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Original Article

Abnormal expression of microRNA-370 regulates Endoglin expression in preeclampsia

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Abstract: Aims: The present study is to measure the expression of Endoglin and microRNA (miR)-370 in the peripheral blood and placental tissues in patients with preeclampsia (PE). The regulation of Endoglin by miR-370 is also investigated to elucidate the clinical significance of miR-370 in the occurrence and development of PE. Methods: A total of 25 PE patients and 25 normal pregnant women were included in the present study. HTR-8/SVneo cells were cultured for transfection and following studies. To measure the levels of Endoglin mRNA and miR-370, quantitative real-time polymerase chain reaction was used. To determine Endoglin protein expression, Western blotting was carried out. Enzyme-linked immunosorbent assay was employed to detect the concentration of Endoglin in serum. The effects of miR-370 on the invasion ability and proliferation of HTR-8/SVneo cell cells were examined using Transwell assay and MTT assay, respectively. Results: Endoglin mRNA and miR-370 levels in PE placental tissues were significantly different from those in normal placental tissues. PE patients had higher expression of Endoglin in peripheral blood circulation system than normal subjects. Overexpression of miR-370 interrupted Endoglin gene transcription and translation. In addition, miR-370 overexpression inhibited the motility and proliferation of HTR-8/SVneo cells. Conclusions: The present study demonstrates that abnormal expression of Endoglin in peripheral blood and placental tissues of PE patients is related to miR-370. Abnormal expression of miR-370 affects the invasion and proliferation of HTR-8/SVneo cells by regulating the expression of Endoglin, playing important roles in the occurrence and development of PE.

Keywords: Preeclampsia, microRNA-370, Endoglin

Introduction

Preeclampsia (PE) is a disease that often occurs after the 20th week of pregnancy, being the main cause of maternal death [1]. Most PE cases have transient hypertension, proteinuria or even symptoms like convulsions, coma, and heart failure during pregnancy [1]. Its pathogenesis involves many kinds of pathological processes, such as implantation dysfunction, endothelial injury, inflammation and so on [2, 3]. Of note, placental dysfunction, as a root cause of PE, plays an important role in these pathological processes [4, 5]. Therefore, studies on placental dysfunction will increase the understanding of the occurrence and development of PE. It has been demonstrated that PE is related with abnormal gene expression, but the mechanisms by which the genes are regulated are still unknown.

MicroRNA (miRNA or miR) is a class of non-encoding small-molecule RNA that plays crucial roles in the post-transcriptional regulation of gene expression. miRNA exerts its effect in cell differentiation, proliferation, apoptosis, angiogenesis, inflammation, oxidation-reduction reaction and other endothelial functions by inhibiting protein translation or facilitating mRNA degradation [6-8]. It is reported that a series of miRNA and target genes with unbalanced expression exist in PE placental tissues, and lead to placental tissue cell dysfunction [9]. Another group of researchers discover that eight types of miRNAs, such as miR-210, miR-1, miR-584 and miR-34c-5p, in PE placental tissues have abnormal expression, and the target genes of these miRNAs participate in the formation of organs and systems, disturbance of immune functions, cell adhesion, cell cycles, and signal transduction [9]. Zhang et al. show that the expression of miR-155 in PE placental tissues is higher than that in normal tissues, leading to decreased expression of CYR61 and finally the formation of PE [10]. Therefore, it is suggested that abnormal miRNA expression

spectrum of PE patients is usually correlated with the occurrence of PE. It has been demonstrated that the expression of miR-370 in gastric cancer tissues is elevated, being closely correlated with the staging and metastasis of gastric cancer [11]. In addition, Wu et al. find that up-regulation of miR-370 expression promotes the proliferation of human prostate cancer cells [12]. However, the molecular mechanism by which miR-370 plays a role in PE has never been reported before.

Endoglin, also named CD105, is a type of homodimer cell membrane glycoprotein with a relative molecular weight of 180 kDa [13]. It is originally identified as a kind of human leukemia-associated antigen, and later found to be densely expressed on the surface of proliferating endothelial cells [14]. Endoglin participates in vascular development and remodeling by regulating cell responses to transforming growth factor (TGF)-β [15, 16]. Several studies have shown abnormal expression of Endoglin in placenta and peripheral blood from PE patients, but its mechanism is still unclear [17-19]. In the present study, we determine the expression of miR-370 and Endoglin in PE patients, and try to provide a basis for studying the molecular regulation mechanism of PE.

Materials and methods

Patients

A total of 25 PE patients and 25 normal pregnant women who gave births at Jining No. 1 People's Hospital between February 2015 and December 2015 were included in the present study. The age and gestational week of the two groups were not significantly different from each other ($P > 0.05$). All procedures were approved by the Ethics Committee of Jining No. 1 People's Hospital. Written informed consents were obtained from all patients or their families.

Cells

HTR-8/SVneo cells were defrosted and cultured in DMEM-F12 (1:1) medium supplemented with 20% fetal bovine serum at 37°C under 5% CO_2. At 12 h after initial inoculation, the medium was changed. Afterwards, the medium was changed every 24 h. When reaching 85% confluency, the cells were passaged at a ratio of 1:4. Afterwards, the cells were passaged once every 2 or 3 days.

HTR-8/SVneo cells in log phase were seeded onto microtiter plates. When reaching 70% confluency, the cells were transfected with Lipofectamine 2000 reagent according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). The cells were divided into has-miR-370 mimics group, has-miR-370 inhibitor group, negative control (NC) group and untransfected (blank) group. At 48 h after transfection, the cells were collected for further analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Before total RNA extraction, tissues (100 mg) were ground into powder using liquid nitrogen before addition of 1 ml Trizol (Thermo Fisher Scientific, Waltham, MA, USA) for lysis, and HTR-8/SVneo cells (2×10^5) were trypsinized and lysed by 1 ml Trizol (Thermo Fisher Scientific, Waltham, MA, USA). After lysis, total RNA was extracted using phenol chloroform method. The purity of RNA was determined by A260/A280 using ultraviolet spectrophotometry (Nanodrop ND1000, Thermo Scientific, Waltham, MA, USA). Then, cDNA was obtained by reverse transcription using PrimeScript RT Reagent Kit (Takara, Dalian, China) from 1 μg RNA and stored at -20°C.

To measure miR-370 expression in tissues and cells, SYBR Green qRT-PCR kit (Takara, Dalian, China) was employed, using U6 as internal reference. The PCR system (25 μl) included 12.5 μl SYBR Premix Ex Taq, 1 μl PCR Forward Primer (miR-370, 5'-GCCTGCTGGGGTGGAACCTGGT-3'), 1 μl Uni-miR qPCR Primer, 2 μl template, and 8.5 μl ddH_2O. Each sample was tested in triplicate. PCR conditions were: initial denaturation at 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 20 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of miR-370.

To determine Endoglin mRNA expression in tissues and cells, SYBR Green qRT-PCR kit (Takara, Dalian, China) was also carried out, using GAPDH as internal reference. The PCR system (20 μl) included 10 μl SYBR EX Taq-Mix, 0.5 μl upstream primer (5'-CATCACCTTTGGTGCCTTCC-3'), 0.5 μl downstream primer (5'-CTATGCCATGCTGCTGGTGGA-3'), 1 μl cDNA and 8 μl ddH_2O. Each sample was tested in triplicate. PCR conditions were: initial denaturation at 95°C for 10 min; 40 cycles of 95°C for 1

min, 60°C for 40 s, 72°C for 30 s, and 72°C for 1 min. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of Endoglin mRNA.

Western blotting

Tissues (50 mg) were ground using liquid nitrogen, while HTR-8/SVneo cells were trypsinized and collected. Then, precooled Radio-Immunoprecipitation Assay (RIPA) lysis buffer (600 μl; 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% TritonX-100, 1% sodium deoxycholate; Beyotime Institute of Biotechnology, Shanghai, China) was added to the samples. After lysis for 50 min on ice, the mixture was centrifuged at 12,000 g/min and 4°C for 5 min. The supernatant was used to determine protein concentration by bicinchoninic acid (BCA) protein concentration determination kit (RTP7102, Real-Times Biotechnology Co., Ltd., Beijing, China). Protein samples (50 μg) were then mixed with equal volume of 2× sodium dodecyl sulfate loading buffer before denaturation in boiling water bath for 5 min. Afterwards, 10 μl samples were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (300 mA, 1.5 h) and blocked with 50 g/L skimmed milk at room temperature for 1 h. Then, the membranes were incubated with Endoglin primary antibody (1:1000; Abcam, Cambridge, UK) and GAPDH primary antibody (1:2000; Abcam, Cambridge, UK) at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 for 3 times of 15 min, the membranes were incubated with goat anti-rabbit-horseradish peroxidase conjugate secondary antibody (1:1000; Abcam, Cambridge, UK) for 1 h at room temperature before washing with phosphate-buffered saline with Tween 20 for 3 times of 15 min. Then, the membrane was developed with enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image lab (Bio-Rad, Hercules, CA, USA) software was used to acquire and analyze imaging signals. The relative content of Endoglin protein was expressed as Endoglin/GAPDH ratio.

Enzyme-linked immunosorbent assay (ELISA)

The serum level of Endoglin was measured using ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA). The procedure was carried out according to the manufacturer's manual. Absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) within 15 min after stopping the reactions.

Transwell assay

HTR-8/SVneo cell invasion ability was evaluated using growth factor-depleted Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA). In Matrigel chambers, 500 μl serum-free DMEM medium was added and kept for 1 h at room temperature. In the lower chamber, 750 μl DMEM medium supplemented with 20% serum was added. After trypsinization, the transfected cells were resuspended to a density of 4×10^5 cells/ml using DMEM containing 0.1% bovine serum albumin. After adding 500 μl cell suspension into the invasion chamber, the cells were incubated at 37°C and 5% CO_2 for 18 h. Cells that invaded into the next side of the chamber were fixed with 100% methanol for 10 min. After being stained using 0.1% crystal violet, the number of cells was counted under a microscope.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

At 24, 48, or 72 h after transfection, MTT (20 μl per well) was added onto HTR-8/SVneo cells before being incubated at 37°C for 4 h. After discarding supernatant, 150 μl MTT was added into each well. Absorbance of each well was measured at 492 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Cell proliferation inhibition rate = (1-absorbance values in experimental groups/absorbance value in control group) ×100.

Statistical analysis

All results were analyzed using SPSS16.0 statistical software (IBM, Armonk, NY, USA). All data were expressed as means ± SD. Differences between two groups were compared using t test. $P < 0.05$ indicates statistical significance.

Results

Endoglin mRNA and miR-370 levels in PE placental tissues are significantly different from those in normal placental tissues

To measure the levels of Endoglin mRNA and miR-370, and the expression of Endoglin pro-

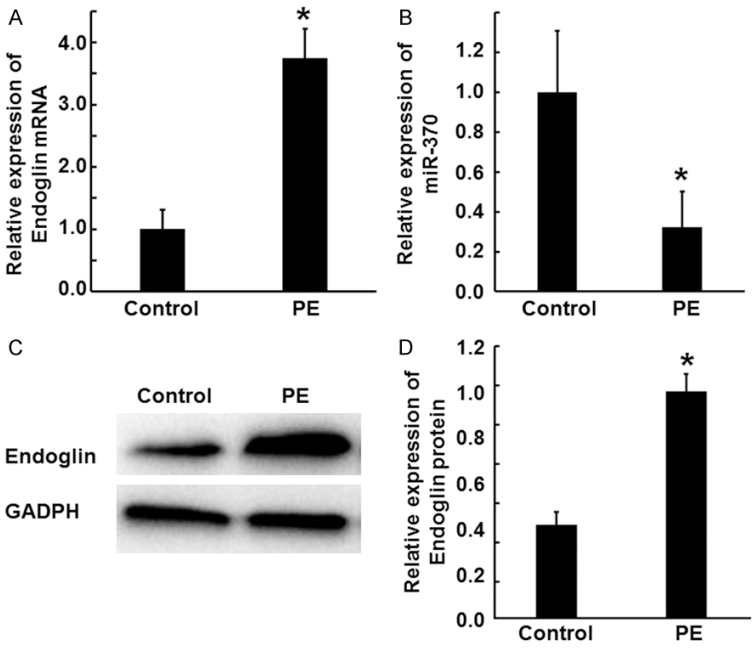

Figure 1. Expression of Endoglin and miR-370 in placental tissues. Levels of (A) Endoglin mRNA and (B) miR-370 in normal and PE placental tissues determined by qRT-PCR. (C) Western blots of Endoglin protein and (D) Quantification of Endoglin protein expression in normal and PE placental tissues. *, $P < 0.05$ compared with control.

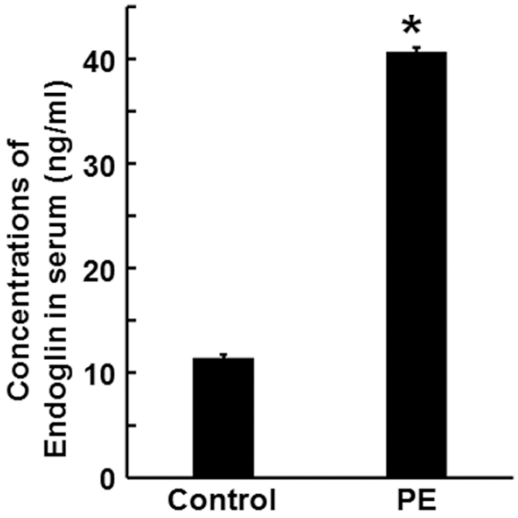

Figure 2. Concentrations of Endoglin in the peripheral blood of normal subjects and PE patients. ELISA was used to measure the serum concentrations of Endoglin protein. *, $P < 0.05$ compared with control.

tein in placental tissues, qRT-PCR and Western blotting were employed, respectively. The data showed that Endoglin mRNA level in PE placental tissue was significantly higher than that in normal placental tissue ($P < 0.05$) (**Figure 1A**). In addition, the expression of miR-370 in PE placental tissue was significantly lower than that in normal placental tissue ($P < 0.05$) (**Figure 1B**). Consistent with Endoglin mRNA level, the protein expression of Endoglin in PE placental tissue was significantly higher than that in normal placental tissues ($P < 0.05$) (**Figure 1C** and **1D**). The results suggest that Endoglin and miR-370 expressions in PE placental tissues are significantly different from those in normal placental tissues.

PE patients have higher expression of Endoglin in peripheral blood circulation system than normal subjects

To determine the level of serum Endoglin, ELISA was used. The data showed that the level of Endoglin in serum from PE patients was significantly higher than that from control group ($P < 0.05$) (**Figure 2**). The result indicates that PE patients have higher expression of Endoglin in peripheral blood circulation system than normal subjects.

Overexpression of miR-370 interrupts Endoglin gene transcription and translation

To predict the target gene of hsa-miR-370, TargetScan (http://www.targetscan.org/) was employed. Endoglin was confirmed to be a potential target of hsa-miR-370 (**Figure 3A**). To confirm the effect of miR-370 on Endoglin expression, HTR-8/SVneo cells were transfected with miR-370 mimics or miR-370 inhibitor. After transfection with miR-370 mimics, the level of miR-370 in HTR-8/SVneo cells was significantly elevated compared with NC and blank groups ($P < 0.05$). By contrast, cells transfected with miR-370 inhibitor had significantly reduced miR-370 expression ($P < 0.05$) (**Figure 3B**). Furthermore, Endoglin protein and mRNA levels in HTR-8/SVneo cells transfected with miR-370 mimics were significantly lower than those in NC group or blank group, while those in cells transfected with miR-370 inhibitor were significantly higher than those in NC or blank

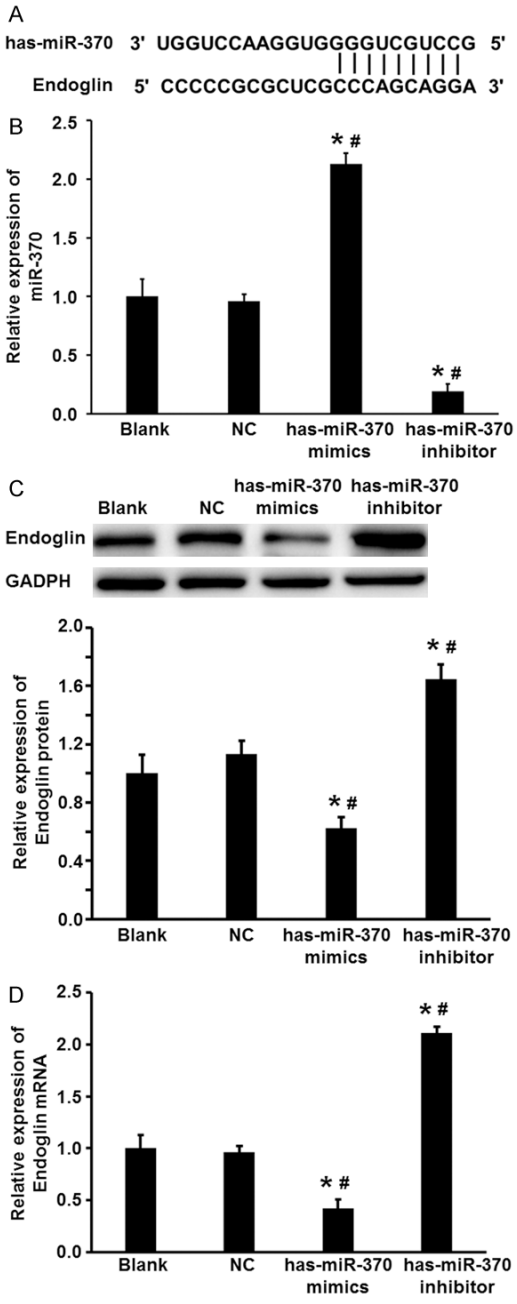

Figure 3. Effect of miR-370 on the expression of Endoglin. (A) Interactions between miR-370 and Endoglin predicted using TargetScan (http://www.targetscan.org/). (B) Relative expression of miR-370 in HTR-8/SVneo cells transfected with miR-370 mimics or miR-370 inhibitor. (C, D) Relative expression of Endoglin (C) protein and (D) mRNA in HTR-8/SVneo cells transfected with miR-370 mimics or miR-370 inhibitor. *, $P < 0.05$ compared with blank; #, $P < 0.05$ compared with NC.

groups (**Figure 3C** and **3D**). These results suggest that overexpression of miR-370 interrupts Endoglin gene transcription and translation.

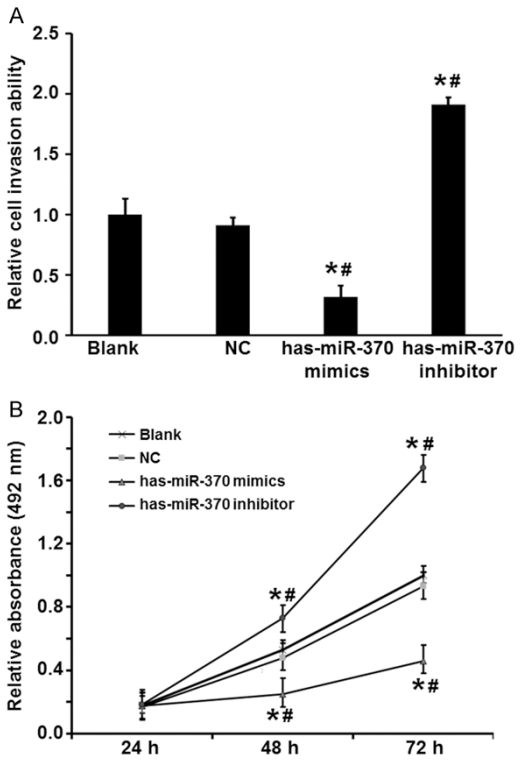

Figure 4. Effect of miR-370 on the motility and proliferation ability of HTR-8/SVneo cells. A. Relative invasion ability of HTR-8/SVneo cells transfected with miR-370 mimics or miR-370 inhibitor, determined using Transwell assay. B. Relative proliferation ability of HTR-8/SVneo cells transfected with miR-370 mimics or miR-370 inhibitor measured using MTT assay. Absorbance of cell suspension was measured at 492 nm. *, $P < 0.05$ compared with blank; #, $P < 0.05$ compared with NC.

Overexpression of miR-370 inhibits the motility of HTR-8/SVneo cells

To test the effect of miR-370 on HTR-8/SVneo cell invasion ability, Transwell assay was performed. Compared with NC and blank groups, overexpression of miR-370 significantly reduced the invasion ability of the cells, while inhibition of miR-370 enhanced their invasion of ability ($P < 0.05$) (**Figure 4A**). The result indicates that overexpression of miR-370 inhibits the motility of HTR-8/SVneo cells.

miR-370 is a kind of miRNA that suppresses the proliferation of HTR-8/SVneo cells

To investigate the effect of miR-370 on HTR-8/SVneo cell proliferation, MTT assay was carried out. The data showed that overexpression of miR-370 significantly inhibited the proliferation

of the cells, but inhibition of miR-370 significantly enhanced cell proliferation ($P < 0.05$) (**Figure 4B**). The result suggests that miR-370 is a kind of miRNA that suppresses the proliferation of HTR-8/SVneo cells.

Discussion

As a unique disease in pregnancy, PE is mainly clinically characterized by hypertension and proteinuria [20]. It is believed that dysfunction of placental trophoblast cells is the root cause of PE [3]. Abnormal differentiation and apoptosis of trophoblast cells are the common causes of the dysfunction [21, 22], but the exact mechanism is still unclear.

miRNA plays important roles in the regulation of biological functions [6]. Functional study of miR-152 shows that miR-152 molecules with high pathological expression in PE placental tissues participate in the pathological process of PE by down-regulating the expression of human leukocyte antigen-G (HLA-G), leading to the occurrence of excessive immune responses [23]. Therefore, miRNA facilitates the occurrence of PE by regulating its target genes that are important in the pathological process of PE. In the present study, we discover that miR-370 is down-regulated in PE lacental tissues. In addition, serum Endoglin concentration in PE patients is higher than that in normal subjects, suggesting that elevated Endoglin level may be an important mechanism for the occurrence of PE. We also find that Endoglin expression in PE placental tissues is significantly increased, being consistent with a previous report [18]. This suggests that elevated serum Endoglin concentration may be resulted from placenta.

Using bioinformatics, miR-370 is predicted to directly target Endoglin gene. It might be that miR-370 participates in PE process by negatively regulating its target Endoglin gene. In oral squamous cell carcinoma [24] and malignant bile duct cell carcinoma [25], the expression of miR-370 in tumor cells is significantly reduced compared with normal cells. However, there has been no report about studies on miR-370 in PE. Our results show that the expression of miR-370 in PE placental tissues is reduced. Furthermore, miR-370 also affects tumor cell proliferation, colony formation, tumor formation, metastasis and invasion. In bile duct cell carcinoma cell, miR-370 inhibits the proliferation of tumor cells by down-regulating the expression of its target gene MAP3K8 [27]. In oral squamous cell carcinoma, miR-370 significantly inhibits the migration and invasion of tumor cells [26]. Our results show that HTR-8/SVneo cells transfected with miR-370 mimics or miR-370 inhibitor have elevated and reduced miR-370 expression levels, respectively. In addition, overexpression of miR-370 in HTR-8/SVneo cells inhibits the expression of Endoglin. By contrast, inhibition of miR-370 elevates the expression of Endoglin. Subsequently, cell biology analyses demonstrate that overexpression of miR-370 reduces the invasion ability and proliferation of HTR-8/SVneo cells, while inhibition of miR-370 enhances those. In conclusion, the present study provides an understanding of the effect of miRNA in PE, which will help making the strategy for the treatment of this unique disease in pregnancy.

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Disclosure of conflict of interest

None.

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