAs H19 and SNHG12 were associated with dysfunction of HMEC-1

endothelial cells and mouse primary brain microvascular endothelial cells by regulating the activation of AMPK and AMPK-induced production of matrix metalloproteinases, VEGF and ET-1 [26,27].

The renin–angiotensin system (RAS) is reported to be involved in vascular remodeling and endothelial dysfunction through inducing vascular growth, apoptosis and low-grade inflammation [28]. Ang II, which can induce NF-κB activation, is the main effector of the RAS and play critical roles in the progression of hypertension. Ang II-induced vascular dysfunction can be prevented by PPARγ activators [29]. ET-1 is a powerful vasoconstrictor and a potent mitogen for vascular smooth muscle cells. Local vascular generation of ET-1 may contribute to elevated peripheral resistance in hypertension [30]. Vascular ET-1 levels was upregulated when Ang II increases, which contribute to Ang II-stimulated vascular growth and hypertension [31]. In our results, it is showed that lncRNA AK094457 inhibited pPPARγ but increased Ang II and ET-1 level, which implies that lncRNA AK094457 suppresses the activation of PPARγ which may accelerate Ang II-induced vascular dysfunction. The results also showed that the suppression of AK094457 obviously increased endothelial cells viability and migration ability, which in turn suggested that the inhibition of AK094457 may be helpful for the recovery of endothelial function.

This is the first study that suggested the mechanisms of AK094457 involved in the development of hypertension. LncRNA AK094457 suppresses the activation of PPARγ and accelerates Ang II-induced vascular dysfunction, whereas the inhibition of AK094457 will ease endothelial damage. LncRNA AK094457 may be a new target for the treatment of hypertension. Whether it can be applied as hypertensive drugs needs further study.

Declaration of competing interest

None.

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