MicroRNA-128a-induced apoptosis in HTR-8/SVneo trophoblast cells contributes to pre-eclampsia

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\textbf{ABSTRACT}

\textbf{Introduction:} Pre-eclampsia (PE) can endanger the survival of the mother and fetus. Currently, the pathogenesis of PE is not completely understood and no fundamental therapeutics are available. The present study was performed to determine the function of miR-128a in HTR-8/SVneo trophoblast cells and to ascertain its underlying role in the pathogenesis of PE.

\textbf{Methods:} We investigated the function of miR-128a in HTR-8/SVneo cells by overexpressing. We analyzed the apoptosis of HTR-8/SVneo cells by performing apoptosis assays and measured the loss of mitochondrial membrane potential (Δψm), the generation of reactive oxygen species (ROS) and caspase activity. In addition, miR-128a target genes were predicted.

\textbf{Results:} Using computer-based programs, we identified Bax as a direct target of miR-128a. In the apoptosis assays of HTR-8/SVneo cells, miR-128a decreased the Δψm, depleted ATP levels and increased ROS generation, cytochrome c release as well as caspase activation. Further studies showed that miR-128a induced the apoptosis of HTR-8/SVneo cells by down-regulating Bax through the mitochondrial apoptosis pathway.

\textbf{Conclusions:} miR-128a is an up-regulated miRNA in patient with PE. Our study demonstrated that the miR-128a-induced apoptosis of HTR-8/SVneo cells may contribute to PE and miR-128a may be a novel potential therapeutic target for PE.

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1. Introduction

As a major cause of rising maternal and perinatal morbidity and mortality [1], Pre-eclampsia (PE) is associated with dysfunction of the heart, brain and liver as well as other organs and usually develops after 20 weeks of gestation [2,3]. The precise pathogenesis of PE remains unclear. Currently, there is no single pathophysiological factor that sufficiently explains the etiology and pathogenesis of PE. There have been numerous theories on the pathogenesis of PE, such as placental dysfunction [4], impaired trophoblast invasion [5], abnormal spiral artery remodeling [6], endothelial dysfunction [7] and increased apoptosis of trophoblast cells [8]. Among these, increased trophoblast cell apoptosis in the placenta will be discussed in connection to the pathogenesis of PE.

MicroRNAs(miRNAs) are a new class of small, non-coding, single-strand, highly conserved RNA molecules that modulate gene expression via mRNA degradation and translational repression [9]. Previous reports have shown that miRNAs participate in the development and progression of diverse diseases, such as cancer [10] and pregnancy-related diseases. Recent studies have indicated that several miRNAs are involved in the pathogenesis of PE. These miRNAs contribute to PE through diverse biological processes, including proliferation, apoptosis, invasion, angiogenesis and mitochondrial respiration [10–13].

Several studies have emphasized the importance of miR-128 in the regulation of cell proliferation [14], invasion [15] and apoptosis [16]. Moreover, the expression level of miR-128a is higher in the placenta of patient with severe PE [17]. However, the function of miR-128a in PE is poorly understood. No studies to date have addressed the effects of miR-128a on trophoblast cell function and whether miR-128a has diagnostic or prognostic value for PE is still

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unknown. Hence, it is necessary to investigate the potential role of miR-128a in the physiopathologic mechanism of PE.

The aim of present study was to explore the function of miR-128a in HTR-8/SVneo cells. Therefore, we analyzed apoptosis level, caspase activation, intracellular ATP and ROS levels, \( \Delta \psi/\m \), mitochondrial DNA (mtDNA) copy number and mitochondrial function-related genes expression in HTR-8/SVneo cells after overexpressing miR-128a. We also revealed the regulatory mechanisms associated with miR-128a during this process and found that Bax was a direct target of miR-128a. These results showed that miR-128a induced the apoptosis of HTR-8/SVneo cells by down-regulating Bax and the mitochondrial apoptosis pathway. These findings highlight the importance of miR-128a in the pathogenesis of PE and provide new insights into the onset of PE.

2. Materials and methods

2.1. Ethics approval and clinical sample collection

Human placentas were collected from Nanjing Medical University affiliated Nanjing Maternal and Child Health Hospital under a protocol approved by the Institutional Ethics Committee of Nanjing Medical University (the ethical approval reference number [2013] 5). Informed consents were obtained from all the patients. All placental tissues were collected immediately following delivery at term by cesarean section and washed with sterile phosphate-buffered saline before being snap-frozen in liquid nitrogen and stored at \(-80^\circ\) C for future RNA extraction. Severe pre-eclampsia was defined as either severe hypertension (systolic blood pressure of \( >160\) mmHg and/or diastolic blood pressure of \( >110\) mmHg on \( \geq 2\) occasions \( 6\) h apart) or severe proteinuria (\( >5\gg 24\) h urine specimen or \( >3\) on \( \geq 2\) random samples collected \( 4\) h apart), according to the International Society for the Study of Hypertension in Pregnancy [2]. Normal pregnancy was defined as not having preeclampsia or any other complications, such as maternal history of hypertension and/or renal or cardiac disease, maternal infection, smoking, multiple pregnancies, premature rupture of membranes or fetal anomalies.

2.2. Cell culture and transfection

HTR-8/SVneo cells (purchased from the Cell Bank of the Chinese Academy of Sciences, China) were maintained in RPMI-1640 medium (Gibco BRL, New York, USA) supplemented with 10% fetal bovine serum (FBS), 100 \( \mu\)g/mL streptomycin and 100 U/mL penicillin under standard culture conditions (\( 37^\circ\) C and 5% CO\( _2\) incubator) [18].

The lentiviral vector LV-GFP-miR128a (expressing human miR-128a gene) and an empty lentiviral negative control (NC) were purchased from GenePharma (Shanghai, China). HTR-8/SVneo cells (\( 1 \times 10^5\)) were infected with miR-128a or NC in the presence of 5 \( \mu\)g/mL polybrene (Sigma-Aldrich, USA) according to the manufacturer’s protocol. The medium was replaced with fresh growth medium after 24 h. Forty-eight hours after infection, the cells were used for further experiments.

2.3. miRNA target prediction

Currently, several target prediction software programs have been developed to identify potential miRNA-target pairs. miR-128a target genes and miRNA binding sites were predicted using three different programs: TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org) and miBase (http://www.mirbase.org). Putative target genes that were predicted by at least 2 of the databases mentioned above were subjected to further validation.

2.4. Apoptosis assay

Apoptotic cells were detected by flow cytometry using a commercially available kit (KeyGen Biotech, China). Cells were trypsinized using trypsin without EDTA, washed with cold PBS, resuspended in 500 \( \mu\)L of binding buffer, and double-stained with propidium iodide and fluorescein isothiocyanate (FITC)-conjugated annexin V according to the manufacturer’s instructions. The samples were analyzed using a BD FACSCan flow Cytometer and BD FACS Diva software (BD Biosciences, USA). A minimum of 10,000 events was counted. The tests were repeated in triplicate.

2.5. Measurement of caspase activation

Caspase 3, 8 and 9 activities were measured using a Caspase Activity Assay Kit (Beyotime Biotech, China) according to the manufacturer’s protocol. Briefly, cells were collected by centrifugation at 600\( g\) for 5 min at 4\( ^\circ\) C, washed with cold PBS and resuspended in lysis buffer for 15 min on ice. Lysates were centrifuged at 16,000\( g\) for 15 min at 4\( ^\circ\) C. The protein concentrations in the extracts were determined using the Bradford method. Then, 80 \( \mu\)L of reaction buffer, 10 \( \mu\)L of protein solution and 10 \( \mu\)L of 2 mM caspase colorimetric substrate (Ac-DEVD-pNA for Caspase 3, Ac-LEHD-pNA for Caspase 8, and Ac-LEHD-pNA for Caspase 9) were mixed sequentially and incubated at 37\( ^\circ\) C for 2 h. The release of pNA was quantified with a spectrophotometer at an absorbance of 405 nm.

2.6. Determination of intracellular ROS and ATP levels

Intracellular ROS production was detected using a reactive oxygen species assay kit (Beyotime Biotech, China) that monitors the oxidation of 2,7\'-dichlorofluorescein diacetate (DCFH-DA) to the fluorescent 2,7\'-dichlorofluorescein (DCF); these assays were performed according to the manufacturer’s protocol. After 48 h of infection, the cells were collected and incubated with serum-free medium containing 10 mM DCFH-DA at 37\( ^\circ\) C for 30 min. The cells were washed with RPMI-1640 three times, and then the fluorescence intensity was monitored by flow cytometry (BD Bioscience, USA) with an excitation of 488 nm and an emission of 525 nm. ROS generation was quantified based on the median fluorescence intensity of 10,000 cells. The values are expressed as the mean absorbance normalized to the negative control.

Intracellular ATP levels were determined using an ATP Assay Kit (Beyotime, China), which is based on firefly luciferase activity, according to the manufacturer’s instructions. The cells were lysed in 200\( \mu\)L of lysis buffer and centrifuged at 12,000\( g\) for 10 min to collect the cell supernatant. An equal volume (100 \( \mu\)L) of the ATP detection working solution was added to each well of a black 96-well plate, and the plate was incubated for 5 min at room temperature. Then, 50 \( \mu\)L samples of cell lysate were added to the wells, and luminescence was immediately measured using a luminometer. Standard curves were generated, and the protein concentration of each sample was measured using the BCA protein assay. ATP content was calculated using an ATP standard curve, and intracellular ATP levels are expressed as a percentage of the control group. The assays were repeated six times.

2.7. Flow cytometric measurement of mitochondrial membrane potential (\( \Delta \psi/\m \))

Mitochondrial membrane potential was examined using a JC-1 mitochondrial membrane potential assay kit (Beyotime Biotech, China). After the cells were infected for 48 h, they were incubated at 37\( ^\circ\) C for 20 min with 10 \( \mu\)g/mL JC-1 in accordance with the manufacturer’s instructions. The excess staining solution was
removed by two washes with JC-1 staining buffer. The cells were centrifuged and then resuspended in 500 μL of JC-1 staining working solution. The fluorescence intensities were monitored by flow cytometry (excitation at 488 nm and emission at 530 nm). Data from a minimum of 10,000 cells per sample were acquired and analyzed using CellQuest software. The experiment was repeated three times.

2.8. Quantitative real-time PCR

Total RNA was extracted from the cells and placenta tissues using TRIzol (Invitrogen, USA). RNA (1 μg) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, USA) and random primers according to the manufacturer’s instructions. Real-time PCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems, CA) on an ABI 7900 system (Applied Biosystems, USA). Melting curve analyses were performed to evaluate primer specificity. The expression level of mitochondrial function-related gene was quantified with real-time PCR. The primers used for real-time PCR are listed in Table 1. GAPDH was used as the endogenous control. The expression level of miR-128a was quantified with a TaqMan miRNA reverse transcription kit and miRNA assay (Applied Biosystems, USA) that includes specific RT primers and a TaqMan probe. The real-time PCR reactions were performed according to the manufacturer’s recommendations with an ABI Prism 7900 system. The expression level of miR-128a was normalized to that of U6 snRNA. Relative expression levels were calculated using the comparative Ct method (2-△△Ct). Each assay was performed in triplicate and was repeated at least three times.

DNA was extracted from cells using the standard protease K and phenol-chloroform method. The mitochondrial DNA (mtDNA) copy number was determined by real-time qPCR [19]. The mtDNA copy numbers in negative control cells and in miR-128a-overexpressing HTR-8/SVneo cell was estimated using two primer pairs, one specific for mtDNA (ND1) and the other specific for nuclear DNA (18S). Ct values can be used to measure mtDNA copy number.

2.9. Western blotting

Cytosolic and mitochondrial fractions were isolated from control and miR-128a-infected cells using a Mitochondrial Isolation Kit according to manufacturer’s protocol (Beyotime Biotech, China). Whole cells and fractionated cells were lysed with RIPA buffer (Sigma-Aldrich, USA). The protein concentration was determined using the bicinchoninic acid (BCA) assay. Samples containing equal amounts of protein (50 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to PVDF membranes. The membranes were blocked with 5% skim milk in Tris-buffer containing 0.1% Tween-20 (TBST) for 1 h and then incubated overnight at 4 °C with the following primary antibodies: anti-Bax, anti-cytochrome c, and anti-β-actin (1:1000 dilution). The membranes were subsequently washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (1:5000 dilution) for 1 h at room temperature. Integrated density values were calculated using Alphalmager 3400 (Alpha Innotech, San Leandro, CA, USA). β-actin was used as a control to verify equal protein loading. Cox IV was used to check the purity of the cytosolic and mitochondrial fractions. All the antibodies were supplied by Cell Signaling Technology. All the experiments were repeated at least three times. Image J was used to quantify the western blot data.

3. Luciferase assays

HTR-8/SVneo cells were plated in 24-well plates and then co-transfected with 400 ng of Bax 3’ UTR reporter (wild-type or mutated), 400 ng of miR-128a expression vector or negative control and 20 ng of Renilla luciferase vector (pRL-TK; Promega, Madison, USA) using Lipofectamine 2000 (Invitrogen, USA) as described by the manufacturer. Forty-eight hours after transfection, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA). All the transfection experiments were performed in triplicate, and firefly luciferase activity was normalized to Renilla activity.

3.1. Statistical analysis

The results were presented as mean ± standard deviation (SD). Comparisons between 2 groups were conducted using Student’s t-test. Analysis of variance (ANOVA) was used where appropriate. Statistical analyses were performed using SPSS 20.0 software. Values of P < 0.05 were considered statistically significant.

4. Results

4.1. Clinical characteristics

Clinical data were collected from all the pregnant woman participated in our study. We selected 25 patients who met the standard criteria for severe pre-eclampsia [2]. Meanwhile,

<table>
<thead>
<tr>
<th>Table 1</th>
<th>The primer sequences used for the quantitative real-time PCR.</th>
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<tbody>
<tr>
<td>Gene</td>
<td>Forward (5’→3’)</td>
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<tr>
<td>Bax</td>
<td>GGGTGTCGCCCTTTTCTA</td>
</tr>
<tr>
<td>DDX1</td>
<td>CTTTCGGGATTCCTCA</td>
</tr>
<tr>
<td>SNAP25</td>
<td>ACCAGTGTGTACAGTACTGA</td>
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<tr>
<td>UCP-1</td>
<td>TCGTGTGACGGCTGTTCC</td>
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<td>Cyto-c</td>
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<tr>
<td>PPAR-a</td>
<td>GTTTGCCCTTTAAGGAAATACCA</td>
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<tr>
<td>PGC-1a</td>
<td>CACCAAAACCCACAGAGACA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCAACACACACACTTCCAGGCTT</td>
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<tr>
<td>DNI</td>
<td>CCTTAAACACGCCCCACATT</td>
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<tr>
<td>H1s</td>
<td>TAGACCGACAGAATGCCTGT</td>
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<table>
<thead>
<tr>
<th>Table 2</th>
<th>Characteristics of the study populations.</th>
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<tbody>
<tr>
<td>Parameters</td>
<td>PE (n = 25)</td>
</tr>
<tr>
<td>Maternal age at delivery (year)</td>
<td>28.52 ± 3.21</td>
</tr>
<tr>
<td>Proteinuria (g/24h)</td>
<td>5.17 ± 2.28</td>
</tr>
<tr>
<td>Gestational age (week)</td>
<td>34.73 ± 3.46</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>166.42 ± 23.21</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>104.56 ± 11.98</td>
</tr>
<tr>
<td>Fetal birth weight (g)</td>
<td>2386.42 ± 786.27</td>
</tr>
</tbody>
</table>

PE, severe pre-eclampsia; values are mean ± SD.
30 pregnant women with a normal term pregnancy were recruited as the control group. Table 2 showed the patients’ detailed clinical characteristics. FGR (fetal growth restriction) had occurred in certain patients with PE, and the incidence of FGR was significantly different between two groups (Table 2).

4.2. MiR-128a was highly expressed in placental tissue from patients with severe pre-eclampsia

We examined the expression level of miR-128a in placental tissues from patients with severe pre-eclampsia by real-time qPCR and compared the results with those from normal placental tissue. The expression level of miR-128a was significantly higher (2.2-fold, P < 0.01) in placentas from patients with severe pre-eclampsia compared to those in the control group (Fig. 1, P = 0.0014).

4.3. Effect of miR-128a on mtDNA copy number and mitochondrial function-related gene expression

The mtDNA copy number was significantly lower in HTR-8/SVneo cells infected with miR-128a compared with negative control-infected cells (P < 0.05; Fig. 2A). PGC-1α (peroxisome proliferator-activated receptor coactivator-1) mRNA was significantly down-regulated in HTR-8/SVneo cells overexpressing miR-128a compared with negative control cells (P < 0.05; Fig. 2B). The expression of cytochrome C, UCP-1 (uncoupling protein 1) and PPAR-α (proliferator-activated receptor α) also markedly decreased in HTR-8/SVneo cells infected with miR-128a (P < 0.05, ANOVA; Fig. 2B).

4.4. miR-128a induced the apoptosis of HTR-8/SVneo cells via caspases

To explore the effects of miR-128a on the induction of apoptosis in HTR-8/SVneo cells, we labeled HTR-8/SVneo cells with annexin V-FITC and PI and analyzed them by flow cytometry. As depicted in Fig. 3A, the percentage of annexin V-positive cells increased from 3.89 ± 0.32% to 39.67 ± 1.57% after overexpressing miR-128a in HTR-8/SVneo cells. Caspase activation is critical for the initiation and execution of apoptosis [20]. To confirm the involvement of caspases in miR-128a-induced apoptosis, we measured the activities changes of caspases 3, 8 and 9. As shown in Fig. 3B, the activities of caspases 3, 8 and 9 increased by 5.0-, 3.2- and 4.6-fold, respectively, in miR-128a-overexpressing HTR-8/SVneo cells compared to negative control cells after 48 h. These results strongly suggested that miR-128a overexpression might induce apoptosis via caspase activation in HTR-8/SVneo cells.

4.5. miR-128a induced mitochondrial dysfunction in HTR-8/SVneo cells

Mitochondria is essential cellular organelles and is described as “cellular power plants”. To ascertain the effect of miR-128a on mitochondrial dysfunction in HTR-8/SVneo cells, we analyzed the ΔΨm, ATP levels, ROS generation and cytochrome c release. One sensitive indicator of mitochondrial membrane integrity is the mitochondrial membrane potential, which is an indicator of apoptosis. A previous report indicated that mitochondrial dysfunction is often related to the induction of apoptosis [21]. To assess the role of mitochondria in miR-128a-induced apoptosis, we determined whether miR-128a caused a loss of the ΔΨm using the specific fluorescence probe JC-1 and flow cytometry. Fig. 4A showed that the ΔΨm decreased by 22.7% in miR-128a-infected HTR-8/SVneo cells. This effect was statistically significant (P < 0.05).

Intracellular ATP level is a sensitive indicator of mitochondrial function. To determine whether ATP levels played any role in miR-128a-induced mitochondrial dysfunction, we measured the intracellular ATP levels. As shown in Fig. 4B, the intracellular ATP levels decreased sharply from 5.51 ± 0.46 to 2.12 ± 0.32 nmol/mg in miR-128a-infected HTR-8/SVneo cells compared to negative control cells.

The loss of ΔΨm is often accompanied by ROS production. An earlier report showed that ROS production leads to mitochondrial damage, which may facilitate the further release of ROS into the cytoplasm [22]. To ascertain whether ROS participated in miR-128a-induced apoptosis, we measured ROS levels using DCFH-DA and flow cytometry. As shown in Fig. 4C, after infecting HTR-8/SVneo cells with miR-128a or 48 h, the fluorescence intensity increased significantly compared with negative control-infected cells (P < 0.01). The ROS levels in miR-128a-infected cells were 2.40-fold higher than those in negative control cells (P = 0.0015).

We further evaluated the role of mitochondria in miR-128a-induced apoptosis by investigating the release of cytochrome c into the cytosol. As shown in Fig. 4D, our western blot analysis revealed that cytochrome c protein levels were higher in miR-128a-overexpressing HTR-8/SVneo cells than in control cells. These
results indicated that the overexpression of miR-128a decreased intracellular ATP levels, disrupted the ΔΨm, induced ROS generation and triggered cytochrome c release, thereby activating the mitochondrial apoptosis pathway in HTR-8/SVneo cells.

4.6. Prediction of miR-128a target genes

To elucidate the mechanisms of action of miR-128a in trophoblast cells, we predicted miR-128a target genes using the online computer programs TargetScan, miRanda, and miRBase. Using these miRNA target prediction tools, we identified miR-128a-5p binding site sequences in the 3′-UTR of the transcripts encoding Bax, DCX and SNAP25 (Fig. 5A). To ascertain whether miR-128a regulated these genes, we analyzed the mRNA and protein expression levels of these genes after overexpressing miR-128a in HTR-8/SVneo cells. Using quantitative real-time PCR, we found that Bax mRNA levels decreased by 2.2-fold \( (P = 0.013) \) after overexpressing miR-128a (Fig. 5B). Similarly, Bax protein expression was dramatically decreased in response to miR-128a overexpression in HTR-8/SVneo cells as determined by Western blot analysis (Fig. 5C). However, there were no differences in the mRNA expression of DCX or SNAP25 between the two groups (Fig. 5B; \( P < 0.05, \) ANOVA). The protein expression of DCX and SNAP25 did not change after overexpressing miR-128a (data not shown). Real-time qPCR analysis of miR-128a expression revealed a 14.7-fold increase after overexpressing miR-128a compared to the negative control (Fig. 5D). The Dual-Luciferase Reporter Assay System was utilized to further verify whether Bax was a direct target of miR-128a. We constructed reporter plasmids containing the 3′ UTR of Bax (wild-type or mutated, Fig. 5E). These Bax reporter constructs were cotransfected with miR-128a or a negative control into HTR-8/SVneo cells. Luciferase activity significantly decreased when miR-128a was overexpressed \( (P < 0.001; \) Fig. 5F). However, the activity of the mutated reporters construct was unaffected by the simultaneous transfection with miR-128a (Fig. 5F). These results indicated that Bax was a direct target of miR-128a.

5. Discussion

Pre-eclampsia is a pregnancy-specific complex disease caused by a combination of risk factors. Previous reports showed that PE was associated with abnormal placental development and expression changes of specific miRNAs had been reported during placenta development. Earlier studies have demonstrated that many miRNAs are associated with PE: miR-29b induced the apoptosis of trophoblast cells [10], miR-155 regulated angiotensin II type 1 receptor expression in pregnant women with PE [23] and miR-101 induced the apoptosis of HTR-8/SVneo trophoblast cells [24]. However, the molecular mechanisms of miRNAs in PE remain unclear. Our findings suggested that miR-128a expression levels
were significantly higher in the placentas from patients with severe pre-eclampsia than in those from normal controls (Fig. 1). Similar to our findings, Adlakha and Saini [17] showed that let-7b, miRNA-104, miRNA-302, miRNA-128a, miRNA-182 and miRNA-133b were more than 2-fold overexpressed in patients with severe pre-eclampsia. However, the molecular mechanisms of miR-128a in the pathogenesis of PE are unknown. Earlier research showed that overexpressing miR-128 in human embryonic kidney cells induced apoptosis [16] and inhibited cell proliferation in glioma cells [25]. In a present study, we found that overexpressing miR-128a induced HTR-8/SVneo apoptosis, which may trigger the onset of PE.

Apoptosis is controlled by a wide range of cellular signals. To explore the signaling pathways involved in miR-128a-induced HTR-8/SVneo apoptosis, we investigated the mitochondria-associated apoptosis pathway. Mitochondria are essential organelles and known as "cellular power plants" because of their role as efficient ATP generators. Mitochondria participate in apoptosis through different mechanisms such as cytochrome c release from the mitochondria into the cytosol [26], the loss of Δψm [27], ATP depletion [28], ROS production [29] and caspase activation [30].

The present study clearly demonstrated that overexpressing miR-128a induced apoptosis in HTR-8/SVneo cells. As shown in Fig. 2, the mtDNA copy number and the mRNA expression levels of mitochondrial function-related genes (UCP-1, PPARα, PGC1α and cytochrome c) suggested that miR-128a may lead to mitochondrial dysfunction. Cytochrome c is localized to the mitochondrial inner membrane and released into the cytosol under apoptotic stimulation [31]. Western blot analyses revealed that miR-128a markedly increased cytochrome c release from the mitochondria into the cytosol (Fig. 4D). Apoptosis is associated with decreased ATP levels and we observed that miR-128a induced reduced ATP levels (Fig. 4B). Previous studies have shown that increased ROS levels and a decreased Δψm plays relevant roles in apoptotic pathways [24,26]. Our present results were consistent with previous studies, the intracellular ROS levels significantly increased (Fig. 4C) and Δψm markedly decreased after over-expressing miR-128a in HTR-8/SVneo cells (Fig. 4A). Caspase activation is a biomarker of the induction of apoptosis [32]. The caspases are produced in cells as catalytically inactivezymogens and must undergo proteolytic activation during apoptosis. To confirm the involvement of caspases in miR-128a-induced apoptosis, we measured the activities changes of caspases 3, 8 and 9. Our data suggested that up-regulating miR-128a lead to the activation of caspases 3, 8 and 9 (Fig. 3B). The present study strongly indicated that miR-128a induced HTR-8/SVneo apoptosis via the mitochondrial apoptosis pathway.

The luciferase assays revealed that miR-128a down-regulated Bax expression (Fig. 5F). As shown in Fig. 5A, the 3'UTR of Bax mRNA was completely complementary to the seed region of miR-128a. Our data from real-time qPCR and Western blotting analyses indicated that overexpressing miR-128a reduced the expression of Bax both at the mRNA and protein levels in HTR-8/SVneo cells (Fig. 5B and C). These data confirmed that Bax was a miR-128a target gene. Bax is a pro-apoptotic member of the Bcl-2 family that induces apoptosis when overexpressed. In contrast, miR-128a down-regulated Bax and triggered apoptosis in HTR-8/SVneo cells. Consistent with our study, Khan et al. [16] showed that down-regulating Bax induces apoptosis in human embryonic kidney cells. In addition, Lewis et al. also showed that Bax promotes rather than
inhibits the apoptosis of cells in mice infected with Sindbis virus [33]. Furthermore, they demonstrated that the anti-apoptotic versus pro-apoptotic functions of Bax were determined by cell-specific factors. Previous reports have shown that Bax is significantly increased in patient with PE [34,35]. In vitro, we found that miR-128a overexpression in HTR-8/SVneo cells decreased Bax expression. The dual-luciferase reporter assay verified that Bax was a direct target of miR-128a. In vivo, Bax is regulated by numerous factors and pathways, and down-regulation by miR-128a may not be the major factor. Whether miR-128a is involved in inducing apoptosis by targeting Bax remains a contentious subject. In view of these conflicting roles of Bax in the apoptotic process (as an inducer or inhibitor), the mechanisms by which Bax is regulated should be further investigated.

In summary, the results of the present study showed that the up-regulation of miR-128a induced apoptosis through the mitochondrial pathway in HTR-8/SVneo cells, which may contribute to PE. In addition, our findings demonstrated that the miR-128a target gene Bax appeared to be associated with this process. Taken together, the regulation of Bax by miR-128a provides new insights into the pathogenesis of PE, and miR-128a could be a novel prognostic marker or potential target for PE therapeutics.

Acknowledgments

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