

Serum from patients with hypertension promotes endothelial dysfunction to induce trophoblast invasion through the miR-27b-3p/ATPase plasma membrane Ca²⁺ transporting 1 axis

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Abstract. Pregnancy-induced hypertension is often accompanied by preeclampsia. The present study investigated whether microRNA (miR)-27b-3p affected the occurrence of preeclampsia by regulating the function of endothelial cells. Expressions levels of miR-27b-3p and ATPase plasma membrane Ca²⁺ transporting 1 (ATP2B1) were determined using reverse-transcription quantitative PCR. miR-27b-3p targeting ATP2B1 was predicted using bioinformatics and further confirmed by dual-luciferase reporter assays. Cell Counting Kit-8, Transwell and Matrigel tube formation assays were performed to detect the effects of miR-27b-3p on proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs), respectively. Moreover, HTR8/SVneo cells were co-cultured with HUVECs to detect the invasion of trophoblast cells, and the expression levels of vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-2 and MMP-9 of HUVECs and HTR8/SVneo were detected by western blotting. Expression levels of miR-27b-3p were upregulated in the serum of patients with hypertension and preeclampsia, which could target and regulate the expression of ATP2B1. The expression levels of miR-27b-3p were increased and those of ATP2B1 were reduced in HUVECs from hypertensive serums. Moreover,

miR-27b-3p mimics reduced the expression level of ATP2B1, and miR-27b-3p inhibitor reversed the effect of hypertensive serum on ATP2B1 expression. Furthermore, patients with hypertension showed increased endothelial dysfunction, reduced trophoblastic invasion and the expressions of VEGF, MMP-2 and MMP-9, and miR-27b-3p mimics and silencing of ATP2B1 produced similar results to HUVECs. The miR-27b-3p inhibitor reversed the effect of hypertensive serum, and silencing of ATP2B1 inhibited the improvement of miR-27b-3p inhibitor to HUVECs and HTR-8/SVneo cells in proliferation, migration and tube formation. The current findings suggested that miR-27b-3p promoted proliferation, migration and tube formation of HUVECs and enhanced invasion of trophoblast cells, via regulation of ATP2B1. Thus, miR-27b-3p could be considered as a molecular risk factor in the pathogenesis and development of preeclampsia.

Introduction

Preeclampsia is a pregnancy-specific hypertensive disorder that may lead to the death of newborns and pregnant women (1). Patients with preeclampsia are likely to have placental dysplasia, which seriously affects the health of pregnant women and fetuses. Pregnant women with chronic hypertension have a significantly higher risk of developing preeclampsia compared with healthy pregnant women. For those with a family history of preeclampsia, the incidence of chronic hypertension is further greatly increased (2). In addition, the risk of patients with preeclampsia having chronic hypertension is estimated to be higher in the future compared with now (3,4). Researchers confirmed that the ATP2B1 gene is a gene susceptible to chronic hypertension among different races (5-7).

The ATP2B1 gene, located at 12q21.3, encodes the ATPase plasma membrane Ca²⁺ transporting 1 (ATP2B1) protein, which belongs to the P-type ion transport ATPase family of proteins and is widely expressed in mammals (8). In 2009, through use of a genome-wide association study, Levy *et al* (9) conducted a large cohort study on two European populations, and found a single nucleotide polymorphism locus associated with chronic hypertension; the SNPrs2681472 locus located

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Abbreviations: ATP2B1, ATPase plasma membrane Ca²⁺ transporting 1; HUVECs, human umbilical vein endothelial cells; FBS, fetal bovine serum; RT-qPCR, reverse-transcription quantitative PCR; TFP, trifluoperazine

Key words: microRNA-27b-3p, ATPase plasma membrane Ca²⁺ transporting 1, HUVECs, hypertension, preeclampsia

in the ATP2B1 gene. SNPrs2681472 polymorphism of ATP2B1 gene has been previously reported to be associated with early-onset preeclampsia among Chinese pregnant women (10). The SNPrs2681472 polymorphism of ATP2B1 gene may participate in the regulation of hypertension and preeclampsia by affecting ATP2B1 gene expression levels (5).

In recent years, the effects of microRNAs (miRNAs/miRs) on hypertension and preeclampsia have been widely studied. miRNAs are non-coding RNAs 21-25 nucleotides in length, and are involved in ~30% of the regulation of gene expressions in the human genomes (11). A previous study by Wang *et al* (12) found that compared with normal pregnant women, the expression levels of nine miRNAs, namely, miR-223, -195, -17, -18a, -218, -19b1, -379, -92a1 and miR-411, are reduced in placenta tissues of patients with severe preeclampsia compared with controls. Moreover, the expression of another seven miRNAs (miR-30a-3p, -210, -524, -518b, -18a, -17-3 and -411) are also reduced, and miR-151 and miR-193b expression levels are increased in the tissues derived from patients with preeclampsia (13). Previous studies showed that miR-125b-5p, -100-5p and -199a-5p have a low level of expression in the peripheral blood of patients with pregnancy-induced hypertension and preeclampsia (14). Compared with normal pregnant women, the levels of total miRNAs and hsa-miR-210 in peripheral blood of pregnant women with hypertensive are significantly increased. hsa-miR-210 has been seen as a suitable biomarker for indicating pregnancy-induced hypertension (15). Moreover, miR-210 is involved in the pathogenesis of preeclampsia (16). Recently, it has been found that miR-27b-3p participates in various pathological processes, including in tumor angiogenesis, lipid metabolism, inflammatory response and the oxidative stress response (17).

The present study was designed to explore the relationship between hypertension and preeclampsia, and the role of miR-27b-3p. miR-27b-3p was found to be upregulated in patients with hypertension and patients with preeclampsia. The effects of miR-27b-3p on HUVECs stimulated by the serum of patients with hypertension were investigated. Moreover, the effects of miR-27b-3p on HTR-8/SVneo cells co-cultured with HUVECs cells were explored, following stimulation by the serum of patients with hypertension. These results confirmed that miR-27b-3p might be a diagnostic marker for hypertension and preeclampsia.

Materials and methods

Patients. Normal pregnant women (n=21; age 33.5±4; body mass index 22.3±3 kg/m²; vaginal delivery), women with preeclampsia (n=21; age 34.1±5; body mass index 24.6±4 kg/m²; vaginal delivery), female patients with hypertension (n=13; 51.5±4) and female healthy volunteers (n=13; 52.5±4), who attended The Affiliated Hangzhou First People's Hospital, were included. The study was approved by The Affiliated Hangzhou First People's Hospital Ethics Committee (Hangzhou, China). All subjects were informed of the purpose and process of the study, and signed an informed consent form. The inclusion criteria of normal pregnant women are: i) Healthy subjects; ii) delivery after 37 weeks; iii) successful pregnancy without any complications, normal blood pressure and negative proteinuria. The diagnostic criteria for preeclampsia are:

i) Hypertension after 20 weeks of pregnancy and ii) positive proteinuria. Systolic blood pressure (>140 mmHg) and/or diastolic blood pressure (>90 mmHg) served as diagnostic criteria for hypertension, with proteinuria ≥0.3 g/24 h. Patients with a history of diabetes, chronic kidney disease and/or heart disease were excluded. The venous blood of pregnant women was collected (3 ml) at birth and centrifuged at 12,000 x g for 8 min at 4°C. The serums were stored at -80°C.

Cell culture and treatment. HUVECs and HTR-8/SVneo cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The cells were grown in RPMI-1640 media containing 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 ng/ml streptomycin (Sigma-Aldrich; Merck KGaA) in a humid incubator at 37°C with CO₂. When the density of cells (1x10⁶ cells/ml) reached 70-80%, the cells either remained untransfected or were transfected with miR-27b-3p inhibitor control (cat. no. 4464076, Thermo Fisher Scientific, Inc.), miR-27b-3p inhibitor (cat. no. 4464084, Thermo Fisher Scientific, Inc.) or miR-27b-3p mimics (cat. no. 4464066, Thermo Fisher Scientific, Inc.) using Lipofectamine[®] RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.). After recovery in fresh medium for 24 h, the cells transfected with either the miR-27b-3p inhibitor control or miR-27b-3p inhibitor were cultured in the medium added with 10% serum from patients with hypertension for 24 h as Serum + control (C) cells and Serum + inhibitor (I) cells. The untransfected cells or those transfected with miR-27b-3p mimics, mimics control (MC) and inhibitor control (IC) were cultured in fresh medium for 24 h as C cells, mimic (M) cells, MC and IC cells.

Revers-transcription-quantitative PCR (RT-qPCR) assay. Total miRNAs were separated from tissues and cells using a miRNeasy Mini kit (cat. no. 217004; Qiagen GmbH) following the manufacturer's instructions, and 1 µg of miRNAs was used to obtain cDNAs with a miScript II reverse transcription kit (cat. no. 218160; Qiagen GmbH) at 37°C for 15 min and 98°C for 5 min. In addition, total cellular RNA was isolated using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the SMART MMLV Reverse Transcriptase (cat. no. 639523; Takara Bio, Inc.) was used to reverse-transcribe mRNAs into cDNAs at 37°C for 15 min and 98°C for 5 min. Then, the levels of miRNAs were detected using a miScript SYBR-Green PCR kit (cat. no. 218075; Qiagen GmbH) according to the manufacturer's instructions (18), and the mRNA levels were measured using SYBR Premix Ex Taq kit (cat. no. RR820A; Takara Bio, Inv.) in an ABI7300 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). U6 served as an endogenous control of miRNA, and GAPDH was used to normalize the mRNAs. The sequences of primers used are listed in Table I. The reaction conditions were as follows: Denatured at 95°C for 10 min followed by 40 cycles at 95°C for 10 sec, at 60°C for 20 sec and at 72°C for 30 sec. Gene expression levels were calculated using 2^{-ΔΔC_q} method (19).

Western blotting. Total cell proteins were extracted by RIPA buffer (cat. no. 9806; Cell Signaling Technology, Inc.), and protein concentration was measured by BCA. A total of 50 µg protein/lane was separated by 12% SDS-PAGE, and then transferred to PVDF

Table I. Primer base sequences.

Primer name	Sequence (5'-3')
miR-27b-3p	Forward: CTCAACTGGCGTGGAGTCGGCAATTCAGTTGAGGCAGAACT
miR-27b-3p	Reverse: AACTCCAGCTGGGTTACAGTGGCTAAG
ATP1B2	Forward: TGTGTTGGCAAGAGAGATGAAGA
ATP1B2	Reverse: GGGTCACATTCAGGAACTTTACA
GAPDH	Forward: CACCCACTCCTCCACCTTTG
GAPDH	Reverse: CCACCACCCTGTTGCTGTAG
U6	Forward: CTCGCTTCGGCAGCAC
U6	Reverse: AACGCTTCACGAATTTGCGT

miR, microRNA.

membrane by electroporation. The membrane was blocked for 1 h using 5% skimmed milk powder dissolved in PBS at room temperature. The membranes were incubated overnight with anti-ATP1B2 (1:1,000; cat. no. ab185210; Abcam), anti-vascular endothelial growth factor (VEGF; 1:1,000 cat. no. ab53465; Abcam), anti-matrix metalloproteinase (MMP)-2 (1:1,000; cat. no. ab97779; Abcam), anti-MMP-9 (1:1,000; cat. no. ab38898; Abcam) and anti-GAPDH (1:2,000; cat. no. ab9485; Abcam) at 4°C. Then the membranes were washed three times with PBST including 0.05% Tween-20, incubated the HRP-conjugated rabbit anti-mouse IgG H&L antibody (1:2,000; cat. no. ab6728; Abcam) at room temperature for 1 h, and then washed three times with PBST. Finally, the membranes were visualized using ECL detection reagents (Thermo Fisher Scientific, Inc.) and then semi-quantified using ImageJ software (version 1.48; National Institutes of Health).

Dual-luciferase reporter gene assay. The HUVECs were cultured in 24-well plates and co-transfected with 200 ng/ μ l miR-27b-3p mimics, miR-27b-3p inhibitor or miRNA control, and 200 ng of pGL3-ATP2B1-3'UTR or pGL3-ATP2B1-mutant (mut, 5'-AUGUCUUGGUGAAUCGUCGG-3')-3'UTR using Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After transfection for 36 h, luciferase activity was measured with the dual-luciferase assay system (Promega Corporation). Luciferase activity of genes was normalized to that of the *Renilla* luciferase. All analyses and experiments were performed in triplicate.

Cell Counting Kit (CCK)-8 assay. The HUVECs (5×10^3 cells/ml) were inoculated into a 96-well plate. miRNA control, miR-27b-3p inhibitor or miR-27b-3p mimics was transfected into the cells, followed by stimulation with 10% serum from patients with hypertension or in combination with Trifluoperazine (TFP; MedChemExpress), which is an inhibitor of ATP2B1 (20). After cell culture for 48 h, 10 μ l CCK-8 reagent was added into the cells and incubated together for 4 h. The absorbance value was detected at 490 nm by a microplate reader (Bio-Rad Laboratories, Inc.).

Cell migration assay. A cell suspension of serum-free medium was prepared at 1×10^5 cells/ml, and inoculated into the upper

chamber of a Transwell dish in 100 μ l/well, while complete medium including the 10% FBS was added to the lower chamber. After incubation for 48 h at 37°C, the cells remaining in the upper chamber were wiped off using a cotton swab, and the invading cells were fixed with 4% polyformaldehyde at room temperature for 15 min and stained by crystal violet. Cell migration was calculated from five randomly selected visual fields with a light microscope at x200 magnification (XDS-800D; Shanghai Caikon Optical Instrument Co., Ltd.). The average number of transplanted cells was counted manually in five randomly selected fields.

Tube formation assay. The bottom of the culture plate was added with 0.3 ml growth factor-reduced Matrigel and rested for 10 h at 3°C. The HUVECs were cultured on upper side of the Matrigel for 12 h, then the culture medium was discarded, and cells were cultured for 24 h in the complete culture medium with 10% FBS. The formation of tubules was observed under an inverted light microscope (XDS-800D; Shanghai Caikon Optical Instrument Co., Ltd.).

Trophoblast invasion assay. For the trophoblast invasion assays, each Transwell chamber 100 μ l of growth factor-reduced Matrigel (placed in a 24-hole plate) was added in the upper chamber, and then solidified in an incubator at 37°C for 2 h. Trophoblast HTR-8/SVneo cells (5×10^3 cells) were seeded into the upper chamber. The transfected HUVECs (2×10^4 cells) under the aforementioned conditions were added into the lower chamber for 24 h at 37°C. Next, the chamber was removed, and the remaining cells in the upper chamber were wiped off using cotton swabs, while those which had invaded the lower chamber were fixed and stained by hematoxylin and eosin at room temperature for 15 min, observed under an inverted light microscope (XDS-800D; Shanghai Caikon Optical Instrument Co., Ltd.) at x200 magnification and images were captured. Invasion was evaluated by counting the cells in five randomly selected fields.

Bioinformatics TargetScan7.2 (targetscan.org) predicted that miR-27b-3p could target the 3'-UTR of ATP2B1.

Statistical analysis. The data were analyzed by GraphPad Prism 5.0 software, and shown as mean \pm standard deviation

Table II. Clinical characteristics.

Variable	Normal pregnant women, n=21	Preeclampsia, n=21	Hypertension, n=13	Healthy volunteers, n=13
Maternal age, years	33.5±4	34.1±5	-	-
Age, years	-	-	51.5±4	52.5±4
Gestational age, weeks	37.5±1.6	36.8±2.3	-	-
Body mass index, kg/m ²	22.3±3	24.6±4	22.6±3.8	21.1±2
Mode of delivery	Vaginal	Vaginal	-	-
Baby birth weight, kg	3.2±0.33	2.9±0.49	-	-

with ≥ 3 independent experiments. Statistical significance was determined by unpaired Student's t-test or one-way ANOVA followed by Tukey's post hoc test according to the number of experimental groups analyzed. $P < 0.05$ was used to indicate a statistically significant difference.

Results

miR-27b-3p is upregulated in serum of patients with hypertension and preeclampsia. The clinical characteristics of patients were described in Table II. Expression levels of miR-27b-3p were detected in higher concentrations in the serum of patients with hypertension compared with those in the serum of healthy females (Fig. 1A). Furthermore, miR-27b-3p expression levels were also found to be higher in the serum of patients with preeclampsia compared with those in normal pregnant women (Fig. 1B).

ATP2B1 is a target gene of miR-27b-3p. ATP2B1 was a hypertensive-susceptible gene, and its expression was downregulated in preeclampsia women (21). Moreover, downregulation of ATP2B1 could increase blood pressure. TargetScan predicted that miR-27b-3p could target the 3'-UTR of ATP2B1 (Fig. 1C). Subsequently, luciferase reporter assays were performed, and demonstrated that the miR-27b-3p mimic reduced luciferase activity in the ATP2B1 wild-type (wt) group, and that miR-27b-3p inhibitor increased luciferase activity in the ATP2B1 wt group compared with the control and mut groups. These results demonstrated that miR-27b-3p targeted ATP2B1 to reduce its expression (Fig. 1D).

Expression levels of ATP2B1 and miR-27b-3p are differentially regulated in HUVECs stimulated with the serum of patients with hypertension. A previous study demonstrated that hypertension can induce dysfunction of HUVECs (22). In the present study, miR-27b-3p and ATP2B1 were predicted to be involved in the regulation of HUVECs biological functions. Subsequent results indicated that the miR-27b-3p expression levels were increased and those of ATP2B1 were reduced when HUVECs were stimulated with the serum of patients with hypertensive (Fig. 1E and F). The expression of miR-27b-3p decreased after miR-27b-3p inhibitor transfection, and increased after miR-27b-3p mimics transfection (Fig. 2A and B). Furthermore, the protein and mRNA expression levels of ATP2B1 were downregulated in HUVECs transfected with miR-27b-3p mimics, which was

consistent with the results that HUVECs were stimulated by the serum of patients with hypertension (Fig. 2C and D). In addition, adding the serum of patients with hypertension after the transfection of the miR-27b-3p inhibitor into HUVECs restored the down-regulation of ATP2B1 caused by the hypertensive serum stimulation alone (Fig. 2C and D). The present results therefore revealed that the serum of patents with hypertension reduced the expression of ATP2B1 in HUVECs.

Effect of miR-27b-3p on angiogenesis of HUVECs in vitro. The dysfunction of endothelial cells could cause blockage of angiogenesis, which was the main cause of preeclampsia (23). The effects of miR-27b-3p on endothelial angiogenesis were examined *in vitro*, and it was observed that serum from hypertensive patients inhibited the proliferation of endothelial cells. Furthermore, such an effect was reversed by the transfection of an miR-27b-3p inhibitor into HUVECs, but the subsequent addition of an ATP2B1 inhibitor, TFP, alongside the miR-27b-3p inhibitor served to inhibit the proliferation of endothelial cells again (Fig. 3A). This demonstrated that miR-27b-3p mimics or TFP treatment of HUVECs inhibited the cell proliferation, and similar effects were observed on cell migration and tubular formation (Fig. 3B and C). Moreover, miR-27b-3p mimics and inhibiting ATP2B1 decreased the expressions of VEGF, MMP-9 and MMP-2 in HUVECs. miR-27b-3p inhibitor increased the expressions of VEGF, MMP-9 and MMP-2, while inhibition of ATP2B1 could reverse the aforementioned effects on HUVECs (Fig. 3D). These results indicated that miR-27b-3p inhibited endothelial cell angiogenesis through targeting ATP2B1.

miR-27b-3p promotes trophoblast invasion in a co-culture system of endothelial cells and trophoblast cells. Endothelial cells have an important role in the invasion of trophoblast cells, which possess a critical function in maintaining normal pregnancy, and any disturbance between these two cells will induce the occurrence of preeclampsia (24). In the present study, the invasion of trophoblast cells was detected by a co-culture system of endothelial cells and trophoblast cells. The data revealed that the invasion of co-cultured trophoblast HTR-8/SVneo cells was inhibited when HUVECs were treated with the serum from patients with hypertension, miR-27b-3p mimics or TFP; however, the aforementioned effects of the hypertensive serum were reversed by an miR-27b-3p inhibitor. In addition, the invasion of trophoblast HTR-8/SVneo cells was again inhibited by the addition of TFP (Fig. 4A). In

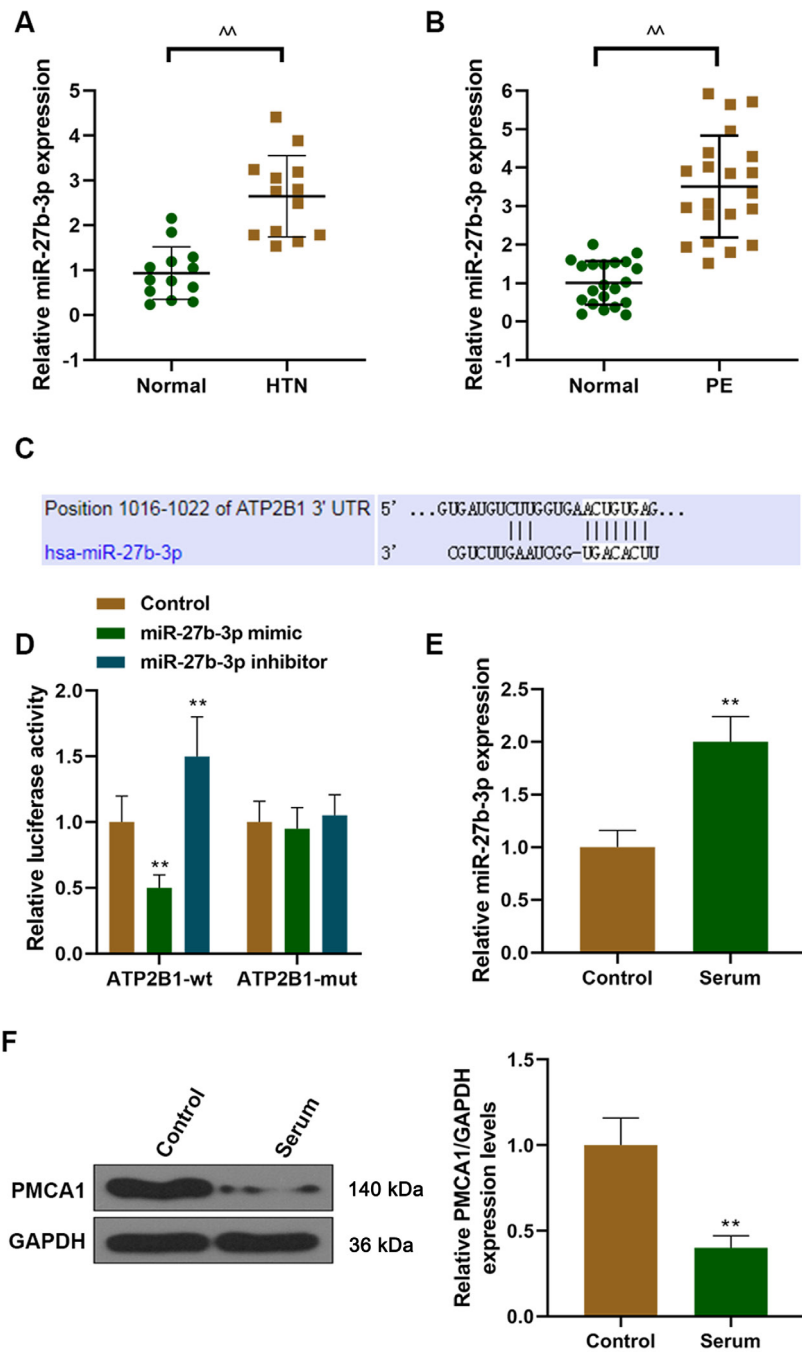


Figure 1. miR-27b-3p is upregulated in patients with hypertension and patients with PE, and miR-27b-3p targets ATP2B1. The level of miR-27b-3p was measured in (A) patients with hypertension and (B) patients with PE using RT-qPCR. (C) A predicted target site for miR-27b-3p was identified in the 3'-UTR of ATP2B1. (D) Luciferase activity was determined using a dual-luciferase reporter assay kit. (E) After HUVECs treated with the serum from patients with hypertension, the level of miR-27b-3p was detected using RT-qPCR. (F) After HUVECs were treated with the serum of patients with hypertension, the expression of ATP2B1 was detected using western blotting. [^]P<0.01 vs. normal, ^{**}P<0.01 vs. control. ATP2B1, ATPase plasma membrane Ca²⁺ transporting 1; HTN, hypertension; miR, microRNA; mut, mutant; PE, preeclampsia; RT-qPCR, reverse transcription-quantitative PCR; UTR, untranslated region; wt, wild-type; HUVECs, human umbilical vein endothelial cells.

addition, miR-27b-3p mimics and inhibition of ATP2B1 decreased the expression of VEGF, MMP-9 and MMP-2 in a co-culture of HTR-8/SVneo and HUVEC cells. miR-27b-3p inhibitor could increase the expressions of VEGF, MMP-9 and MMP-2, and ATP2B1 inhibition was found to reverse the effect in a co-culture of HTR-8/SVneo and HUVEC cells hypertension model (Fig. 4B). These results demonstrated that endothelial miR-27b-3p inhibited the invasion of trophoblast HTR-8/SVneo cells through targeting ATP2B1.

Discussion

Preeclampsia, which is one of the most important complications during pregnancy, seriously threatens the health of mothers and infants (25,26). Studies have showed that endothelial dysfunction and placental trophoblast invasion play important roles in the pathogenesis of preeclampsia (27,28). Preeclampsia is often accompanied by hypertension, and vascular endothelial cell dysfunction is closely related to hypertension (29,30).

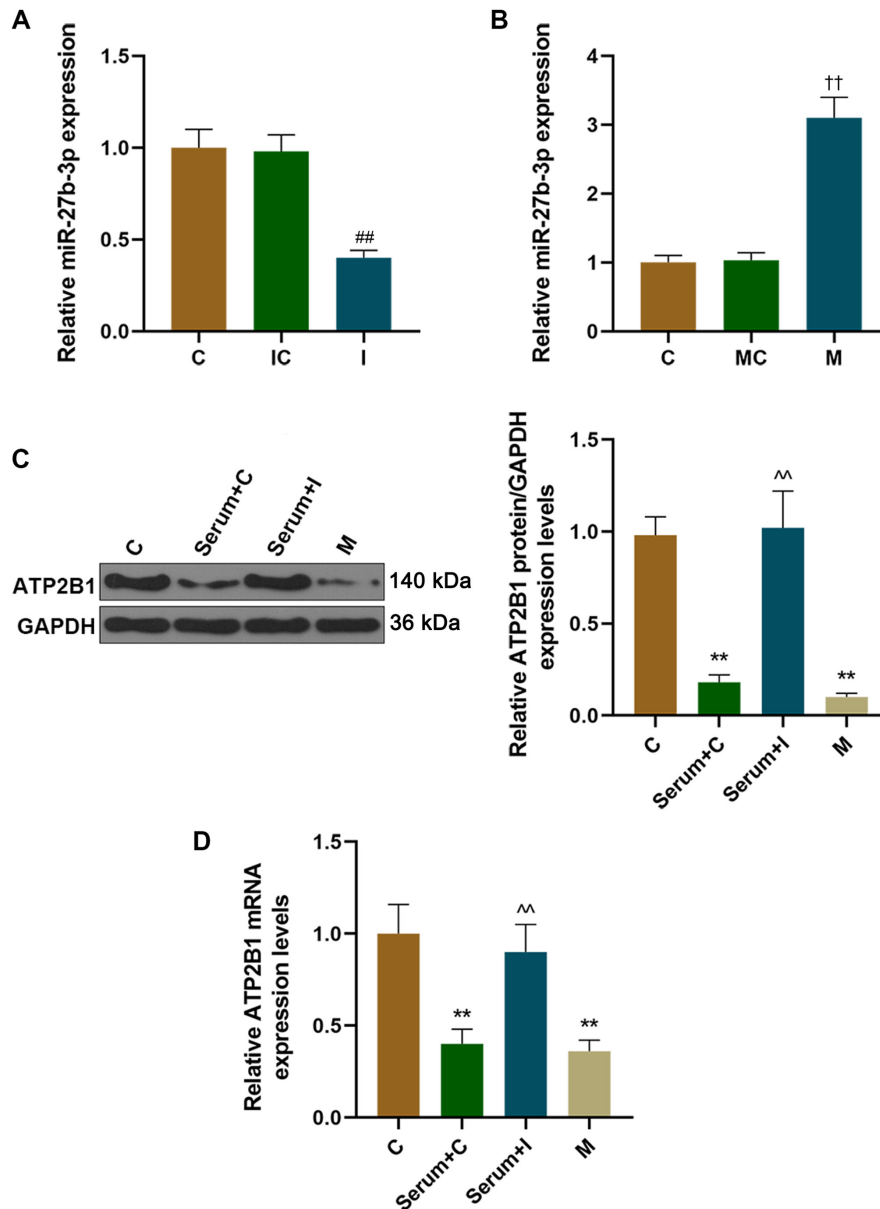


Figure 2. The expression of ATP2B1 was reduced in HUVEC cells following treatment with the serum of patients with hypertension, and miR-27b-3p regulated the expression of ATP2B1. The transfection efficiency of (A) miR-27b-3p inhibitor or (B) mimics were detected using RT-qPCR. After HUVECs were treated with the serum of patients with hypertension and/or miR-27b-3p inhibitor or mimics, the protein and mRNA expressions of ATP2B1 were detected using (C) western blotting and (D) RT-qPCR. †† $P < 0.01$ vs. MC, ## $P < 0.01$ vs. IC; ** $P < 0.01$ vs. C, ∧ $P < 0.01$ vs. Serum+C. ATP2B1, ATPase plasma membrane Ca^{2+} transporting 1; C, control; M, mimic; miR, microRNA; I, inhibitor; MC, mimic control; IC, inhibitor control; RT-qPCR, reverse transcription-quantitative PCR; HUVECs, human umbilical vein endothelial cells.

As miRNAs are highly-expressed under hypertension, they may have clinical value in early diagnosis of hypertension and prognosis of complications (31,32). Detection of the expression levels of miRNAs in peripheral blood mononuclear cells of healthy individuals and those with hypertension showed that the expressions of miR-1, miR-21, -208b and -499 were increased, and the expressions of miR-26b and -133a were reduced in patients with hypertension (33). Migration and vascularization of the three types of endothelial cells are regulated by miR-505/FGF18 (34). miR-27b-3p is a multifunctional miRNA molecule that plays an important role in numerous physiological and pathological processes (35-37), and is closely related to cell proliferation, invasion, migration and apoptosis (38-40). The current study found that miR-27b-3p

expression was elevated in the serum of patients with hypertension and preeclampsia.

The ATP2B1 gene is known to be a risk gene for hypertension (41), and hypertension is a risk factor for preeclampsia (42). The risk of preeclampsia in patients with chronic hypertension is known to be increased after the incidence of pregnancy (43). ATP2B1 acts as a sodium/calcium exchanger to transport intracellular Ca^{2+} to the outside of the cells, thereby maintaining a low intracellular Ca^{2+} concentration in the resting state, which then affects the function of endothelial cells and angiogenesis (44,45). Higher nitric oxide production and endothelial nitric oxide synthase activity, which are related with reduced ATP2B1 activity, could induce endothelial cell injury (46). The present

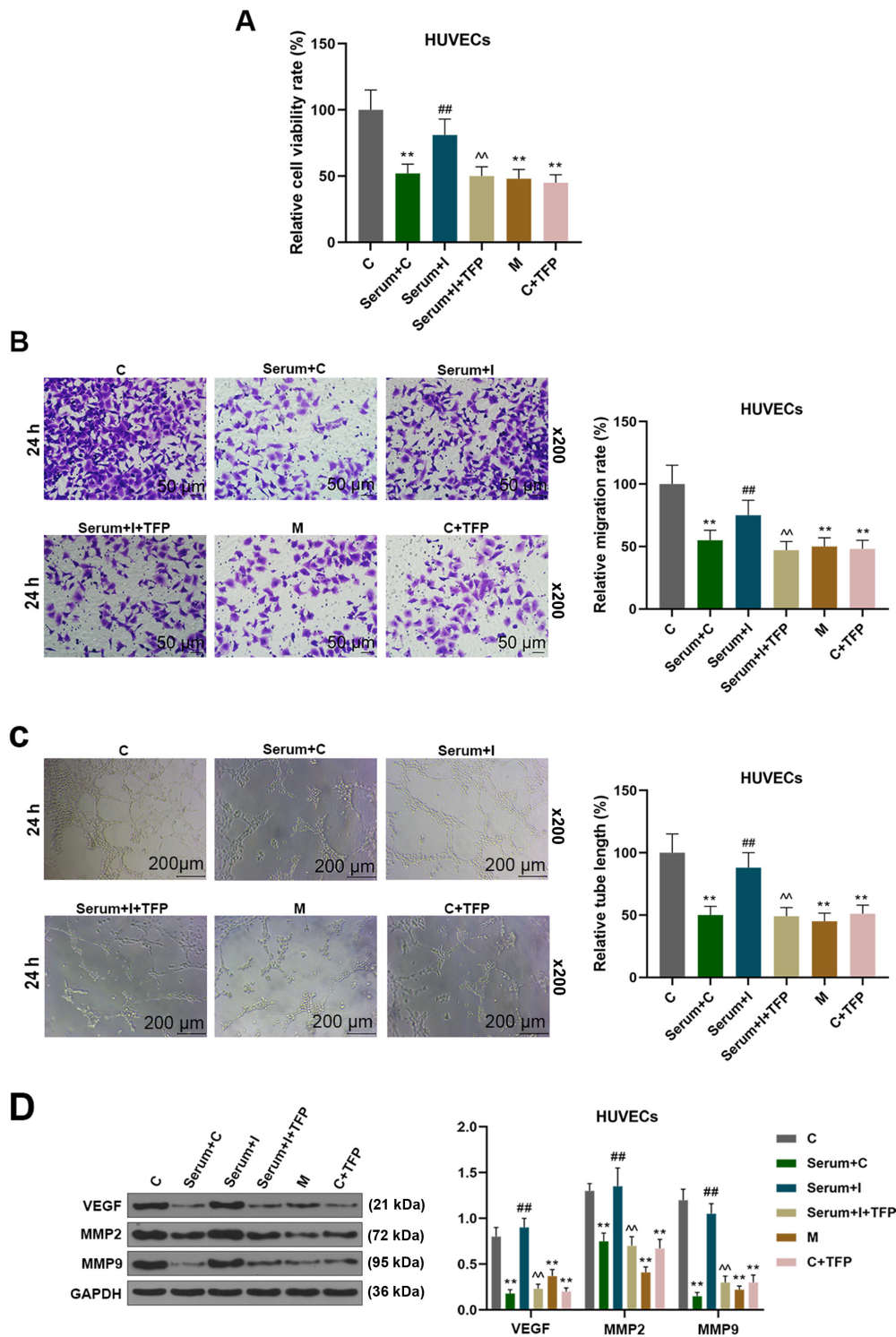


Figure 3. miR-27b-3p inhibited the process of angiogenesis of HUVECs. The experimental groups were as follows: i) HUVECs were transfected with C miRNA; ii) HUVECs transfected with C were stimulated by the serum of patients with hypertension; iii) HUVEC cells were transfected with miR-27b-3p I, followed by stimulation with the serum of patients with hypertension; iv) HUVECs were transfected with miR-27b-3p I, followed by stimulation with the serum of patients with hypertension and TFP; v) HUVECs transfected with miR-27b-3p M; vi) HUVECs transfected with C miRNA and treated with TFP (A) HUVECs viability was determined by CCK-8. (B) Cell migration was detected by Transwell assay. (C) Endothelial cell tube formation was determined on a layer of growth factor-reduced Matrigel. (D) The expressions of VEGF, MMP-9 and MMP-2 were detected by western blot analysis. **P<0.01 vs. C, ##P<0.01 vs. Serum+C, ^P<0.01 vs. Serum+miR-27b-3p I. C, control; CCK-8, Cell Counting Kit-8; I, inhibitor; M, mimic; TFP, trifluoperazine; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; HUVECs, human umbilical vein endothelial cells.

results showed that ATP2B1 was targeted by miR-27b-3p and low-expression levels in HUVECs were stimulated by hypertensive patients' serum, which could help to reduce cell migration and invasion.

Preeclampsia a disease that occurs during pregnancy, with superficial placenta implantation and uterine spiral artery remodeling disorder as its main pathological mechanisms (25,47). This process is an orderly activity, where

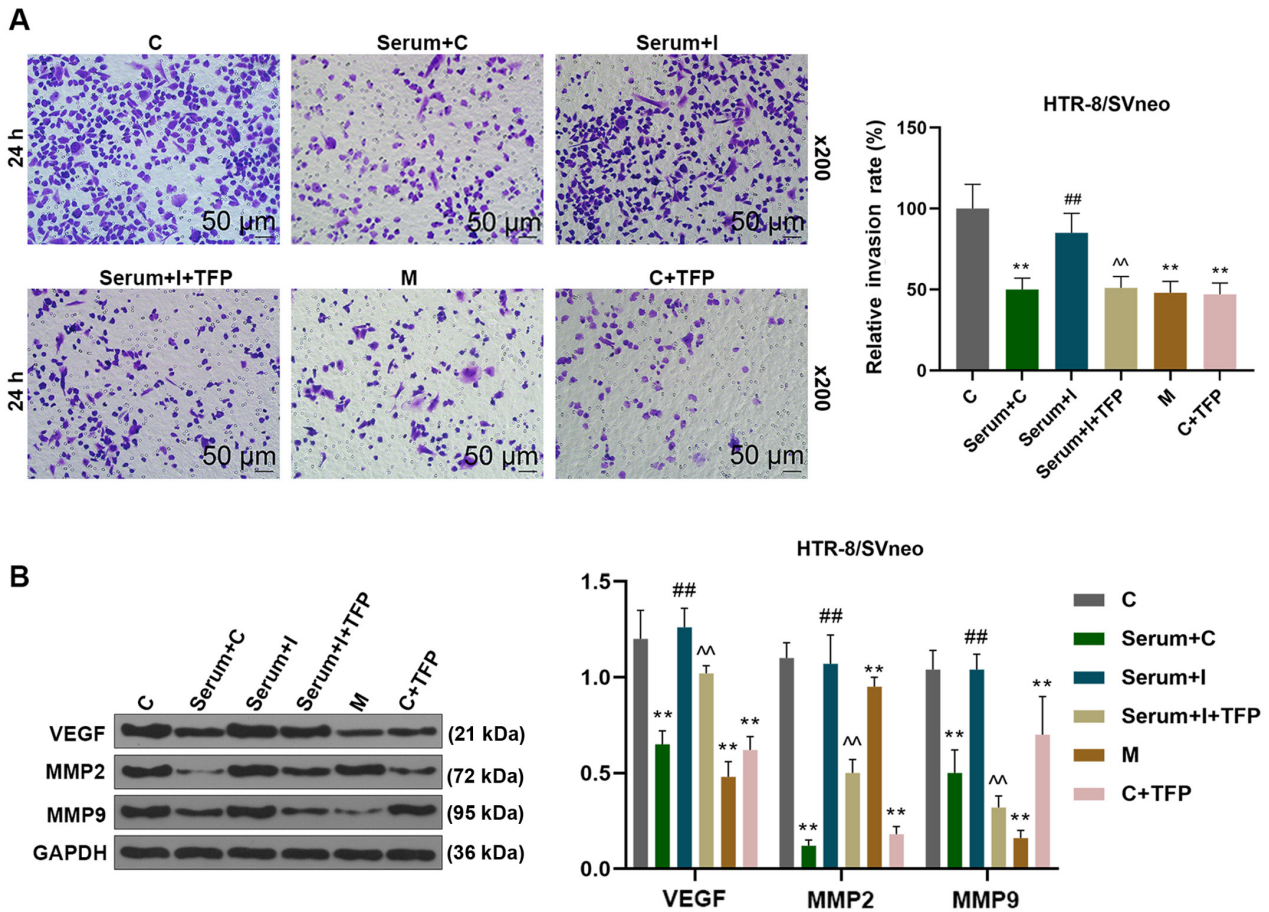


Figure 4. miR-27b-3p inhibited endothelial cell tube formation and trophoblast invasion by suppressing the expression of ATP2B1. The HUVEC experimental groups were as follows: Transfected with i) C miRNA; ii) C miRNA and stimulated by serum from patients with hypertension; iii) miR-27b-3p I, and stimulated with serum from patients with hypertension; iv) miR-27b-3p I, followed by stimulation with serum from patients with hypertension and TFP; v) miR-27b-3p M and vi) C miRNA and treated with TFP. (A) HUVECs were co-cultured with trophoblastic HTR-8/SVneo cells in Transwell plates for 5 h. HTR-8/SVneo cells invasion was detected and analyzed using ImageJ software. (B) After HTR-8/SVneo cells were isolated, the expression levels of VEGF, MMP-9 and MMP-2 were detected using western blotting. ** $P < 0.01$ vs. C, ## $P < 0.01$ vs. Serum+C, ^ $P < 0.01$ vs. Serum+miR-27b-3p I. miR, microRNA; ATP2B1, ATPase plasma membrane Ca^{2+} transporting 1; TFP, trifluoperazine; C, control; I, inhibitor; M, mimic; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; HUVECs, human umbilical vein endothelial cells.

active trophoblast cells and endothelial cells form a linkage through cell proliferation, invasion and tube formation (48,49). Angiogenesis plays an important role in tissue repair and organ growth. Imbalance in angiogenesis will easily lead to the occurrence and development of several diseases, including preeclampsia (50).

VEGF is a highly specific mitogen that promotes vascular endothelial cell division, and is closely related to angiogenesis (51). The high expression of VEGF can promote vascular endothelial cell division and the expression of MMP to increase the infiltration and promotion of cell migration and invasion and angiogenesis of endothelial cells (52,53). Moreover, degradation of extracellular matrix by MMPs is known to be a rate-limiting step for trophoblast invasion (54). miR-378 has been shown to bind to VEGF and promote angiogenesis (55). Abnormal expression of miR-10b and -200c can regulate the expression of soluble Fms-like tyrosine kinase-1, and induce vascular endothelial injury and vascular remodeling disorder (56). Wang *et al* (12) found that the expression of miR-16 in decidua-derived mesenchymal stem cells (dMSCs) of patients with severe preeclampsia is significantly increased. miR-16 was negatively correlated with

cyclin E1 and VEGF-A in dMSCs from severe preeclampsia. Furthermore, miR-16 is known to regulate the migration and tube formation of endothelial cells (12). A recent study has shown that miR-27b suppressed endothelial cell proliferation and migration by targeting Smad7 in Kawasaki disease (57).

In the present study, a miR-27b-3p inhibitor was shown to ameliorate the impairment of endothelial cells known to be induced by hypertensive serum, angiogenesis and trophoblast invasion, all of which are characteristics of preeclampsia (58). Moreover, miR-27b-3p inhibited the process of angiogenesis of HUVECs and invasion of trophoblasts with ATP2B1 inhibition, and also inhibited the expression of VEGF, MMP-9 and MMP-2 in HUVECs. Furthermore, similar changes to VEGF, MMP-9 and MMP-2 expression were observed when testing the HTR-8/SVneo trophoblast cells. The results indicated that serum from patients with hypertension promoted endothelial dysfunction to induce trophoblast invasion through the miR-27b-3p/ATP2B1 axis.

The limitation of the present study is that the main signaling pathways need further study (such as Akt signaling). Furthermore, *in vivo* experiments would be beneficial in order to clarify the effects of miR-27b-3p/ATP2B1 on preeclampsia

in a natural setting and determine if miRNA expression could be successfully used as a biomarker.

Taken together, expression of miR-27b-3p was upregulated in patients with hypertension and patients with preeclampsia. Downregulation of miR-27b-3p could inhibit the dysfunction of endothelial cells induced by the serum of patients with hypertension and promote the invasion of trophoblast cells through upregulating ATP2B1, thus inhibiting the development of preeclampsia. In addition, the present data revealed that miR-27b-3p might be a novel predictive risk factor of endothelial dysfunction and hypertension. The current findings may improve our understanding of the pathogenesis of preeclampsia.

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Availability of data and materials

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

Authors' contributions

LZ made substantial contributions to conception and design. LZ and ZL authors were involved in the data acquisition, analysis and interpretation, along with drafting the article or critically revising it for important intellectual content. LZ and ZL authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved. LZ and ZL authors approved the final version of the manuscript for publication.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The present study was approved by The Affiliated Hangzhou First People's Hospital Ethics Committee (approval no. YY201802016C). All subjects were informed of the purpose and process of the study and signed an informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Alrahmani L and Willrich MAV: The complement alternative pathway and preeclampsia. *Curr Hypertens Rep* 20: 40, 2018.
- Stegers EA, von Dadelszen P, Duvekot JJ and Pijnenborg R: Pre-eclampsia. *Lancet* 376: 631-644, 2010.
- Kelly RS, Giorgio RT, Chawes BL, Palacios NI, Gray KJ, Mirzakhani H, Wu A, Blighe K, Weiss ST and Lasky-Su J: Applications of metabolomics in the study and management of preeclampsia: A review of the literature. *Metabolomics* 13: 13, 2017.
- Seely EW and Maxwell C: Cardiology patient page. Chronic hypertension in pregnancy. *Circulation* 115: e188-e190, 2007.
- Tabara Y, Kohara K and Miki T: Millennium Genome Project for Hypertension: Hunting for genes for hypertension: The Millennium Genome Project for Hypertension. *Hypertens Res* 35: 567-573, 2012.
- Tabara Y, Kohara K, Kita Y, Hirawa N, Katsuya T, Ohkubo T, Hiura Y, Tajima A, Morisaki T, Miyata T, *et al*: Global Blood Pressure Genetics Consortium: Common variants in the ATP2B1 gene are associated with susceptibility to hypertension: The Japanese Millennium Genome Project. *Hypertension* 56: 973-980, 2010.
- Cho YS, Go MJ, Kim YJ, Heo JY, Oh JH, Ban HJ, Yoon D, Lee MH, Kim DJ, Park M, *et al*: A large-scale genome-wide association study of Asian populations uncovers genetic factors influencing eight quantitative traits. *Nat Genet* 41: 527-534, 2009.
- Kohara K, Tabara Y, Nakura J, Imai Y, Ohkubo T, Hata A, Soma M, Nakayama T, Umemura S, Hirawa N, *et al*: Identification of hypertension-susceptibility genes and pathways by a systemic multiple candidate gene approach: The millennium genome project for hypertension. *Hypertens Res* 31: 203-212, 2008.
- Levy D, Ehret GB, Rice K, Verwoert GC, Launer LJ, Dehghan A, Glazer NL, Morrison AC, Johnson AD, Aspelund T, *et al*: Genome-wide association study of blood pressure and hypertension. *Nat Genet* 41: 677-687, 2009.
- Wang Y, Zhang Y, Li Y, Zhou X, Wang X, Gao P, Jin L, Zhang X and Zhu D: Common variants in the ATP2B1 gene are associated with hypertension and arterial stiffness in Chinese population. *Mol Biol Rep* 40: 1867-1873, 2013.
- Doridot L, Houry D, Gaillard H, Chelbi ST, Barbaux S and Vaiman D: miR-34a expression, epigenetic regulation, and function in human placental diseases. *Epigenetics* 9: 142-151, 2014.
- Wang Y, Fan H, Zhao G, Liu D, Du L, Wang Z, Hu Y and Hou Y: miR-16 inhibits the proliferation and angiogenesis-regulating potential of mesenchymal stem cells in severe pre-eclampsia. *FEBS J* 279: 4510-4524, 2012.
- Xu P, Zhao Y, Liu M, Wang Y, Wang H, Li YX, Zhu X, Yao Y, Wang H, Qiao J, *et al*: Variations of microRNAs in human placentas and plasma from preeclamptic pregnancy. *Hypertension* 63: 1276-1284, 2014.
- Hromadnikova I, Kotlabova K, Hympanova L and Krofta L: Gestational hypertension, preeclampsia and intrauterine growth restriction induce dysregulation of cardiovascular and cerebrovascular disease associated microRNAs in maternal whole peripheral blood. *Thromb Res* 137: 126-140, 2016.
- Jr JR, Alasztics B, Molvarec A, Nagy B and Biró O: 11 expression analysis of circulating exosomal hsa miR 210 in hypertensive disorders of pregnancy: Biomarkers, prediction of preeclampsia. *Pregnancy Hypertens An Int J Wom Cardiovasc Health* 6: 183-183, 2016.
- Liu C, Zhou Y and Zhang Z: miR-210: An important player in the pathogenesis of preeclampsia? *J Cell Mol Med* 16: 943-944, 2012.
- Tao J, Zhi X, Zhang X, Fu M, Huang H, Fan Y, Guan W and Zou C: miR-27b-3p suppresses cell proliferation through targeting receptor tyrosine kinase like orphan receptor 1 in gastric cancer. *J Exp Clin Cancer Res* 34: 139, 2015.
- Ruzzo A, Graziano F, Vincenzi B, Canestrari E, Perrone G, Galluccio N, Catalano V, Loupakis F, Rabitti C, Santini D, *et al*: High let-7a microRNA levels in KRAS-mutated colorectal carcinomas may rescue anti-EGFR therapy effects in patients with chemotherapy-refractory metastatic disease. *Oncologist* 17: 823-829, 2012.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
- Thongon N, Nakkrasae LI, Thongbunchoo J, Krishnamra N and Charoenphandhu N: Enhancement of calcium transport in Caco-2 monolayer through PKCzeta-dependent Cav1.3-mediated transcellular and rectifying paracellular pathways by prolactin. *Am J Physiol Cell Physiol* 296: C1373-C1382, 2009.
- Little R, Cartwright EJ, Neyses L and Austin C: Plasma membrane calcium ATPases (PMCA) as potential targets for the treatment of essential hypertension. *Pharmacol Ther* 159: 23-34, 2016.

22. Gonçalves-Rizzi VH, Possomato-Vieira JS, Nascimento RA, Caldeira-Dias M and Dias-Junior CA: Maternal hypertension and feto-placental growth restriction is reversed by sildenafil: Evidence of independent effects of circulating nitric oxide levels. *Eur J Pharmacol* 822: 119-127, 2018.
23. Yan X, Yang Z, Chen Y, Li N, Wang L, Dou G, Liu Y, Duan J, Feng L, Deng S, *et al*: Endothelial cells-targeted soluble human Delta-like 4 suppresses both physiological and pathological ocular angiogenesis. *Sci China Life Sci* 58: 425-431, 2015.
24. Beltrame JS, Scotti L, Sordelli MS, Cañumil VA, Franchi AM, Parborell F and Ribeiro ML: Lysophosphatidic acid induces the crosstalk between the endovascular human trophoblast and endothelial cells in vitro. *J Cell Physiol* 234: 6274-6285, 2019.
25. Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA and McLaughlin MK: Preeclampsia: An endothelial cell disorder. *Am J Obstet Gynecol* 161: 1200-1204, 1989.
26. Huppertz B: Placental origins of preeclampsia: Challenging the current hypothesis. *Hypertension* 51: 970-975, 2008.
27. Brennan L, Morton JS, Quon A and Davidge ST: Postpartum vascular dysfunction in the reduced uteroplacental perfusion model of preeclampsia. *PLoS One* 11: e0162487, 2016.
28. Shen L, Diao Z, Sun HX, Yan GJ, Wang Z, Li RT, Dai Y, Wang J, Li J, Ding H, *et al*: Up-regulation of CD81 inhibits cytotrophoblast invasion and mediates maternal endothelial cell dysfunction in preeclampsia. *Proc Natl Acad Sci USA* 114: 1940-1945, 2017.
29. Granger JP, Alexander BT, Llinas MT, Bennett WA and Khalil RA: Pathophysiology of hypertension during preeclampsia linking placental ischemia with endothelial dysfunction. *Hypertension* 38: 718-722, 2001.
30. Sánchez-Aranguren LC, Prada CE, Riaño-Medina CE and Lopez M: Endothelial dysfunction and preeclampsia: Role of oxidative stress. *Front Physiol* 5: 372, 2014.
31. McDermott AM, Kerin MJ and Miller N: Identification and validation of miRNAs as endogenous controls for RQ-PCR in blood specimens for breast cancer studies. *PLoS One* 8: e83718, 2013.
32. Cengiz M, Karatas OF, Koparir E, Yavuzer S, Ali C, Yavuzer H, Kirat E, Karter Y and Ozen M: Differential expression of hypertension-associated microRNAs in the plasma of patients with white coat hypertension. *Medicine (Baltimore)* 94: e693, 2015.
33. Karolina DS, Tavintharan S, Armugam A, Sepsramaniam S, Pek SLT, Wong MTK, Lim SC, Sum CF and Jeyaseelan K: Circulating miRNA profiles in patients with metabolic syndrome. *J Clin Endocrinol Metab* 97: E2271-E2276, 2012.
34. Yang Q, Jia C, Wang P, Xiong M, Cui J, Li L, Wang W, Wu Q, Chen Y and Zhang T: MicroRNA-505 identified from patients with essential hypertension impairs endothelial cell migration and tube formation. *Int J Cardiol* 177: 925-934, 2014.
35. Yang Z, Xiao Z, Guo H, Fang X, Liang J, Zhu J, Yang J, Li H, Pan R, Yuan S, *et al*: Novel role of the clustered miR-23b-3p and miR-27b-3p in enhanced expression of fibrosis-associated genes by targeting TGFBR3 in atrial fibroblasts. *J Cell Mol Med* 23: 3246-3256, 2019.
36. Yu J, Lv Y, Wang F, Kong X, Di W, Liu J, Sheng Y, Lv S and Ding G: miR-27b-3p inhibition enhances browning of epididymal fat in high-fat diet induced obese mice. *Front Endocrinol (Lausanne)* 10: 38, 2019.
37. Lv X, Li J, Hu Y, Wang S, Yang C, Li C and Zhong G: Overexpression of miR-27b-3p targeting Wnt3a regulates the signaling pathway of Wnt/ β -catenin and attenuates atrial fibrosis in rats with atrial fibrillation. *Oxid Med Cell Longev* 2019: 5703764, 2019.
38. Liu Y, Cai Q, Bao PP, Su Y, Cai H, Wu J, Ye F, Guo X, Zheng W, Zheng Y, *et al*: Tumor tissue microRNA expression in association with triple-negative breast cancer outcomes. *Breast Cancer Res Treat* 152: 183-191, 2015.
39. Chen D, Si W, Shen J, Du C, Lou W, Bao C, Zheng H, Pan J, Zhong G, Xu L, *et al*: miR-27b-3p inhibits proliferation and potentially reverses multi-chemoresistance by targeting CBLB/GRB2 in breast cancer cells. *Cell Death Dis* 9: 188, 2018.
40. Song C, Yao J, Cao C, Liang X, Huang J, Han Z, Zhang Y, Qin G, Tao C, Li C, *et al*: PPAR γ is regulated by miR-27b-3p negatively and plays an important role in porcine oocyte maturation. *Biochem Biophys Res Commun* 479: 224-230, 2016.
41. Xi B, Tang W and Wang Q: Polymorphism near the ATP2B1 gene is associated with hypertension risk in East Asians: A meta-analysis involving 15 909 cases and 18 529 controls. *Blood Press* 21: 134-138, 2012.
42. Drost JT, Maas AH, van Eyck J and van der Schouw YT: Preeclampsia as a female-specific risk factor for chronic hypertension. *Maturitas* 67: 321-326, 2010.
43. Sibai BM: Diagnosis and management of gestational hypertension and preeclampsia. *Obstet Gynecol* 102: 181-192, 2003.
44. Daily JW, Kim BC, Liu M and Park S: People with the major alleles of ATP2B1 rs17249754 increases the risk of hypertension in high ratio of sodium and potassium, and low calcium intakes. *J Hum Hypertens* 31: 787-794, 2017.
45. Ryan ZC, Craig TA, Filoteo AG, Westendorf JJ, Cartwright EJ, Neyses L, Strehler EE and Kumar R: Deletion of the intestinal plasma membrane calcium pump, isoform 1, Atp2b1, in mice is associated with decreased bone mineral density and impaired responsiveness to 1, 25-dihydroxyvitamin D3. *Biochem Biophys Res Commun* 467: 152-156, 2015.
46. Long Y, Chen SW, Gao CL, He XM, Liang GN, Wu J, Jiang CX, Liu X, Wang F and Chen F: ATP2B1 gene silencing increases NO production under basal conditions through the Ca²⁺/calmodulin/eNOS signaling pathway in endothelial cells. *Hypertens Res* 41: 246-252, 2018.
47. Zhang Y, Fei M, Xue G, Zhou Q, Jia Y, Li L, Xin H and Sun S: Elevated levels of hypoxia-inducible microRNA-210 in pre-eclampsia: New insights into molecular mechanisms for the disease. *J Cell Mol Med* 16: 249-259, 2012.
48. Zhou Y, McMaster M, Woo K, Janatpour M, Perry J, Karpanen T, Alitalo K, Damsky C and Fisher SJ: Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. *Am J Pathol* 160: 1405-1423, 2002.
49. Cross JC, Hemberger M, Lu Y, Nozaki T, Whiteley K, Masutani M and Adamson SL: Trophoblast functions, angiogenesis and remodeling of the maternal vasculature in the placenta. *Mol Cell Endocrinol* 187: 207-212, 2002.
50. Zhang M, Muralimanoharan S, Wortman AC and Mendelson CR: Primate-specific miR-515 family members inhibit key genes in human trophoblast differentiation and are upregulated in preeclampsia. *Proc Natl Acad Sci USA* 113: E7069-E7076, 2016.
51. Melincovici CS, Boşca AB, Şuşman S, Mărginean M, Mişu C, Istrate M, Moldovan IM, Roman AL and Mişu CM: Vascular endothelial growth factor (VEGF) - key factor in normal and pathological angiogenesis. *Rom J Morphol Embryol* 59: 455-467, 2018.
52. VanKlompberg MK, Manjarín R, Donovan CE, Trott JF and Hovey RC: Regulation and localization of vascular endothelial growth factor within the mammary glands during the transition from late gestation to lactation. *Domest Anim Endocrinol* 54: 37-47, 2016.
53. Thangapazham RL, Sharma A, Gaddipati JP, Singh AK and Maheshwari RK: Inhibition of tumor angiogenesis by Brahma Rasayana (BR). *J Exp Ther Oncol* 6: 13-21, 2006.
54. Zhong T, Chen J, Ling Y, Yang B, Xie X, Yu D, Zhang D, Ouyang J and Kuang H: Down-Regulation of neuropathy target esterase in preeclampsia placenta inhibits human trophoblast cell invasion via modulating MMP-9 levels. *Cell Physiol Biochem* 45: 1013-1022, 2018.
55. Lee DY, Deng Z, Wang CH and Yang BB: MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. *Proc Natl Acad Sci USA* 104: 20350-20355, 2007.
56. Shen X, Fang J, Lv X, Pei Z, Wang Y, Jiang S and Ding K: Heparin impairs angiogenesis through inhibition of microRNA-10b. *J Biol Chem* 286: 26616-26627, 2011.
57. Rong X, Ge D, Shen D, Chen X, Wang X, Zhang L, Jia C, Zeng J, He Y, Qiu H, *et al*: miR-27b suppresses endothelial cell proliferation and migration by targeting Smad7 in Kawasaki disease. *Cell Physiol Biochem* 48: 1804-1814, 2018.
58. Adu-Gyamfi EA, Fondjo LA, Owiredu WKBA, Czika A, Nelson W, Lamptey J, Wang YX and Ding YB: The role of adiponectin in placentation and preeclampsia. *Cell Biochem Funct* 38: 106-117, 2020.

