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Highly expressed miR-182-5p can promote preeclampsia progression by degrading RND3 and inhibiting HTR-8/SVneo cell invasion

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Abstract. – OBJECTIVE: The role of miR-182-5p in preeclampsia was studied, and its mechanism was also explored.

PATIENTS AND METHODS: Fifty patients with preeclampsia were assigned to the study group and 50 normal pregnant women to the control group. The age, weight, blood pressure, urinary protein, and weight of newborns were compared between the two groups. The placental tissues of the above-mentioned subjects were collected, and quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) assay was used to detect the expression of miR-182-5p. MiR-182-5p was overexpressed or knocked down using a cell transfection assay in HTR-8/SVneov cell, which is a kind of human chorionic trophoblast cell. Changes in cell migration and invasiveness before and after transfection were determined by wound healing test and transwell assay, respectively. Western blot was performed to analyze the change of RND3 protein level before and after transfection. The biological prediction of the relationship between miR-182-5p and RND3 was performed and a dual luciferase reporter gene experiment was designed to verify the results. Finally, a rescue experiment was conducted to investigate whether RND3 could affect the role of miRNA-182-5p in the capacity of cell migration and invasion.

RESULTS: Preeclampsia patients had higher systolic blood pressure, diastolic blood pressure, and urinary protein than normal pregnant women, while neonatal weight decreased compared with normal pregnant women. MiRNA-182-5p was highly expressed in placental tissues of patients with preeclampsia. After miRNA-182-5p was overexpressed, the migration and invasion of HTR-8/SVneo cells were significantly attenuated, and the mRNA and protein levels of RND3 were markedly downregulated, and vice versa. The dual luciferase reporting assay confirmed that miRNA-182-5p could bind to 3'UTR of RND3. In addition, the results of rescue experiment showed that overexpressing miRNA-182-5p could markedly inhibit the migration and invasion of HTR-8/SVneo cells; however, when RND3 was simultaneously overexpressed, the inhibitory effect of miRNA-182-5p was partially reversed.

CONCLUSIONS: The highly expressed miRNA-182-5p in patients with preeclampsia promoted the development of preeclampsia, the possible mechanism of which might be that the increased miRNA-182-5p expression could inhibit the migratory and invasive ability of trophoblast cells through targeted degrading RND3 protein.

Key Words:
MiR-182-5p, RND3, Preeclampsia, Cell migration, Cell invasion.

Introduction

Preeclampsia (PE) is a pregnancy-specific disease characterized by high blood pressure and proteinuria after 20 weeks of gestation. It is an important cause of neonatal and maternal death worldwide, which is accompanied by a systemic multiple organ damage[1-3]. The prevalence of PE in pregnant women is 52%-8%[4], and about 25% of patients may occur fetal growth restriction[1]. Studies[5,6] have found that PE can increase the risks of pulmonary edema, assisted ventilation, amniotic fluid embolism, acute renal failure, and death in pregnant women. The risk of neonatal adverse outcomes such as eclampsia-induced prematurity, respiratory distress syndrome, and death also increases, severely affecting maternal and neonatal health. At present, there are no effective

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preventive measures, so pregnancy should be terminated in advance if necessary, which lead to a poor prognosis of pregnant women and perinatal infants. However, the pathogenesis of PE is not yet clear, so it is urgently needed to further study.

MicroRNAs (miRNAs) are a type of endogenous single-stranded small RNAs that are ubiquitously present. They are approximately 22 nucleotides in length and are evolutionarily conserved[7]. They play an important role in the transcriptional regulation of genes through binding to the target gene by way of complementary base pairing, then target gene expression is inhibited post-transcriptionally[4,8]. MiRNAs are involved in the regulation of about one-third of protein-coding genes in human cells. Multiple mRNAs can serve as target genes for the same miRNA. Therefore, miRNAs and target molecules form a complex regulatory network that affects the vital activities of entire cell. MiRNAs can act as regulatory molecules to inhibit the translation process of proteins and thus participate in a series of life activities including cell proliferation, differentiation, and apoptosis[9,10].

Studies have shown that certain miRNAs in placenta and serum of patients with PE present differential expression compared with normal pregnant women[11], suggesting that miRNA may be involved in the development of preeclampsia. In the placenta of patients with PE, the expression of miR-182-5p is higher than that of healthy placenta[12]. In this work, the role of miR-182-5p in preeclampsia was initially studied, and its mechanism was preliminarily explored.

Patients and Methods

Sample Collection

50 cases diagnosed with preeclampsia from July 2013 to July 2015 were selected as subjects of experimental group in The First People's Hospital of Jining. Meanwhile, 50 normal pregnant women in the same period were randomly selected into control group. The blood pressure and 24-hour urinary protein content of all the subjects were monitored, and the newborns were weighed and recorded. 2 pieces of placental tissue without calcification and bleeding with size of 1.0 cm × 1.0 cm × 0.6 cm were collected in maternal face of the attachment area for placental umbilical cord. After bleaching the blood, they were placed in a sterile enzyme-free cryopreservation and saved at - 80°C refrigerator. This study has been approved by the The First People's Hospital of Jining Ethics Committee. All the participants have signed the informed consent. All selected subjects were diagnosed without internal and surgical diseases, drug taken history during pregnancy, or *in vitro* fertilization. Clinical information of the patients is shown in Table I.

Cell Culture and Transfection Experiments

HTR-8/SVneo cells, the preeclamptic cells, were cultured in Dulbecco's Modified Eagle Medium (DMEM) complete medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) along with 1% penicillin and streptomycin, and then were placed in a 37°C, 5% CO_2 incubator. Before transfection, cells in logarithmic growth phase were seeded in 6-well plates 1 day before. When the cell density reached 60-80%, miR-182-5p-mimics or its control, miR-182-5p-inhibitor or its control, miR-182-5p-mimics and pcDNA-RND3 or its control were respectively transfected into cells following the instructions of the lipofatamine 2000 kit (Invitrogen, Carlsbad, CA, USA). After 48 hours, the transfection efficiency was analyzed by quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) experiments and the cells were collected for follow-up study.

Table I. Clinical characteristics of normal and preeclamptic pregnancies.

			p value
Variable	**PE (n=50)**	**Normal (n=50)**	**Control *vs.* PE**
Maternal age	29.8±4.2	30.5±3.2	>0.05
Proterinuria (g/day)	>0.3	<0.3	<0.01
Systolic blood pressure, mmHg	160±15.7	105±12.3	<0.01
Diastolic blood pressure, mmHg	124±14.3	71±8.5	<0.01
Body weight of infant (g)	2517±532	3601±452	<0.05

RNA Extraction and qRT-PCR Assay

A small piece of placenta tissue was ground into powder in liquid nitrogen, and the HTR-8/SVneo cells were collected after transfection for 48 h. Total RNA of the above tissue and cells were extracted in strict accordance with the procedures of the TRIzol reagent instructions (Invitrogen, Carlsbad, CA, USA). The UV spectrophotometer was used to determine the RNA concentration and purity, and it was considered eligible when the absorbance ratio of 260 nm/280 nm was between 1.8 and 2.1. The cDNA was obtained according to the reverse-transcription kit, and the qRT-PCR reaction solution was formulated according to the operating instructions of SYBR fluorescence quantitative premix kit. Amplification conditions were as follows: 95°C denaturation 30 s, 95°C 5 s, 60°C 31 s, with a total of 40 cycles. U6 was used as the internal reference of miR-182-5p while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was as the internal reference of RND3. This experiment was repeated 3 times and 3 parallel assays were performed each time. The result was represented by 2-ΔΔCt. Primer sequences were as follows: miR-182-5P: F: AGCCGTTTGGCAATGGTAGAACTC; R: GTGCAGGGTCCGAGGT; U6: F: GCTTCGGCAGCACATATACTAAAAT; R: CGCTTCAGAATTTGCGTGTCAT; RND3: F: CTATGACCAGGGGGCAAATA; R: GGCATCGTGATGGACTCCG; GAPDH: F: CGCTCTCTGCTCCTCCTGTTC; R: ATCCGTTGACTCCGACCTTCAC

Western Blot Assay

A small piece of embryo tissue was ground in liquid nitrogen to a powder and lysed with 1 mL of radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China). HTR-8/SVneo cells were collected 48 hours after transfection and lysed with RIPA lysate either. Then, the tissue or the cell lysate were ultrasonated on ice and centrifuged to collect the supernatant. Bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) was used for protein concentration. Protein samples were separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes by wet transfer (Millipore, Billerica, MA, USA). After blocking with 5% skim milk at room temperature for 1 hour, the corresponding primary antibodies were added to incubate the protein bands cut from the membrane and placed at 4°C overnight. After the bands were washed 3 times with Tris-buffered saline and tween 20 (TBST), the corresponding secondary antibody was added to the bands and incubated for 1 hour at room temperature. The membrane was washed again 3 times with TBST and developed with enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA). GAPDH was used as an internal reference of RND3 protein, and the experiment was repeated three times.

Wound Healing Test

Transfected HTR-8/SVneo cells were seeded in 6-well plates at 1 x 10^6 cells per well. The next day, a gun of 200 μL was used to draw a mark in the well, and the floating cells were eluted with phosphate-buffered saline (PBS). The cells were photographed under a microscope, the wound distance was measured, and the culture was continued at 37°C in a 5% CO_2 incubator. After 48 hours, the wound distance was observed and measured again, and then, the wound healing ability of the cells was calculated.

Transwell Assay

First, Matrigel diluted by pre-cooled serum-free DMEM medium was placed on a culture chamber. Then, HTR-8/SVneo cells (1×10^5cells) in 100 μL of serum-free medium were seeded in the upper chamber, while the lower one was supplemented with 600 μL of DMEM containing 10% serum, with 3 replicates per group. Subsequently, the chamber was incubated at 37°C with 5% CO_2. After 48 hours, the culture chamber was taken out and fixed in 2.5% glutaraldehyde for 15 minutes, and then treated with 0.5% Triton X-100 for 3 minutes. The hematoxylin was used to dye cell nucleus for 15 min. Finally, the inverted chamber was observed and counted under an optical microscope and photographed. The method was repeated three times.

Dual Luciferase Reporter Assay

Biology was applied to predict that miR-182-5p can bind to 3'UTR of RND3, suggesting that RND3 may be the target gene for miR-182-5p. Luciferase reporter vector plasmids containing wild-type or mutant 3'UTR of RND3 (RND3 WT and RND3 MT) were constructed and then co-transfected with miR-182-5p mimics or its control in cells following the Lipofectamine 2000 instructions. The luciferase activity of cells in each group was detected 48 h after transfection.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL,

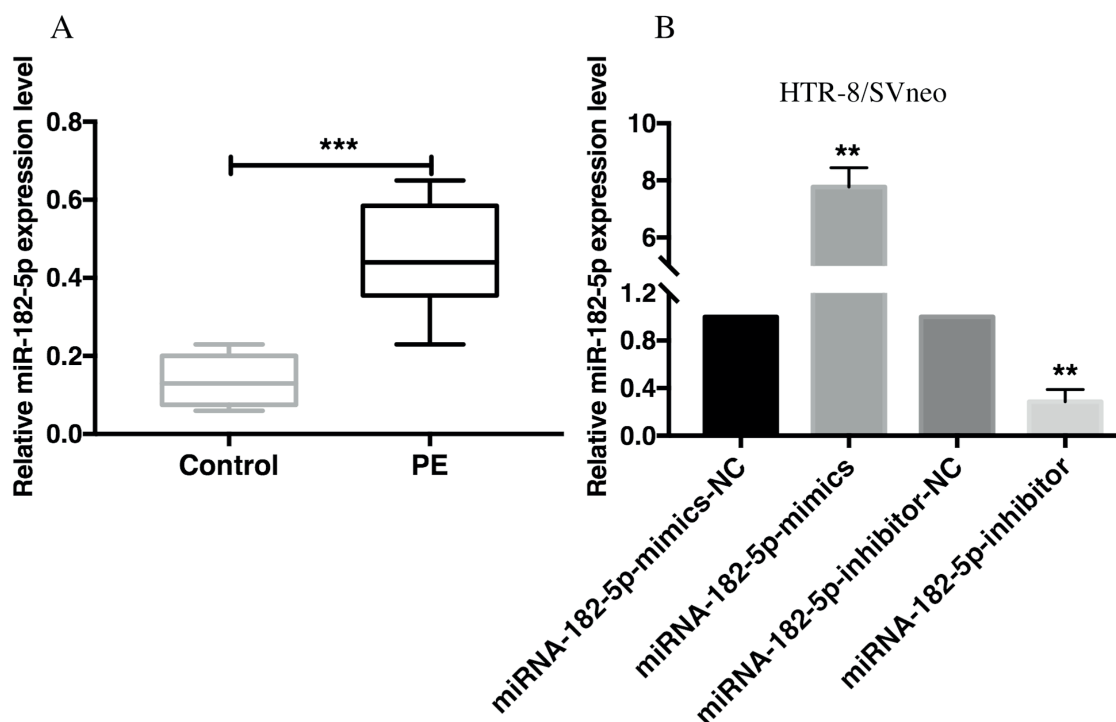

Figure 1. miR-182-5p was highly expressed in the placenta of preeclampsia patients. ***A,*** miR-182-5p was highly expressed in preeclampsia placenta. ***B,*** After overexpressing and inhibiting miR-182-5p in HTR-8/SVneo cells, a corresponding change presented.

USA) was used for data analysis. Measured data were expressed as mean ± standard deviation (mean ± SD), and the *t*-test was used for the comparison between groups. $p<0.05$ was considered statistically significant.

Results

MiRNA-182 was Highly Expressed in Placenta of Preeclampsia Patients

Preeclampsia patients and normal pregnant women in the same period were selected as the study objects. We then monitored the 24 h proteinuria, blood pressure, and neonatal weight of the subjects in the above two groups. The analysis found that there was no significant difference in the age of the subjects of two groups. However, the 24-hour proteinuria, systolic blood pressure, and diastolic blood pressure of PE patients were found higher than those of normal pregnant women, and the weight of newborns was significantly lower than that of normal pregnant women (Table I). After the placenta was delivered from the pregnant subjects, placental tissue was collected to detect the expression of miRNA-182-5p, and the result showed that it was significantly elevated in the preeclampsia group (Figure 1A).

MiRNA-182-5p Inhibited HTR-8/SVneo Cell Invasion and Migration

MiRNA-182-5p was highly expressed in the placenta of preeclampsia patients, suggesting that it might be involved in the development of preeclampsia. We further designed experiments to initially explore its role. First, miRNA-182-5p-mimics or its NC and miRNA-182-5p-inhibitor or the NC were transfected into HTR-8/Svneo, the human trophoblast cells, respectively to enhance miRNA-182-5p level or inhibit its expression (Figure 1B). Then, the migratory and invasive capacity of HTR-8/SVneo before and after transfection were evaluated. It was found that overexpressed miRNA-182-5p strikingly weakened the migratory ability of HTR-8/SVneo cells, while the opposite result was observed after miRNA-182-5p was inhibited (Figure 2A). Meanwhile, the changes of cell invasive ability also presented the same trend (Figure 2B).

MiRNA-182-5p Could Targeted Bind to RND3

MiRNA-182 could regulate the migration and invasion of HTR-8/SVneo cells. To further explore its mechanism of action, we used biological methods to predict the target gene of miRNA-182-5p, namely RND3. The results indicated that overexpressing miR-182-5p could enhance the mRNA level of RND3 in cells, while inhibiting miR-182-5p decreased RND3 level (Figure 3A). Result of Western blot assay showed that the protein level of RND3 in HTR-8/SVneo was similar before and after transfection (Figure 3B-C). Bioinformatics suggested that miRNA-182-5p could bind to the 3'UTR of RND3 (Figure 3D), which was then confirmed by dual luciferase reporting assay (Figure 3F). The above results demonstrated that miR-182-5p may change the migratory and invasive ability of HTR-8/SVneo cells by regulating the expression of RND3.

RND3 Reversed the Effects Exerted by Overexpression of miR-182-5p

To further confirm that RND3 is a key factor in miRNA-182-5p action, we designed a rescue experiment. The results indicated that simultaneous overexpression of RND3 and miRNA-182-5p may partially reverse the inhibitory effects of overexpressed miRNA-182-5p on migratory (Figure 4A) and invasive capacity of (Figure 4B) HTR-8/SVneo. It was suggested that RND3 was a vital factor in the role of miRNA-182-5p in preeclampsia diseases.

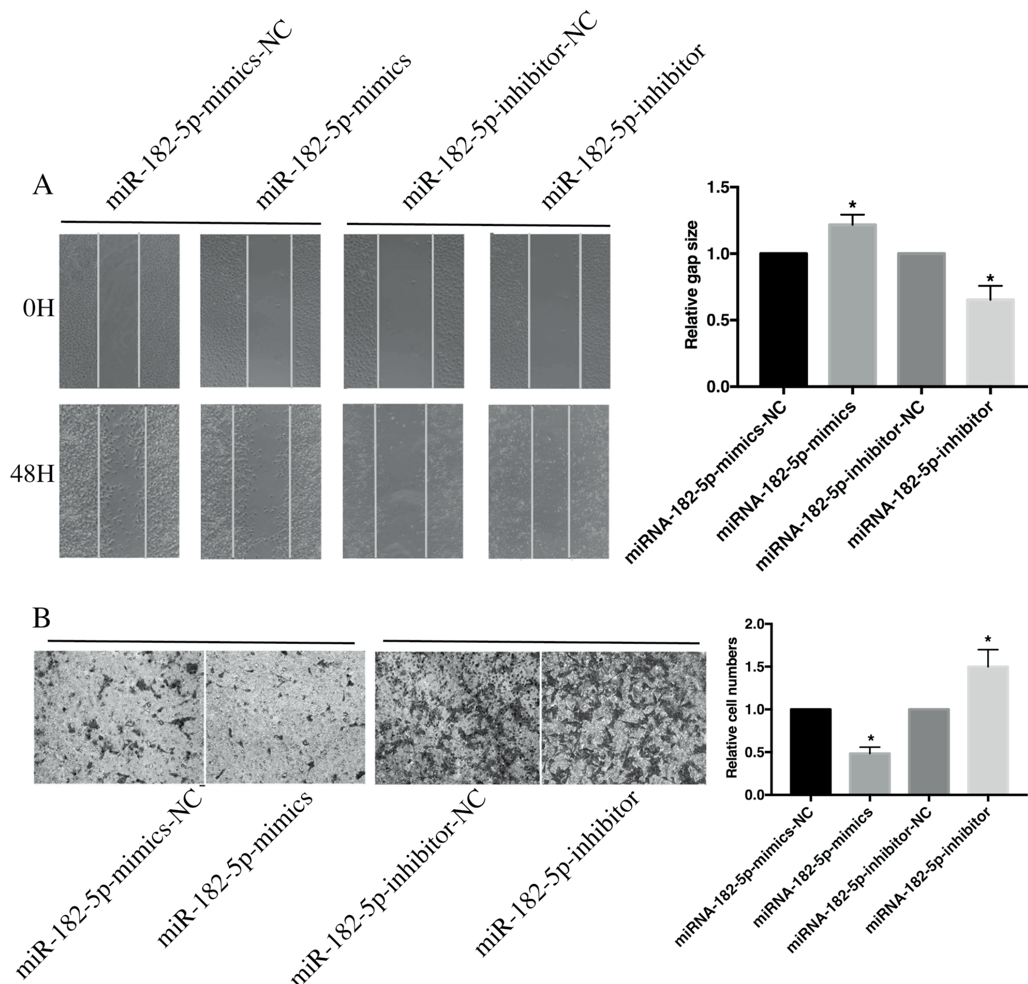

Figure 2. miR-182-5p inhibited invasion and migration of HTR-8/SVneo cells. ***A,*** Wound healing test showed that after overexpression of miR-182-5p, the cell migration ability was significantly weakened, while the opposite result was observed after miR-182-5p was inhibited. ***B,*** Transwell assay showed that after overexpression of miR-182-5p, the invasive ability of the cells was significantly weakened, while the opposite result was observed after miR-182-5p was inhibited.

Discussion

Inadequate invasion of trophoblast cells and impaired infiltration are the most prominent pathological features in patients with preeclampsia, and are also central links in the occurrence and development of preeclampsia[13,14]. The processes of trophoblast cells invading the endometrium include adhesion, migration, and invasion, which are the most basic biological processes of embryo implantation. Trophoblast cells are commonly regulated by autocrine factors of themselves and paracrine factors of uterine. The migratory and invasive characteristics of trophoblast cell are similar to those of tumor cells, but the difference is that the trophoblast cells have strict temporal and spatial constraints and are finely regulated, which meanwhile are limited to the early gestation of the uterus[15-17]. The invasion number of trophoblast cells is related to the severity of preeclampsia. Inadequate invasiveness can lead to complications during pregnancy, such as spontaneous abortion, intrauterine growth restriction, etc.[18].

Many studies have shown that Rho E is closely related to tumor metastasis and its role in different tumors is different. Rho E, also known as RND3, is a protein with CDS length of 735 bp and a molecular weight of approximately 29 kDa, which encodes 244 amino acids[19]. It plays a promotive role in prostate cancer, gastric cancer, melanoma, and other tumors. Previous evidence found that the expression of RND3 was increased in prostate cancer cells with high metastatic potential[20-22]. RND3 was found to be able to promote invasion of human invasive melanoma cells[23]. Similarly, RND3 is upregulated in gastric cancer tissues and cells and is positively correlated with metastasis of gastric cancer. In addition, upregulated Rho E was found to enhance the invasive ability of gastric cancer cells[24]. However, it plays a role in inhibiting tumor metastasis in breast cancer and sarcoma. Previous works have suggested that high expression of RND3 in breast cancer cells increases the tight junctions number of mammary epithelial tight junctions[25]. In mice researches, RND3 inhibited the metastatic ability of tumor cells[26]. The study in mice with sarcoma also showed that metastasis occurred in the Rho E/RND3 overexpressed group, while metastatic lesions of the lung and adrenal gland appeared in 6/8 in the control group. The above results suggested that RND3 is closely related to tumor metastasis.

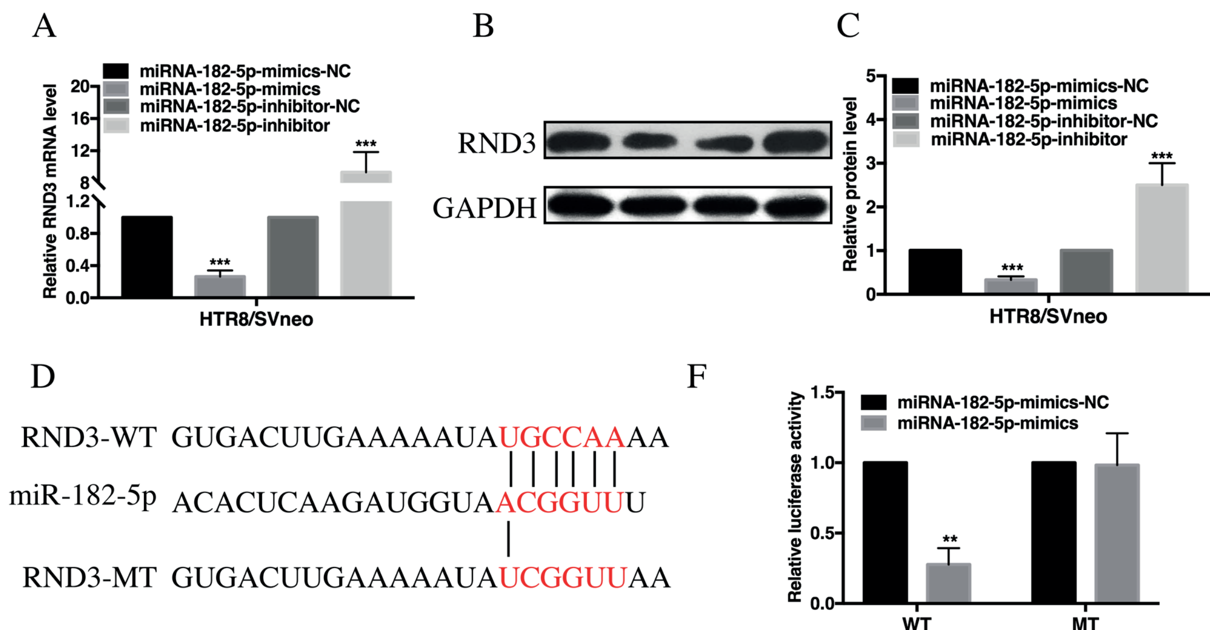

Figure 3. MiR-182-5p could targeted bind to RND3. ***A,*** After overexpression of miR-182-5p, the expression of RND3 mRNA was significantly down-regulated. After knockout of miR-182-5p, the expression of RND3 mRNA was significantly up-regulated. ***B,*** and ***C,*** After overexpression of miR-182-5p, the expression of RND3 protein was significantly down-regulated. After knockout of miR-182-5p, the expression of RND3 protein was significantly up-regulated. ***D,*** and ***E,*** Dual luciferase reporter assays showed that miR-182-5p could bind to 3'UTR of RND3, thereby reducing its expression.

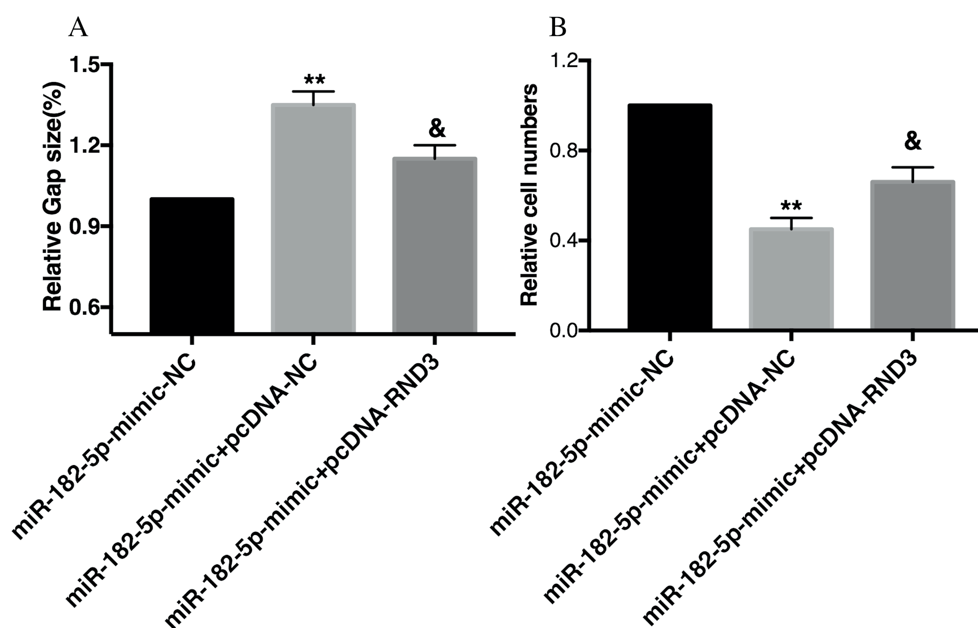

Figure 4. RND3 reversed the effects exerted by overexpression overexpression of miR-182-5p. ***A,*** Wound healing test demonstrated that after overexpression of miR-182-5p, the ability of cell migration was significantly attenuated. After overexpression of RND3, cell migration was significantly upregulated. ***B,*** Transwell assay showed that after overexpression of miR-182-5p, the invasive ability of the cells was significantly reduced. After overexpression of RND3, the invasive ability of cells was significantly upregulated.

The migrated and invasive characteristics of trophoblast cells are similar to those of tumor cells. In this investigation, the expression of miR-182-5p in placenta of patients with preeclampsia and normal pregnant women was detected. It was found that miR-182-5p was highly expressed in the placenta of patients with preeclampsia, and cell experiments confirmed that it could regulate the ability of migration and invasion of trophoblast cells. Biological predictions have found that miR-182-5p can bind to the RND3 3' UTR region, and the dual luciferase reporter gene confirmed this targeted binding of RND3. The results of transfection and rescue experiments further suggested that miR-182-5p may play a role through RND3, and RND3 may also partly reverse the promotive effect of miRNA-182-5p on migration and invasion of trophoblast cells.

Conclusions

We demonstrated that miR-182-5p accelerated the development of preeclampsia, and its possible mechanism might be the regulation of the migration and invasion of trophoblast cells by changing the expression of the target gene RND3.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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