Abstract. Continuous activation of angiotensin II (Ang II) induces renal vascular endothelial dysfunction, inflammation and oxidative stress, all of which may contribute to renal damage. MicroRNAs (miRs/miRNAs) play a crucial regulatory role in the pathogenesis of hypertensive nephropathy (HN). The present study aimed to assess the differential expression profiles of potential candidate genes involved in Ang II-induced rat renal artery endothelial cell (RRAEC) dysfunction and explore their possible functions. In the present study, the changes in energy metabolism and autophagy function in RRAECs were evaluated using the Seahorse XF Glycolysis Stress Test and dansylcadaverine/transmission electron microscopy following exposure to Ang II. Subsequently, mRNA-miRNA sequencing experiments were performed to determine the differential expression profiles of mRNAs and miRNAs. Integrated bioinformatics analysis was applied to further explore the molecular mechanisms of Ang II on endothelial injury induced by Ang II. The present data supported the notion that Ang II upregulated glycolysis levels and promoted autophagy activation in RRAECs. The sequencing data demonstrated that 443 mRNAs and 58 miRNAs were differentially expressed (DE) in response to Ang II exposure, where 66 mRNAs and 55 miRNAs were upregulated, while 377 mRNAs and 3 miRNAs were downregulated (fold change >1.5 or <0.67; P<0.05). Functional analysis indicated that DE mRNA and DE miRNA target genes were mainly associated with cell metabolism (metabolic pathways), differentiation (Th1 and Th2 cell differentiation), autophagy (autophagy -animal and autophagy -other) and repair (RNA -repair). To the best of the authors' knowledge, this is the first report on mRNA‑miRNA integrated profiles of Ang II -induced RRAECs. The present results provided evidence suggesting that the miRNA -mediated effect on the ‘mTOR signaling pathway’ might play a role in Ang II-induced RRAEC injury by driving glycolysis and autophagy activation. Targeting miRNAs and their associated pathways may provide valuable insight into the clinical management of HN and may improve patient outcome.

Introduction

Recent studies have found that the local renin-angiotensin system (RAS) might contribute to glomerulosclerosis and renal interstitial fibrosis (1). The organ-specific roles exerted by angiotensin II (Ang II), which is the most potent biologically active product of the RAS, have also been studied (2). The RAS is overactivated in vivo once hypertension occurs. Overproduced Ang II directly constricts vascular endothelial cells (ECs), causes changes in diastolic and contractile substances, increases the synthesis and release of endothelium-derived vasoconstrictors such as endothelin-1 (ET-1) and thromboxane A2, reduces the production of endothelium-derived vasodilators such as nitric oxide (NO) and ultimately results in vascular endothelial damage and retention of sodium and water (3,4). Further studies demonstrated that inappropriate activation of intrarenal Ang II plays a central role in the pathogenesis of hypertension and renal injury (5). The role of renal artery ECs in self-regulation is associated with cell autophagy and energy homeostasis. However, activation of the endothelium by elevated blood pressure is followed
by endothelial dysfunction, which eventually leads to endothelial disintegration (6). In this context, dysfunctional ECs may continue generating ATP by glycolysis for a long period of time and keep their mitochondrial membrane potential in a depolarized state that can be reversed. Furthermore, under these circumstances, the autophagy pathway may be activated to maintain glycolytic-dependent ATP production (7). The effect of hypertension on renal vascular endothelium is directly related to hypertensive nephropathy (HN), but few studies have harnessed the power of transcriptome sequencing or microarray analysis to identify the potential vulnerabilities of hypertensive renal artery injury. Therefore, the molecular mechanism of Ang II on renal artery ECs has important research value and significance.

MicroRNAs (miRs/miRNAs) are post-transcriptional regulators of gene expression. These small (20-25 nucleotides long) noncoding RNAs bind to a target recognition site (seed sequence) in the 3' untranslated regions of mRNA transcripts, leading to mRNA degradation and/or inhibition of protein translation, depending on the complementarity of the miRNA with the target mRNA (8). A growing number of miRNAs, including miRNA-let-7b, miRNA-431 and miRNA-29 (9-11), are implicated in the promotion or suppression in the initiation and progression of hypertension. Ang II-mediated STAT3 activation in kidney epithelial cells results in hypertensive kidney disease (12); however, the detailed mechanisms and regulatory role as therapeutic targets of miRNAs underlying renal artery EC injury induced by Ang II remain poorly understood. Hence, the present study focused on the miRNAs involved in renal artery EC dysfunction.

The present study constructed an Ang II-induced rat renal artery EC (RRAEC) injury model. The mitochondrial membrane potential and glycolysis levels were determined to assess mitochondrial function and cellular energy supply of RRAECs exposed to Ang II. The stability of the intracellular mitochondrial membrane potential was assessed with a dansylcadaverine (MDC) Autophagy Assay kit (cat. no. 100777-004; Agilent Technologies, Inc.) at 37°C and 5% CO2. Subsequent assays were performed using 3x104 cells/well. A sensor cartridge was hydrated in a Seahorse XF Calibrate (Agilent Technologies, Inc.) at 37°C in a non-CO2 incubator overnight. XF Glycolysis Stress Test kit medium (cat. no. 103020-100; Agilent Technologies, Inc.) was heated to 37°C, and the pH was adjusted to 7.4±0.1. The cell culture microplate was removed from the 37°C CO2 incubator, and the RRAECs were washed with XF glycolysis assay medium and then placed in a 37°C, non-CO2 incubator for 1 h prior to the assay. Injection ports A, B, and C were loaded with glucose (10 mmol/l), oligomycin (1 µmol/l) and 2-deoxy-glucose (50 mmol/l; included in the Glycolysis Stress assay kit), according to the manufacturer's protocol. The experimental template was set up or imported on the XF controller. The default Mix-Wait-Measure time was 3 min-2 min-3 min. Seahorse Wave software (version 2.2.0; Agilent Technologies, Inc.) was used for data calculation. The XF Glycolysis Stress Test was used to measure glycolysis as the ECAR that is reached by RRAECs after the addition of saturating amounts of glucose. The glycolytic reserve was defined as the difference between glycolytic capacity and glycolysis rate. Prior to glucose injection, ECAR was referred to as non-glycolytic acidification and was caused by processes in the cell other than glycolysis.

Materials and methods

Cell culture and reagents. RRAECs (cat. no. RAEC; Sixin) were cultured in DMEM (cat. no. 10-013-CVR; Corning, Inc.) supplemented with 10% FBS (cat. no. 10099-141; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (cat. no. SV30010; HyClone; GE Healthcare Life Sciences) at 37°C and 5% CO2. Cells from passages 2-8 were used for subsequent experiments. RRAECs were seeded in six-well plates at a density of 2x104 cells/well for 24 h with exposure to Ang II (5x10-7 mol/l; cat. no. A9290; Beijing Solarbio Science & Technology Co., Ltd.) at 37°C, while untreated RRAECs were used as a negative control. The cells were serum-starved overnight before further treatment. All samples were assayed in triplicate.

Detection of mitochondrial membrane potential. JC-1 is a mitochondrion-selective dye widely used to detect mitochondrial membrane potential (ΔΨm), which reversibly changes color from green to red as the membrane potential increases (values of >80-100 mV). The degree of mitochondrial depolarization is measured by the relative proportion of red and green fluorescence (13). Following 24 h of Ang II exposure as described above, the cells were collected and washed with PBS (cat. no. 21-040-CVR; Corning, Inc.). Washed cells were resuspended in JC-1 staining solution (cat. no. M8650; Beijing Solarbio Science & Technology Co., Ltd.) and incubated at 37°C for 20 min. RRAECs were then washed twice with the diluted working solution (JC-1 staining solution: 1X). Mitochondrial membrane potential analysis was performed using a flow cytometer (BD Biosciences) and analyzed by CytExpert for DXFLEX 2.0 (Beckman Coulter, Inc.).

Seahorse XF analysis. The Seahorse XF Glycolysis Stress Test performed using the Seahorse XF(e)24 Cell Energy Metabolic Analyzer (Agilent Technologies, Inc.) directly measures the extracellular acidification rate (ECAR) of living cells in real time, reflecting glycolytic function in cells. Cell density was optimized by inoculating different amounts of RRAECs (1-8x104 cells/well) into a 24-well Seahorse XF Cell Culture Microplate (cat. no. 100777-004; Agilent Technologies, Inc.) at 37°C and 5% CO2. Subsequent assays were performed using 3x104 cells/well. A sensor cartridge was hydrated in a Seahorse XF Calibrate (Agilent Technologies, Inc.) at 37°C in a non-CO2 incubator overnight. XF Glycolysis Stress Test kit medium (cat. no. 103020-100; Agilent Technologies, Inc.) was heated to 37°C, and the pH was adjusted to 7.4±0.1. The cell culture microplate was removed from the 37°C CO2 incubator, and the RRAECs were washed with XF glycolysis assay medium and then placed in a 37°C, non-CO2 incubator for 1 h prior to the assay. Injection ports A, B, and C were loaded with glucose (10 mmol/l), oligomycin (1 µmol/l) and 2-deoxy-glucose (50 mmol/l; included in the Glycolysis Stress assay kit), according to the manufacturer's protocol. The experimental template was set up or imported on the XF controller. The default Mix-Wait-Measure time was 3 min-2 min-3 min. Seahorse Wave software (version 2.2.0; Agilent Technologies, Inc.) was used for data calculation. The XF Glycolysis Stress Test was used to measure glycolysis as the ECAR that is reached by RRAECs after the addition of saturating amounts of glucose. The glycolytic reserve was defined as the difference between glycolytic capacity and glycolysis rate. Prior to glucose injection, ECAR was referred to as non-glycolytic acidification and was caused by processes in the cell other than glycolysis.
were detected with a FACScan flow cytometer at a wavelength of 488 nm, and the total autophagosome rate (without distinguishing between macroautophagy and mitochondrial autophagy) was statistically analyzed. Data were analyzed using cell Quest 3.1 software (Becton, Dickinson and Company).

Observation of autophagosomes by transmission electron microscopy (TEM). As the most reliable method and gold standard for detecting phagocytosis (15), TEM was used to clearly observe the independent bilayer membrane structure of autophagosomes and various autophagic morphological changes at x10,000 magnification. Autophagosomes in the control group and the Ang II group were observed by TEM (JEOL, Ltd.). RRAECs at a density of 1x10⁵ cells/well were seeded in six-well plates with or without exposure to Ang II for 24 h. Subsequently, the cells were gently scraped and centrifuged at a rate of 2,000 x g for 10 min at 37°C in PBS, following which RRAECs were fixed with 2.5% glutaraldehyde containing 0.1 mol/l sodium cacodylate. Samples were fixed using 1% osmium tetroxide, followed by dehydration with an increasing concentration gradient of acetone solution. Samples were then embedded in epoxy resin provided by a SPI-PonTM 812 Epoxy Resin Embedding kit (cat. no. 02635-AB; SPI Supplies). The resin was subsequently polymerized at 37°C for 6 h, 45°C for 12 h and 67°C for 24 h. All cell samples were then cut into 50-nm sections and stained with 3% uranyl acetate for 10 min and lead citrate for 5 min at room temperature. Images were acquired using a TEM. Sample processing and image capture were performed by Weiya Biotechnology Co., Ltd.

RNA extraction and quality control. Total RNA was isolated using the miRNeasy Mini kit (Qiagen GmbH), following which RNA concentration and quality were determined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Inc.) and validated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). The integrity of total RNA was assessed using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Inc.) and a Qubit 2.0 Fluorometer (Qiagen GmbH), following the TruSeq™ RNA Sample Preparation Guide (Qiagen miRNA Library). The products were then purified and enriched with PCR to create the final cDNA library. Purified libraries were quantified by a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Inc.) and validated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) to confirm the insert size and calculate the molar concentration. Clusters were generated by cBot (Illumina, Inc.) with the library diluted to 10 pm and then sequenced on the Illumina HiSeq X™ Ten sequencer (Illumina, Inc.). Library construction and sequencing were performed at Shanghai Sinomics Corporation.

Library construction for miRNA-sequencing (miRNA-seq) and sequencing procedures. Paired-end libraries were synthesized using the QIAseq miRNA Library kit (Qiagen GmbH) following the TruSeq™ RNA Sample Preparation Guide (QIAseq miRNA Library). The products were then purified and enriched with PCR to create the final cDNA library. Purified libraries were quantified by a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Inc.) and validated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) to confirm the insert size and calculate the molar concentration. Clusters were generated by cBot, (Illumina, Inc.) with the library diluted to 10 pm and sequenced on a HiSeq X Ten sequencer (Illumina, Inc.). Library construction and sequencing were performed at Shanghai Sinomics Corporation.

Library construction for RNA-sequencing (RNA-seq) and sequencing procedures. Strand-specific libraries were prepared using the TruSeq® Stranded Total RNA Sample Preparation kit (Illumina, Inc.) following the manufacturer's instructions. Briefly, ribosomal RNA (rRNA) was removed from total RNA using Ribo-Zero rRNA removal beads. Following purification, the mRNA was fragmented into small pieces using divalent cations at 94°C for 8 min. The cleaved RNA fragments were reverse transcribed into first strand cDNA using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. The cDNA fragments then went through an end repair process, the addition of a single ‘A’ base and adapter ligation. The products were then purified and amplified by PCR to create the final cDNA library. Purified libraries were quantified by a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Inc.) and validated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) to confirm the insert size and to calculate the mole concentration. Clusters were generated by cBot, (Illumina, Inc.) with the library diluted to 10 pm and sequenced on a HiSeq X Ten sequencer (Illumina, Inc.). Library construction and sequencing were performed at Shanghai Sinomics Corporation.

Data analysis for gene expression. RNA-seq raw reads were pre-processed by filtering out rRNA reads, sequencing adapters, short-fragment reads and other low-quality reads. Tophat version 2.0.9 (https://ccb.jhu.edu/software/tophat/index.shtml) was used to map the clean reads to the Rnor_6.0.91 reference genome [Rnor_6.0 (GCA_000001895.4)] using alignment with two mismatches. Following genome mapping, Cufflinks version 2.1.1 (http://cole-trapnell-lab.github.io/cufflinks/) was run with a reference annotation to generate fragments per kilobase of exon model per million reads mapped (FPKM) values for known gene models. Differentially expressed genes were identified using edgeR package. The P-value significance threshold in multiple tests was set by the false discovery rate. The fold changes (FCs) were also estimated according to the FPKM of each sample. The differentially expressed genes were selected using the following filtering criteria: P<0.05 and FC>1.5 or <0.67. Different samples and genes were clustered and classified and a heatmap was generated to display the expression of genes in different samples using MeV_4.6.0 software (multiple experiment viewer). Gene Ontology (GO) (http://geneontology.org/) adapted Fisher test, cluster profiler packages in R-3.4.3 and Bioconductor 3.6 (16,17) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) (https://www. kegg.jp/) pathway analysis were used to analyze the functions of the genes in the pathways. Read's sample containing miRNA sequences were compared with existing sequences in a miRNA database (miRBase; http://www.mirbase.org/) and the predicted results of new miRNAs generated by MiRDeep2 version 2.0.0.5 software to calculate the miRNA expression level (count number). miRNA expression was screened by counts per million. DE sequencing software (DESeq 1.30.0; https://bioconductor.org/packages/DESeq/) was used to analyze the expression between samples and identify DE miRNAs with P<0.05 and FC>1.5 or <0.67.

Reverse transcription-quantitative PCR (RT-qPCR) analysis of miRNA and mRNA expression. To verify the sequencing results, 15 DE genes closely related to endothelial dysfunction were selected for RT-qPCR analysis: Nine miRNAs (miR-200a-3p, miR-200b-3p, miR-200c-3p, miR-192-5p,
miRNAs were extracted using the MiPure Cell/Tissue miRNA kit (cat. no. RC201; Vazyme Biotech Co., Ltd.). Purified miRNAs were then reverse transcribed into cDNA using a Mir-X miRNA First-Strand Synthesis kit (cat. no. 638313; Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. Gene-specific primers were designed and purchased from Sangon Biotech, Co., Ltd. qPCR was performed as described above. Data were analyzed using the 2^ΔΔCq method with GAPDH as an internal reference gene. All samples were analyzed in triplicate. The primer sequences of the corresponding miRNAs and mRNAs are listed in Table I.

| Target | Forward sequence (5’-3’) | Reverse sequence (5’-3’) |
|--------|--------------------------|-------------------------|
| rno-miR-29b-3p | TAGCACCATTTGAAATCAGTGTT | Universal |
| rno-miR-200b-3p | TAATACTGCGCTGTATGGATGAC | |
| rno-miR-192-5p | CTGACATGGAATTGAGAAGCC | |
| rno-miR-223-3p | TGTGAAGGGGCTCAAATACCCC | |
| rno-miR-194-5p | TGTAACTGGCTGTATGGATGAC | |
| rno-miR-200a-3p | TAACACTGTCTGGTAAGGTGTTT | |
| rno-miR-494-3p | TGAACATACCCGGGACACCTCT | |
| rno-miR-429 | TAATACTGTCTGGTAATGGATGAC | |
| rno-miR-200c-3p | TAATACTGCGCGTGTAATGGATGAC | |

miR/miRNA, microRNA; Pfkfb3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; Tpd52, tumor protein D52; Xkr8, XK related 8; Elk4, ETS transcription factor ELK4; Selenop, selenoprotein P; Zeb2, zinc finger E-box binding homeobox 2.

**Table I. Primer sequences of miRNAs and mRNAs used in the study.**

**A. miRNA**

| Target | Forward sequence (5’-3’) | Reverse sequence (5’-3’) |
|--------|--------------------------|-------------------------|
| Elk4 | CAGCCAGACTGCAAGTTGCTAA | ATCCAGGCCCCAGACAGATGGATG |
| Zeb2 | CCGATCAACCCGTACACAGGA | CTTCAGGATGTCTTGGACGTT |
| Selenop | AGCCATCAAGATCGCTACTGTT | TGCCCATTTTCTTATGGT |
| Tpd52 | GGTTGCGAGTGTCAGACGCA | GAPGACTGACCGCCACCAAG |
| Xkr8 | GACGGAGTGGAATATGCGAAAT | TAGCTCCAGGCAATGCCACAGA |
| Pfkfb3 | GCACGAGCTACCGAGGGGAGT | CAGTGGAGGTTAGCGAGTCAGT |
| GAPDH | GCCACAGTCAAGCTGAGAATG | ATGGTGGTTAAGAGCCAG |

**B. mRNA**

| Target | Forward sequence (5’-3’) | Reverse sequence (5’-3’) |
|--------|--------------------------|-------------------------|
| miR-223-3p, miR-194-5p, miR-494-3p, miR-429 and miR-29b-3p | TAGCACCATTTGAAATCAGTGTT | Universal |
| miR-200b-3p | TAATCTGCGCTGTATGGATGAC | |
| miR-192-5p | CTGACATGGAATTGAGAAGCC | |
| miR-223-3p | TGTGAAGGGGCTCAAATACCCC | |
| miR-194-5p | TGTAACTGGCTGTATGGATGAC | |
| miR-200a-3p | TAACACTGTCTGGTAAGGTGTTT | |
| miR-494-3p | TGAACATACCCGGGACACCTCT | |
| miR-429 | TAATACTGTCTGGTAATGGATGAC | |
| miR-200c-3p | TAATACTGCGCGTGTAATGGATGAC | |

miR/miRNA, microRNA; Pfkfb3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; Tpd52, tumor protein D52; Xkr8, XK related 8; Elk4, ETS transcription factor ELK4; Selenop, selenoprotein P; Zeb2, zinc finger E-box binding homeobox 2.

miRNAs were extracted using the MiPure Cell/Tissue miRNA kit (cat. no. RC201; Vazyme Biotech Co., Ltd.). Purified miRNAs were then reverse transcribed into cDNA using a Mir-X miRNA First-Strand Synthesis kit (cat. no. 638313; Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. Gene-specific primers were designed and purchased from Sangon Biotech, Co., Ltd. qPCR was performed as described above. Data were analyzed using the 2^ΔΔCq method with GAPDH as an internal reference gene. All samples were analyzed in triplicate. The primer sequences of the corresponding miRNAs and mRNAs are listed in Table I.

**Target gene prediction of DE miRNAs.** The miRanda algorithm (19) was used to predict miRNA target genes. The miRanda algorithm comprehensively predicts miRNA target genes based on miRNA-mRNA sequencing matching or energy stability and uses a dynamic programming algorithm to search for regions where miRNAs have complementary sequences with target mRNAs and form stable double-strands.

**Construction of a network map of miRNAs and target genes.** A global network of DE miRNAs and target genes was constructed using Cytoscape 3.7.2 software (https://cytoscape.org/).

**Statistical analysis.** Data were analyzed with SPSS 17.0 software (SPSS, Inc.) and presented as the mean ± SD from at least three separate experiments. Two-tailed independent sample t-test was used to compare the mean values between
two groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Ang II decreases the mitochondrial membrane potential of RRAECs.** The F value, defined as the proportion of cells with decreased mitochondrial membrane potential, of cells in the Ang II group was significantly higher compared with the control group (P<0.001; Fig. 1A-C). The F value in the Ang II group was 17.99±1.23% while the F value in the control group was 4.64±0.88%. The results suggested that the normal mitochondrial aerobic respiration of RRAECs was partially inhibited in response to mitochondrial dysfunction.

**RRAECs treated with Ang II are highly glycolytic.** The Seahorse XF Glycolysis Stress Test provides a standard and comprehensive method to assess the key parameters of glycolytic function. The basic value represents the non-glycolytic acid production value of the cells. The injection of glucose represents the rate of glycolysis under basal conditions. After adding oligomycin, mitochondrial ATP production was inhibited and the cells were supplied with oxygen via glycolysis. The subsequent increase in ECAR revealed the cellular maximum glycolytic capacity. The final injection was 2-DG, a glucose analog, that inhibits glycolysis by competitively binding to glucose hexokinase, the first enzyme in the glycolytic pathway. The decreased ECAR represents the glycolytic reserve of the cells. (E) Real-time ECARs obtained from Ang II-induced RRAECs (n=5) and control cells (n=5). (F) ECAR showing higher glycolysis, defined as the maximum ECAR reached by cells after the addition of saturating amounts of glucose, in Ang II-induced RRAECs compared with control cells. (G) ECAR showing higher glycolytic capacity, defined as the maximum ECAR reached by cells following the addition of oligomycin, in Ang II-induced RRAECs compared with control cells. (H) ECAR showing higher glycolytic reserve, defined as the capability of cells to respond to an energetic demand, in Ang II-induced RRAECs compared with control cells. **P<0.01 and ***P<0.001. ECAR, extracellular acidification rate; RRAEC, rat renal artery endothelial cell; Ang II, angiotensin II; 2-DG, 2-deoxy-glucose; PE-A, phycoerythrin-area; H1-UR, height1-upper right; H1-LR, height1-lower right.
flux, including glycolysis, glycolytic capacity and glycolytic reserve, by directly measuring the extracellular acidification rate (Fig. 1D and E). The glycolysis value (control group, 8.25±8.12 mpH/min; Ang II group, 22.57±3.76 mpH/min; Fig. 1F), glycolytic capacity value (control group, 18.31±12.01 mpH/min; Ang II group, 62.28±5.03 mpH/min; Fig. 1G) and glycolytic reserve value (control group, 10.06±4.25 mpH/min; Ang II group, 39.71±3.44 mpH/min; Fig. 1H) of RRAECs in the Ang II group were significantly increased compared with the control group. Taken together, these findings suggested that Ang II may cause glycolysis pathway activation.

**Ang II promotes autophagy activation in RRAECs.** The autophagy rate of RRAECs increased by ~4-fold (15.12±1.45) after 24 h of Ang II treatment compared with the autophagy rate of untreated RRAECs (3.87±1.03; Fig. 2A-C).

Autophagosomes were evaluated in RRAECs cultured with or without Ang II treatment. RRAECs in the control group had a smooth nuclear membrane, a uniform distribution of nuclear chromatin and abundant organelles in the cytoplasm (Fig. 2D). By contrast, TEM showed an increase in the formation of autophagic vesicles in RRAECs treated with Ang II (Fig. 2E). Mitochondria were swollen and vacuolized in the cytoplasm of Ang II-treated cells (Fig. 2E). The number of autophagic corpuscles after 24 h of Ang II exposure significantly increased compared with the control group (Fig. 2F). Collectively, the data indicated that Ang II might activate the autophagy pathway in RRAECs.

**Differential expression profiling of mRNAs and miRNAs.** A total of 98,134,568-132,788,030 raw mRNA reads with clean ratios of 98.315-98.503% were collected for libraries.
of RRAECs in the Ang II and control groups. After filtering out low-quality raw reads and trimming the 3′ and 5′ adapter sequences, 96,665,811-130,551,102 clean reads were used for genomic alignment (Table II).

A total of 30,446,150-35,655,373 raw miRNA reads with clean ratios of 47-54% were collected for libraries of RRAECs in the Ang II and control groups. After filtering out low-quality raw reads, 15,852,783-18,500,681 clean reads were used for genomic alignment (Table III).

DE mRNAs are shown in Fig. 3A and C. After 24 h of Ang II exposure, a total of 443 DE mRNAs were identified (66 upregulated and 377 downregulated; FC >1.5 or <0.67; P<0.05). The mRNA with the most significant difference was 5′-nucleotidase cytosolic 1B (FC=10.46876; P=0.007145). DE mRNAs are shown in Fig. 3B and D. After 24 h of Ang II treatment, a total of 58 statistically significant DE miRNAs were detected (55 upregulated and 3 downregulated; FC >1.5 or <0.67; P<0.05). The mRNA with the most significant difference was miRNA-7b (FC=12.44444; P=5.46x10^{-8}). The 17 upregulated and 3 downregulated miRNAs are presented in Table IV.

RT-qPCR validation of significantly DE miRNAs and DE mRNAs. RT-qPCR was performed to validate the RNA-seq data. All nine detected miRNAs were upregulated in the Ang II group compared with the control group (Fig. 4A). The expression results of six mRNAs were consistent with the results of the RNA-seq, with the exception of Elk4 and Xkr8 (Fig. 4B). These data corroborated the results of the mRNA sequencing analysis, indicating that the RNA-seq data was reliable.

Functional analysis of DE mRNAs. GO analysis describes the properties of genes and gene products and provides functional annotation (20). GO covers three aspects of biology: Cellular component (CC), molecular function (MF), and biological process (BP). DE genes were sorted based on the associated P-values, and the top 30 terms are listed in Fig. 5A. After 24 h of Ang II treatment, the most enriched GO terms associated with endothelial dysfunction in each classification were ‘urogenital system development’ (GO ID, GO: 00016655; type, BP; P=7.49x10^{-8}), ‘apical part of cell’ (GO ID, GO: 0045177; type, CC; P=5.66x10^{-14}) and ‘oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor’ (GO ID, GO: 0016616; type, MF; P=3.19x10^{-8}). Various enriched biological processes were also observed in the present study, including ‘glycolytic process’ (Pfkfb3, glycerol-3-phosphate dehydrogenase 1, fructose-bisphosphatase 1 and peroxisome proliferative activated receptor, gamma, coactivator 1 alpha), ‘regulation of nitric oxide biosynthetic process’ (apartate dehydrogenase domain containing, flavin containing dimethylamino monooxygenase 1 and 3, and hydroxysteroid 11b dehydrogenase 1), ‘regulation of systemic arterial blood pressure mediated by a chemical’ [N-deacetylasine and N-sulfotransferase 2, serpin family F member 2, Hydroxysteroid dehydrogenase 1b2 (Hsd11b2) and glutamyl aminopeptidase] (Fig. 5A). The enriched pathways for DE mRNAs were analyzed by KEGG annotation, and the 20 most significantly enriched pathways are listed in Fig. 5B. The main enriched pathways of DE mRNAs after 24 h of Ang II exposure were ‘metabolic pathways’ (62 genes), ‘glycolysis/gluconeogenesis’ (62 genes), ‘glycolysis/gluconeogenesis’ pathways of DE mRNAs after 24 h of Ang II exposure were analyzed by KEGG annotation, and the 20 most significantly enriched pathways are listed in Fig. 5A. The enriched pathways for DE mRNAs were dehydrogenase 1 (Hsd11b2) and glutamyl aminopeptidase.

Prediction and functional analysis of DE miRNA targets. miRNA target prediction was performed to explore the potential regulatory roles of miRNAs in RRAECs. Bioinformatics analysis suggested the presence of 4,294 target genes of DE miRNAs after treatment with Ang II for 24 h. As shown in Fig. 5C, the most enriched GO terms of DE miRNA target genes associated with endothelial dysfunction in each class were ‘regulation of protein serine/threonine kinase activity’ (GO ID, GO: 0071900; type, BP; P=0.000393044), ‘vacuolar membrane’ (GO ID, GO: 0005774; type, CC; P=3.34x10^{-8}) and ‘transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding’ (GO ID, GO: 0001228; type, MF; P=2.40x10^{-8}). KEGG analysis suggested that the pathways of the DE miRNA target genes were mainly enriched in the ‘mTOR signaling pathway’, ‘AMPK signaling pathway’, ‘autophagy-animal’, ‘Proximal tubule bicarbonate reclamation’, ‘MAPK signaling pathway’, ‘insulin resistance’ and ‘Ras signaling pathway’ (Fig. 5D).
mRNAs and DE miRNAs associated with autophagy are presented in Tables SIII and SIV (32-48).

Network map of DE miRNAs and their target genes. A global network of miRNAs and target genes was constructed to determine the functional interactions between the two types of genes (Fig. 7A). Furthermore, to evaluate the contribution of specific miRNAs, the current study focused on the miRNA-200 family, which was highly and specifically expressed in RRAECs cultured with Ang II and may be a potent regulator of renal artery EC dysfunction. Therefore, a local network map of the miRNA-200 family and their corresponding target genes was constructed (Fig. 7B).

Discussion

Targeting unique miRNAs which are highly expressed in ECs may be a promising approach in the development of therapeutic tools for diseases associated with endothelial dysfunction. The present study proposed that Ang II activated autophagy pathway in RRAECs, as well as glycolysis pathways to compensate for the lack of the total cellular ATP level caused by mitochondrial respiratory disorder (49). To test this hypothesis, the differential expression of miRNAs and mRNAs in RRAECs after Ang II exposure was profiled in order to extend the list of potential candidate genes involved in hypertensive renal disease. The top 10 DE mRNAs identified in RNA-seq, including matrix metalloproteinase (MMP)-28, anoctamin-1 (ANO1), and myosin light chain kinase 2 (Mylk2), were closely related to EC dysfunction. MMP-28, the newest member of the MMP family, affects the function of hypertensive target organs by impairing the microvascular endothelium (50). MMP-28 overexpression causes EC apoptosis and capillary degeneration, leading to renal arteriosclerosis by directly cleaving the extracellular domain of vascular endothelial growth factor receptor 2 and also increases the expression and activity of MMP-2, causing
### Table IV. Top 10 upregulated and downregulated miRNAs and mRNAs in the Ang II group compared with the control group.

#### A. miRNA

| Name       | P-value | Fold change | Expression status |
|------------|---------|-------------|-------------------|
| miR-7b     | 5.46x10^-8 | 12.44444   | Upregulated       |
| miR-127-3p | 0.000344 | 10.4        | Upregulated       |
| miR-434-5p | 0.001827 | 9.75        | Upregulated       |
| miR-141-5p | 0.032335 | 8           | Upregulated       |
| miR-205    | 1.11x10^-17 | 7.074074   | Upregulated       |
| miR-215    | 1.82x10^-14 | 6.103448   | Upregulated       |
| miR-203a-3p| 3.29x10^-24 | 5.870866 | Upregulated       |
| miR-133a-3p| 0.00262  | 5.625       | Upregulated       |
| miR-449a-3p| 0.041573 | 5.2         | Upregulated       |
| miR-486    | 4.09x10^-4 | 5.172414   | Upregulated       |
| miR-143-5p | 9.24x10^-10 | 4.684932 | Upregulated       |
| miR-200c-3p| 4.25x10^-8  | 4.228571   | Upregulated       |

#### B. mRNA

| Name       | P-value | Fold change | Expression status |
|------------|---------|-------------|-------------------|
| Nt5c1b     | 0.007145 | 10.46876   | Upregulated       |
| Sectm1b    | 0.029084 | 9.008912   | Upregulated       |
| Irs3       | 0.008376 | 8.137557   | Upregulated       |
| Mmp28      | 0.013961 | 6.498763   | Upregulated       |
| Mrh8       | 0.031247 | 5.287269   | Upregulated       |
| Ipce1      | 0.047342 | 5.064618   | Upregulated       |
| Ano1       | 0.031278 | 5.082963   | Upregulated       |
| Mylk2      | 0.035396 | 4.617866   | Upregulated       |
| Olr1387    | 0.016509 | 4.455626   | Upregulated       |
| Cldn18     | 0.019303 | 4.307229   | Upregulated       |
| Metazoa_SR P | 1.51x10^-9 | 0.003036 | Downregulated     |
| Tmprss2    | 1.37x10^-6 | 0.046255 | Downregulated     |
| Scl6a19    | 0.001815 | 0.0583     | Downregulated     |
| Scl5a8     | 0.000943 | 0.059467   | Downregulated     |
| Bhtm2      | 2.04x10^-7 | 0.062865 | Downregulated     |
| Ank4b      | 0.012484 | 0.065145   | Downregulated     |
| Scl26a1    | 0.000423 | 0.0671     | Downregulated     |
| Pparg1a    | 0.027021 | 0.072853   | Downregulated     |
| Inmt       | 0.011903 | 0.075168   | Downregulated     |
| Tmem207    | 0.003891 | 0.076696   | Downregulated     |

miRNA, microRNA; Ang II, angiotensin II.
Figure 4. Reverse transcription-quantitative PCR validation (n=6) and RNA-seq data (n=3) of DE genes. (A) Relative expression of DE miRNAs in the Ang II group compared with the control group in quantitative PCR validation and RNA-seq, respectively. (B) Relative expression of DE mRNAs in the Ang II groups compared with the control groups in qPCR validation and RNA-seq, respectively. **P<0.01 and *P<0.001. The fold change for each gene (red bar) is the mean of 6 determinations originating from the Ang II groups vs. controls. qPCR, quantitative PCR; miRNA/miR, microRNA; RNA-seq, RNA sequencing; DE, differentially expressed; Pfkfb3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; Tpd52, tumor protein D52; Xkr8, XK related 8; Elk4, ETS transcription factor ELK4; Selenop, selenoprotein P; Zeb2, zinc finger E-box binding homeobox 2.

Figure 5. GO and KEGG enrichment analyses of DE mRNAs and miRNAs. (A) GO enrichment of DE mRNAs in the Ang II group compared with the control group. The top 30 GO terms in three categories are summarized. (B) KEGG enrichment of DE mRNAs in the Ang II group compared with the control group. The top 20 pathways of DE mRNAs are summarized. (C) GO enrichment of DE miRNAs in the Ang II group compared with the control group. (D) KEGG enrichment DE miRNAs in the Ang II group compared with the control group. The top 20 pathways of DE miRNA target genes are summarized. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DE, differentially expressed; miRNA, microRNA; Ang II, angiotensin II; BP, biological process; CC, cellular component; MF, molecular function.
and significantly reduced the mitochondrial membrane potential of RRAECs. Therefore, it may be concluded that ECs tend to rely on glycolysis metabolism to compensate for mitochondrial dysfunction. In addition, fatty acid degradation in ECs is directly related to angiogenesis, which regulates biomass synthesis to a certain extent, especially the production of deoxyribonucleotides required for DNA synthesis during EC proliferation (65).

Ang II has direct effects on renal vascular ECs, causing vasoconstriction of both afferent and efferent arterioles, which results in the development of both glomerular capillary hypertension and reduced glomerular filtration rates. Ang II has been shown to stimulate the adrenal cortex to secrete aldosterone, leading to increased reabsorption of sodium and water into the distal renal tubules. In addition, Ang II induces vascular NADPH oxidase and ET-1 expression in the kidneys (66,67). Therefore, the relevance of RAS components in the determination of hypertensive nephropathy demonstrated therapeutic implications.

miRNAs are known to play a role in the occurrence and development of hypertensive renal damage. RNA-seq analysis and RT-qPCR results showed that miR-200a-3p, miR-200b-3p, miR-200c-3p and miR-429 were highly expressed in the Ang II group compared with the normal group. These miRNAs are members of the miRNA-200 family, which regulates the expression of transcriptional factors through temporal and spatial patterns during the development of the anterior kidney (68). Therefore, an in-depth analysis of miRNA-200 family members can be performed in a subsequent study based on the constructed network map of the above four miRNAs and their respective target mRNAs. Recent reports demonstrated that miRNA-200c not only promotes epithelial mesenchymal transdifferentiation by inhibiting zinc finger E-box binding to homologous cassette (ZEB) 2 (69), but also upregulates cyclooxygenase 2 expression via ZEB1 to mediate human artery vascular dysfunction (70). The present findings demonstrated that the expression of miRNA-200c was significantly upregulated in the Ang II group compared with the normal group (FC=4.228571; P=4.25x10^-8). Further insights into the role of miRNA-200 in ECs will improve the understanding of the molecular mechanisms of Ang II-induced renal artery endothelial dysfunction. A previous study has indicated that exogenous platelet miRNA-223 decreases the expression of insulin-like growth factor 1 receptor in HUVEC and thus promotes advanced glycation end product-induced vascular endothelial cell apoptosis (71). Upregulated expression of miRNA-223 in the Ang II group may provide a promising and novel approach to the treatment of hypertension (72). miRNA-494, which increases lipopolysaccharide-induced apoptosis of human proximal tubular epithelial cells by negatively regulating the cyclic AMP-dependent transcription factor ATF-3 gene, was found to mediate the apoptosis of various cells (73). Whether overexpressed miRNA-494 in the Ang II group (FC=1.550713; P=0.002353) exerts a similar effect requires further investigation.

As small noncoding RNAs, miRNAs exert their regulatory functions by mRNA degradation or translational inhibition (74).
In the current study, the target genes of DE miRNAs were mainly enriched in the ‘mTOR signaling pathway’, ‘AMPK signaling pathway’ and ‘autophagy-animal’. Among these, the mTOR signaling pathway, which regulates the growth and differentiation of cells by sensing the stimulation of growth factors and nutrition, is the main nutrient sensor in the cells and serves a critical role in coordinating intracellular energy metabolism and overall energy levels in vivo (75,76). In addition, mTOR is also a key regulator of autophagy initiation, and mTOR activation inhibits the formation of autophagosomes and negatively regulates autophagy (77,78). Autophagy, which can be regulated by the mTOR pathway, is a biological process in which parts of proteins or organelles encapsulated in a cell membrane are transferred to lysosomes for digestion and degradation to maintain cell homeostasis (79). Furthermore, glycolysis flux, apart from mitochondrial respiratory function, has been shown to be a potential treatment for ischemic kidney damage (80). In ECs, glucose is shuttled through hexokinase into the glycolytic pathway. The product of glycolysis is used not only for the synthesis of glycogen, but also for energy production. A key enzyme for glycolysis, 6-phosphate fructose-2-kinase/fructose-2,6-bisphosphatase 3 is regulated by cellular energy status (the AMPK (81) or growth factor AKT (82) signaling pathways) and participates in protein synthesis regulated by mTOR. The present sequencing results also demonstrated that glycolysis was one of the most important energy metabolism pathways of Ang II-induced renal artery EC dysfunction. Ang II may activate the glycolysis and autophagy pathway in RRAECs through several DE mRNAs or the binding of DE miRNA to its target mRNA. Furthermore, several studies revealed that autophagy may regulate cell glycolysis by selectively degrading hexokinase 2 (83);
glycolysis in turn plays an essential role in autophagy by limiting superoxide levels and maintaining expression of autophagy genes required for autophagic vesicle maturation (84). Therefore, the effect of the miRNA-mediated mTOR signaling pathway on glycolytic metabolism and autophagy is indicative of the pathological mechanism of Ang II-induced renal artery EC dysfunction.

In conclusion, miRNAs that regulate multiple target genes provide an effective link between regulator target genes and complex biological processes that can be identified through miRNA-mRNA interactions. Further studies investigating the miRNAs and their associated pathway will likely result in the development of novel approaches for the treatment of hypertensive kidney damage.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YL performed the experiments and drafted the manuscript. YJ designed the study and performed the experiments. WL designed the study, contributed to the discussion and performed important revisions of the article. CH and ZQ contributed to the discussion and performed ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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