miR-203 contributes to pre-eclampsia via inhibition of VEGFA expression

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Abstract. Pre-eclampsia (PE) is a common but complex condition that can occur in pregnancy. It is estimated to affect 3-8% of pregnancies worldwide. PE development is thought to be multifactorial and to involve the dysregulation of microRNA (miR) expression. However, the precise mechanisms of PE development remain unclear. The present study aimed to illustrate the association between miR-203 expression and PE development in samples of human placenta collected from mothers with (n=18) and without (n=20) PE. It was demonstrated that miR-203 expression was significantly increased in the PE placenta compared with the normal placenta samples, while the expression of vascular endothelial growth factor A (VEGFA) was decreased. *In vitro* experiments revealed that miR-203 overexpression significantly downregulated VEGFA expression and inhibited the proliferation, migration and invasion ability of HTR-8/SVneo cells. Suppression of miR-203 expression alleviated these effects. A luciferase reporter assay confirmed the interaction of the 3'-untranslated region of VEGFA with miR-203. Thus, miR-203 may have significant contribution to the development of PE by targeting VEGFA in the human placenta and may have potential as a biomarker or therapeutic target in the treatment of PE.

Introduction

Pre-eclampsia (PE) is a common but complex condition that can occur in pregnant women, characterized by hypertension and proteinuria (1). It has been reported to occur in 3-8% of pregnancies worldwide (2). According to the level of hypertension and proteinuria, PE is classified from mild to severe, using the American Congress of Obstetricians and Gynecologists

Committee on Practice Bulletin-Obstetrics (3). Pre-existing conditions including diabetes mellitus, obesity, renal disease and hypertension are considered to be risk factors for PE development (4). PE is a multi-system disease that threatens both maternal and newborn health (5). PE development is thought to be multifactorial, involving the disruption of vascular homeostasis in the placenta (6), altered placental transcriptome (7), reduced 2-methoxyestradiol level (8) and hypoxia (9). It is well established that delivery of the fetus and placenta is the most effective way to treat PE. However, the exact mechanisms of PE development still remain unclear and it is important to further elucidate the exact pathophysiology of this disease.

microRNAs (miRNAs/miRs) are small, single-stranded non-coding RNA molecules ~22 nucleotides in length. miRNAs suppress the expression of their target mRNAs through RNA silencing and post-transcriptional regulation (10-13). It has been reported that the chromosome 19 miRNA cluster (C19MC), C14MC, miRNA (miR-37-3) and miR-17-92 clusters are involved placenta development (14,15). Accumulating evidence indicates that the abnormal miRNA expression contributes to PE development (16,17). For example, the upregulation of miR-520g can promote PE via the inhibition of trophoblast migration and invasion (18). The human placenta expresses numerous angiogenic factors that may be involved in maternal vascular adaptation to pregnancy, including vascular endothelial growth factor (VEGF). Placental expression of VEGF has been reported in villous trophoblasts, but immunostaining indicated that VEGF is not expressed in the villous endothelium (19,20). VEGFA in particular is a highly specific mitogen for vascular endothelial cell growth. Previous studies have reported that miR-203 is involved in the pathogenesis of tumor growth by targeting VEGFA (21,22). Thus, the potential involvement of miR-203 in trophoblast VEGFA expression and PE development were investigated. In combination with previous research, the present study may aid in the development of miRNAs or miRNA mimics as effective biomarkers or therapeutic agents for the treatment of placental diseases.

Materials and methods

Ethics statement. Human placental tissue used in this research was collected from 38 patients that had provided written

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informed consent. The present study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (Wuhan, China; permit no. 2016016).

Human placenta collection. Human placental tissues were collected from artificial labor and caesarean sections at the Renmin Hospital of Wuhan University (Wuhan, Hubei, China) between June 2016 and February 2017. All samples were frozen in liquid nitrogen for 5 min and stored at -80°C for subsequent analyses. Placental tissue was collected from pregnant women who met the following inclusion criteria: Monocyesis, normal pregnancy at the time of sample collection and no pre-existing clinical conditions, including diabetes, chronic nephritis, cardiovascular, hepatic or autoimmune disease. Samples were subsequently categorized into the PE or normal group according to the presence or absence of PE. Detailed clinical characteristics of the sample groups are presented in Table I.

Hematoxylin and eosin (H&E) staining. Placental tissue from different treatment groups was sequentially fixed in 4% paraformaldehyde at room temperature for 12 h, dehydrated in 60, 70, 80, 90, 95% and absolute ethanol respectively for 30 min at room temperature, embedded in paraffin and cut into 5 μm sections. Following deparaffinization in xylene for 5 min twice and rehydration sequentially in absolute, 95, 90, 80, 70 and 60% ethanol respectively for 5 min at room temperature, placental tissue sections were stained with H&E. Images were captured under x100 magnification by a Nikon Eclipse Ci microscope (Nikon Corporation, Tokyo, Japan).

Immunohistochemistry (IHC) detection of VEGFA in the placenta. Immunohistochemical detection of VEGFA was conducted on 5-μm-thick paraffin-embedded placental tissue sections using the avidin-streptavidin-biotin method. IHC operation kits was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). The standard IHC protocol was performed according to the manufacturer's instructions. Following blocking in immunnol staining blocking buffer (P0102; Beyotime Institute of Biotechnology) at room temperature for 1 h and incubation with a human VEGFA primary antibody (1:50; ab1316; Abcam, Shanghai, China) at 4°C overnight, sections were washed with PBS and subsequently incubated with the biotin-conjugated goat anti-mouse IgG (TA130008; 1:200; OriGene Technologies, Inc., Beijing, China) at room temperature for 45 min. Visualization was achieved with diaminobenzidine staining (OriGene Technologies, Inc., Beijing, China) for 5 min and counterstaining with hematoxylin solution for 2 min at room temperature. Images were captured with the Nikon Eclipse Ci microscope (Nikon Corporation, Tokyo, Japan) at x100 magnification.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Each human placental tissue sample for RT-qPCR was obtained from the center between placental edge and umbilical cord. The size of the samples was ~1.5x1.5x1.5 cm^3. Total RNA was isolated with TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Quantitative real-time PCR kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Details of the RNA

extraction, cDNA conversion and RT-qPCR assays have been described previously (23). To detect the mRNA expression levels of miR-203 and VEGFA, RT-qPCR was performed in 96-well reaction plates with a total volume of 10 μl, containing the following: 1 μl cDNA template, 0.2 μl each primer, 3.6 μl diethyl pyrocarbonate-H$_2$O and 5 μl SYBR-Green dye using the ABI Step One Plus^{TM} Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primers were designed using Primer Premier version 6.0 (Premier Biosoft International, Palo Alto, CA, USA): miR-203 forward, 5'-GAATTCGGGGATCTGGGCGCAGGGGCC-3' and reverse, 5'-CTCGAGCCGACCTGGAGCGCGGAGC-3'; VEGFA forward, 5'-ACCATGAACTTTCTGCTGTCTTGG GTGCAT-3' and reverse, 5'-TCACCGCCTCGGCTTGTC ACATCTGCAAGT-3'; GAPDH forward, 5'-TTGATGGCA ACAATCTCCAC-3' and reverse, 5'-CGTCCCGTAGACAAA ATGGT-3'; and 6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The PCR cycling parameters used were as follows: Pre-denaturation at 95°C for 30 sec; 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 10 sec and extension at 72°C for 30 sec. GAPDH and U6 mRNA expression levels were measured and applied as quantitative controls to normalize the relative expression of VEGFA and miR-203, respectively. The relative expression of each target amplicon was calculated using the $2^{-\Delta\Delta Cq}$ method relative to the endogenous control (24).

Cell culture and transfection. The HTR-8/SVneo cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). This cell line was established by the transfection of first trimester villous explants of human primary trophoblasts. Cells were cultured in RPMI 1640 (11875-093; Thermo Fisher Scientific, Inc.) medium supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) without antibiotics for 24 h. miR-203 mimic (Thermo Fisher Scientific, Inc.), miR-203 inhibitor (anti-miR-203; Thermo Fisher Scientific, Inc.) and a scramble control miRNA (Shanghai GenePharma Co., Ltd., Shanghai, China) were subsequently transfected into cells seeded on a 6-well plate at 10,000 cells/well, as described previously (21). Sequences were transfected at a final concentration of 10 nM using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc., Shanghai, China) according to the manufacturer's protocol. This concentration was determined based on dose-response experiments. Cells were further cultured for the following cell proliferation and transwell assays and subsequently harvested for RT-qPCR or western blot analysis 48 h after transfection. All experiments were conducted in triplicate.

Cell proliferation. For the cell proliferation assay, transfected HTR-8/SVneo cells were seeded into 96-well plates (5x10^4 cells/well). Cells were cultivated in RPMI 1640 medium for 3 days. CCK-8 (10 μl; American Type Culture Collection; Manassas, VA, USA) was subsequently added to each well and HTR-8/SVneo cells were incubated for 2 h. Cell viability was measured by the absorbance at a wavelength of 450 nm using an ELISA reader (Tecan Group, Ltd., Mannedorf, Switzerland).

Transwell assays. Cell migration and invasion was detected using Transwell permeable supports (Corning Incorporated,

Table I. Clinical characteristics of normal and pre-eclampsia pregnancies.

Characteristic	Pre-eclampsia (n=18)	Normal (n=20)	P-value
Maternal age (years)	30.3±4.6	29.5±4.3	0.58
Gestational age (weeks)	36.5±1.2	38.3±1.9	<0.05
Birth weight (g)	2,984.9±684.2	3,624.6±363.3	<0.05
Placenta weight (g)	559.2±79.4	642.9±108.6	<0.05
Mean arterial pressure (mmHg)	122.5±10.6	94.3±6.4	<0.05
Body mass index (kg/m^2)	23.3±4.3	24.3±3.2	0.42

Corning, NY, USA). HTR-8/SVneo cells were transfected with the miR-203 mimic, anti-miR-203 or the negative control (miRNC) for 24 h and subsequently cultured in 200 μl of serum-free RPMI 1640 prior to transfer onto the upper chambers of 24-well plates (5x10^5 cells/well) with or without a Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) coating. The lower chamber was supplemented with 800 μl of RPMI 1640 with 10% FBS. After 24 h without Matrigel or 48 h with Matrigel, cells in the lower chamber were fixed with absolute methanol for 20 min and stained with 0.1% crystal violet solution (Sigma Aldrich; Merck KgaA, Darmstadt, Germany) for 10 min at room temperature prior to cell counting under a microscope.

Western blot analysis. Western blot analysis was performed as described previously (21). Total protein was extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and Protease Inhibitor Cocktail (Sigma Aldrich; Merck KgaA). A BCA Assay Kit (Thermo Fisher Scientific, Inc.) was applied to detect the concentration of extracted protein. A total of 30 μg was loaded onto each lane, separated using 10% SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Merck KgaA). The membrane was subsequently blocked in 5% non-fat milk for 1 h at room temperature and incubated overnight at 4˚C with the primary antibodies VEGFA (1:1,000; ab1316, Abcam) and GAPDH (sc-47724; 1:3,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following incubation with horseradish peroxidase-conjugated anti-rabbit (A0208; 1:5,000; Beyotime Institute of Biotechnology) and anti-mouse (A0216; 1:5,000; Beyotime Institute of Biotechnology) secondary antibodies at room temperature for 45 min, antibody binding signals were detected using the enhanced chemiluminescence luminol reagent (PerkinElmer Inc., Waltham, MA, USA) and a Chemi-doc image analyzer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein bands were subsequently visualized with ImageJ software version 1.49 (National Institutes of Health, Bethesda, MD, USA). GAPDH was used to normalize the band intensities.

Luciferase reporter assay. The luciferase reporter assay was conducted as described previously (21). The 3'-untranslated region (UTR) sequence of VEGFA was amplified and inserted into the luciferase reporter vector pGL3-enhancer (Promega Corporation, Madison, WI, USA). The primers for VEGFA 3'-UTR were 5'-CAGCTCGAGTGTGTGAGTGGTTGACCT TCCT-3' (forward) and 5'-CCGAAGCTTTCAGGGAGAGAG AGATTGGAAA-3' (reverse). HTR-8/SVneo cells were seeded

into 24-well plates (5x10^5 cells/well) and incubated for 24 h. Wild-type or mutant VEGFA 3'-UTR vectors were subsequently co-transfected with the miR-203 mimic into HTR-8/SVneo cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). After incubation for 48 h, cells were lysed and assayed for luciferase activity with the Luciferase Assay System (Promega Corporation) according to the manufacturer's instructions. Renilla-luciferase was used for normalization.

Statistical analysis. Data analysis was performed with the Stata 13.0 software (StataCorp LP, College Station, TX, USA), using the Student's t-test, one way analysis of variance and the Scheffe post hoc test with Spearman's correlation analysis. Results are presented as the mean ± standard error. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of miR-203 and VEGFA in human placenta. To evaluate the role of miR-203 and VEGFA in the placenta development, miR-203 and VEGFA expression levels in all 38 placental tissue samples were measured using RT-qPCR. miR-203 expression levels were significantly increased in PE placentas compared with normal placentas (Fig. 1A; P<0.01), whereas VEGFA expression levels were significantly decreased (Fig. 1B; P<0.01). The correlation between miR-203 and VEGFA expression was further evaluated and a significant negative correlation (r=0.2217; P=0.0028; Fig. 1C) was identified. These results indicate that miR-203 upregulation may be associated with VEGFA downregulation.

Placenta pathology and distribution of VEGFA expression. To confirm morphological changes in the PE placenta, H&E staining was applied to the normal and PE placenta tissue samples. A disordered structure and a prominent decrease in chorionic villi was observed in the PE placenta in comparison with normal placenta tissue (Fig. 2A and B). IHC revealed abundant VEGF expression in villous trophoblasts and a lack of expression in the villous endothelium. VEGFA expression levels in the PE placentas was clearly downregulated in comparison with normal placenta samples (Fig. 2C and D). These results suggest that VEGFA downregulation in the placenta may be associated with PE occurrence.

miR-203 affects the proliferation, migration and invasion of HTR-8/SVneo cells. The HTR-8/SVneo cell line is established by the transfection of human primary trophoblasts originating
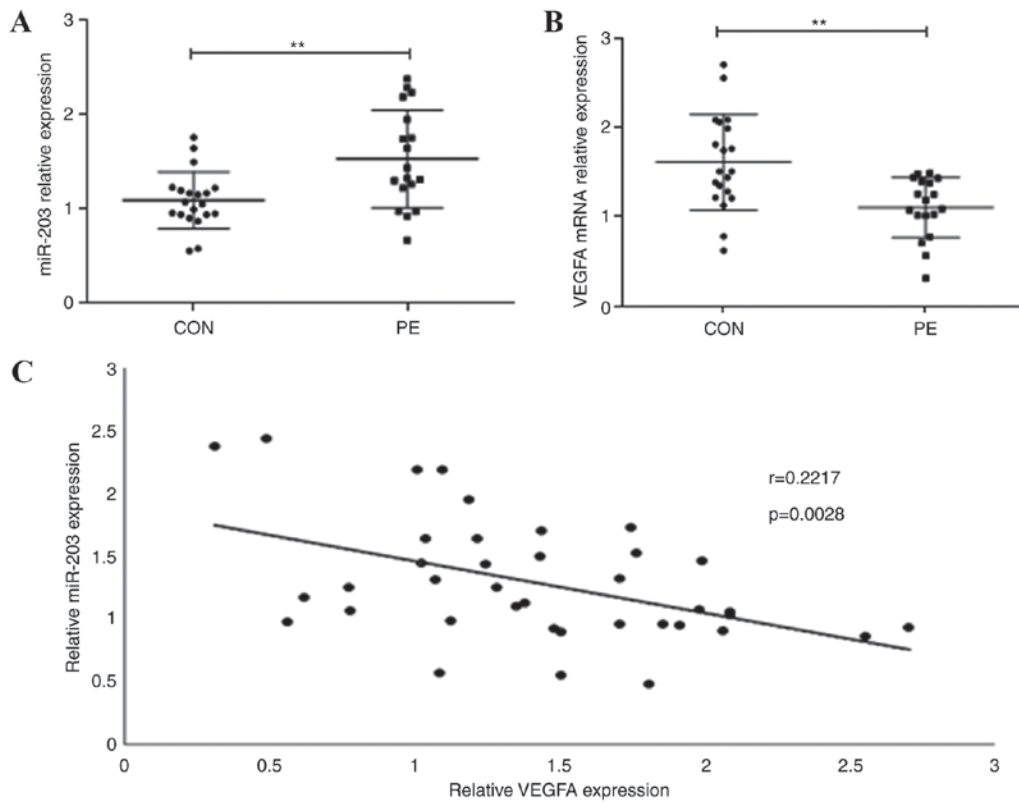
Figure 1. miR-203 and VEGFA mRNA expression levels in human placenta sample. (A) miR-203 and (B) VEGFA mRNA expression levels in the placenta were analyzed and quantified. (C) The correlation between miR-203 and VEGFA expression in 38 human placenta samples using Spearman's correlation analysis. Data is expressed as the mean ± standard error of the mean. **P<0.01, vs. control. miR-203, microRNA-203; VEGFA, vascular endothelial growth factor A; CON, control; PE, pre-eclampsia.
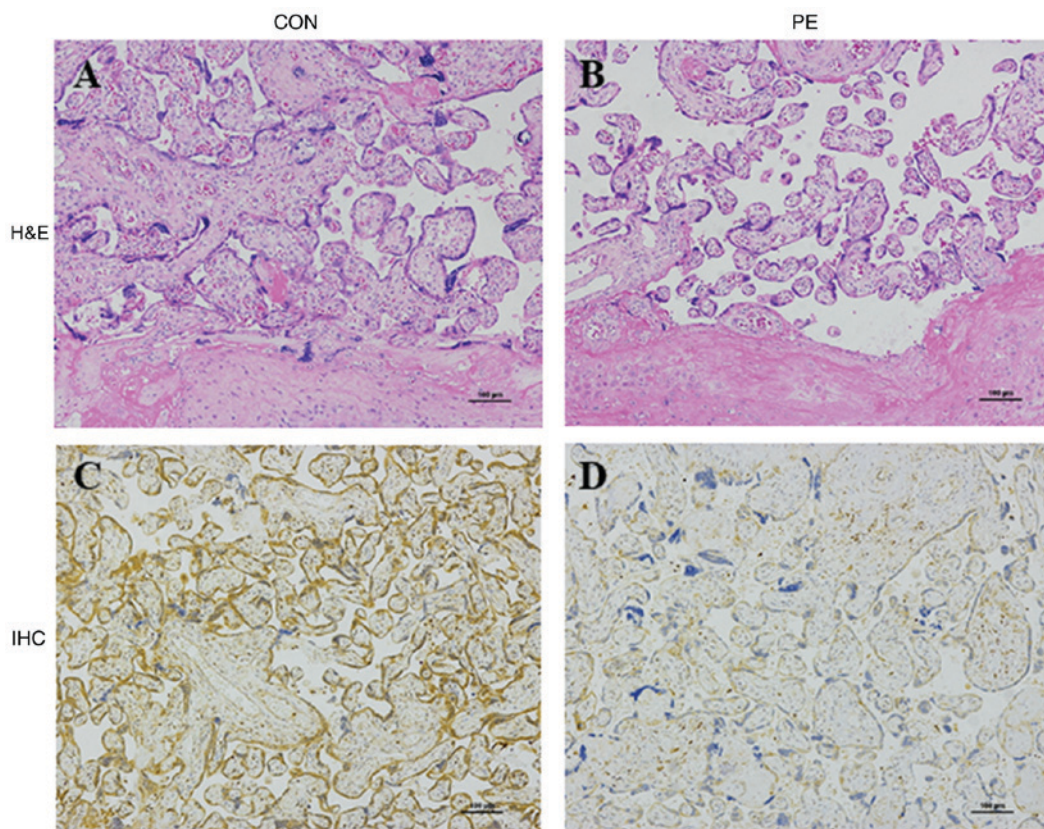

Figure 2. (A) Normal and (B) pre-eclampsia placental histopathology visualized with H&E staining (C) Normal and (D) Pre-eclampsia vascular endothelial growth factor A expression analyzed by IHC. Scale bars, 100 μm. CON, control; PE, pre-eclampsia; IHC, immunohistochemistry; H&E, hematoxylin and eosin.
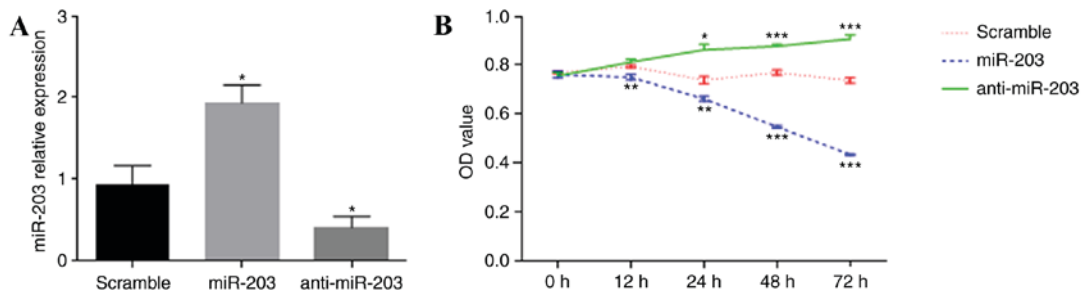
Figure 3. miR-203 inhibits HTR-8/SVneo cell proliferation. (A) Relative miR-203 expression in HTR-8/SVneo cells was measured with reverse transcription-quantitative polymerase chain reaction following cell transfection with scramble control miRNA, miR-203 mimics or anti-miR203 (B) Cell proliferation rate was measured using a CCK-8 assay 48 h after transfection with scramble control miRNA, miR-203 mimics or anti-miR-203. Data is expressed as the mean ± standard error from three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. control. miR-203, microRNA-203; anti-miR-203, miR-203 inhibitor; OD, optical density.
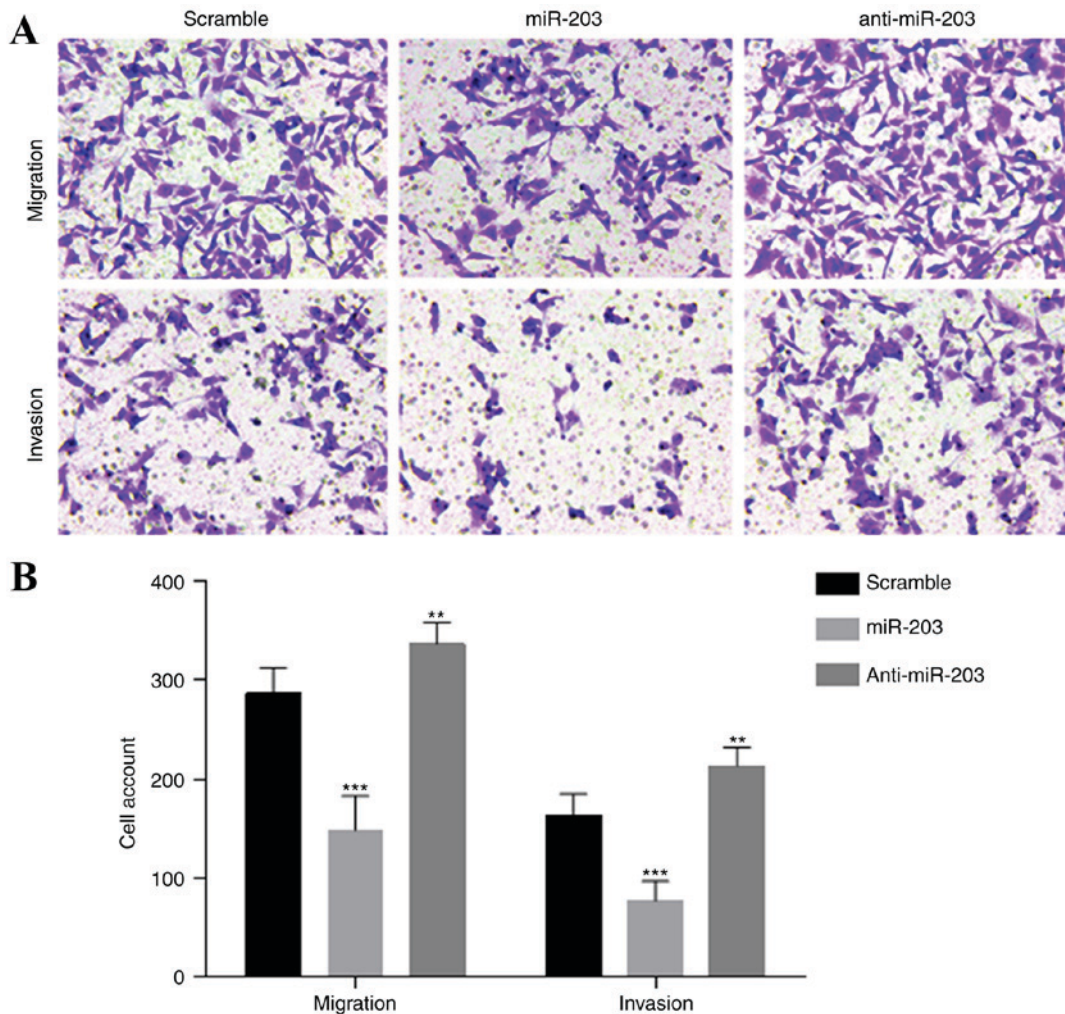

Figure 4. miR-203 inhibits the migration and invasion of HTR-8/SVneo cells. (A) The migration and invasion capacity of HTR-8/SVneo cells was measured with a Transwell migration/invasion assay following transfection with miR-203 mimics, anti-miR-203 or scramble control miRNA for 48 h. Overexpression of miR-203 inhibited the migration and invasion of HTR-8/SVneo cells. (B) The relative ratio of the migration and invasion of cells per field is shown. Data is expressed as the mean ± standard error from three independent experiments. **P<0.01 ***P<0.001 vs. scramble control. miR-203, microRNA-203; anti-miR-203, miR-203 inhibitor.

from first trimester villous explants, with a gene encoding the simian virus 40 large T antigen, resulting in immortalization (25). To determine the effect of miR-203 on cell proliferation in the human placenta, HTR-8/SVneo cells were transiently transfected with miR-203 mimics and anti-miR-203,

and a CCK-8 assay was performed to test the cell proliferation rate. RT-qPCR was used to confirm the overexpression or inhibition of miR-203 expression in HTR-8/SVneo cells (Fig. 3A). Overexpression of miR-203 was demonstrated to reduce the proliferative capacity of HTR-8/SVneo cells, whereas the
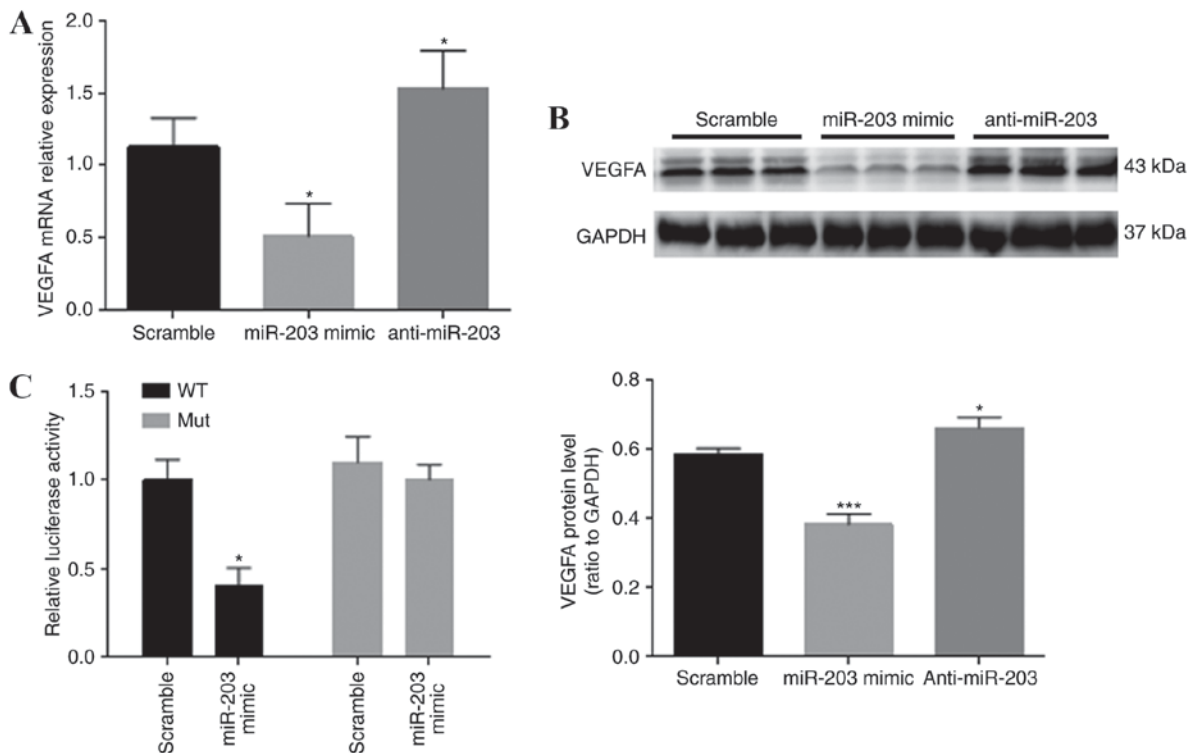
Figure 5. miR-203 directly targets VEGFA in HTR-8/SVneo cells. (A) mRNA and (B) protein expression levels of VEGFA were measured in HTR-8/SVneo cells using polymerase chain reaction and western blot analysis. GAPDH was used as an internal loading control. The relative expression levels of VEGFA mRNA and protein were calculated using Image J software. (C) WT or Mut 3' untranslated region vectors were co-transfected with miR-203 mimics into HTR-8/SVneo cells. The cells were assayed for luciferase activity after incubation for 48 h. The luciferase activity was quantified by the Luciferase Assay System. Each bar represents the mean ± standard error of three independent experiments. *P<0.05 ***P<0.001 vs. control. VEGFA, vascular endothelial growth factor A; miR-203, microRNA-203; anti-miR-203, mir-203 inhibitor; WT, wild-type; Mut, mutated.

inhibition of miR-203 expression resulted in a significant increase in cell proliferation (Fig. 3B). To assess the effects of miR-203 on cell migration and invasion, Transwell migration and invasion assays were performed in the HTR-8/SVneo cells. Overexpression of miR-203 significantly suppressed the migration and invasion abilities of HTR-8/SVneo cells compared to the control cells (Fig. 4A and B). The migration and invasion capacity of HTR-8/SVneo cells in the anti-miR-203 group was also significantly enhanced.

VEGFA is directly targeted by miR-203 in HTR-8/SVneo cells. To evaluate the possible effect of miR-203 on VEGFA expression, VEGFA mRNA and protein expression levels in HTR-8/SVneo cells were examined with RT-qPCR and western blot analysis, respectively (Fig. 5A and B). Both VEGFA mRNA and protein levels were significantly reduced in HTR-8/SVneo cells transfected with the miR-203 mimics. By contrast, the expression levels of VEGFA mRNA and protein in the anti-miR-203 group were significantly upregulated in comparison with the control group. Results indicated that VEGFA mRNA expression levels of VEGFA in the different groups was consistent with the protein expression levels. The luciferase reporter assay was conducted to evaluate the direct relationship between the VEGFA 3'-UTR and miR-203. miR-203 overexpression in HTR-8/SVneo cells significantly suppressed the luciferase activity of the wild-type reporter. This inhibition was eliminated when the target miR-203 was mutated (Fig. 5C).

Discussion

miRNAs are considered to be major contributors to gene expression regulation and various biological processes. It is well established that the proliferation, migration and invasion of trophoblast cells into the pregnant uterus nourishes the developing fetus. Abnormal trophoblast invasion or migration may lead to the development of PE (18,26). The mechanisms of PE development are unclear and are thought to be multifactorial. Following the discovery of a potential association between the dysregulation of placental miRNA expression and PE (27), other studies subsequently demonstrated the expression of numerous miRNAs in the placenta and a minimally overlapping pattern was identified (16). However, contradictory findings concerning miRNA regulation have also been identified (28,29). Thus, the stepwise mechanisms of miRNA promotion of PE development remain incompletely understood. It is essential to elucidate the precise contribution of miRNAs in PE development in order to clarify the pathogenesis of this disease. To the best of our knowledge, this is the first report to demonstrate the participation of miR-203 in PE development through the placenta. The data of the present study suggests that miR-203 has critical involvement in PE development through the downregulation of VEGFA expression and consequentially the proliferation, invasion and migration ability of trophoblast cells in the human placenta.

A previous study confirmed the involvement of miRNA clusters in the development of the placenta (16), particularly the miR-37-3 cluster. It has been reported that expression of

the miR-37-3 cluster decreases during the development of the placenta (30). The results of the present study also revealed a significant upregulation of miR-203 expression in PE placentas in comparison with the normal placenta samples, indicating that miR-203 upregulation may participate in the development of PE. Studies have demonstrated the involvement of miRNAs in the behaviors of endothelial and trophoblast cells via the regulation of the expression of proteins, including metallo-proteinase 2, ephrin-B and VEGFA (30,31). Thus, the present study investigated the expression of VEGFA in the PE placenta and demonstrated a significant downregulation of VEGFA expression in PE placental tissue in comparison with the normal placental tissue with RT-qPCR and IHC, suggesting that VEGFA may participate in PE, which is in line with the results of previous research (32).

The negative effects of miR-203 on HTR-8/SVneo cells was evaluated to further investigate the role of miR-203 in PE development. The results revealed that the overexpression or deletion of miR-203 markedly suppressed or promoted cell proliferation, migration and invasion, respectively. Similar results have been demonstrated in studies investigating miR-29b and miR-519d, which also contribute to PE development by affecting the proliferation, invasion and migration of trophoblast cells (26,33,34). Furthermore, several studies have also reported the ability of miR-203 to function as a tumor suppressor (35-38). Viticchiè et al (37) reported that miR-203 can influence cell proliferation, migration and invasion ability in prostate cancer. Notably, miR-203 has been demonstrated to suppress tumor growth and angiogenesis by targeting VEGFA (21,22). The present study demonstrated that VEGFA mRNA and protein expression levels were significantly reduced in HTR-8/SVneo cells transfected with miR-203 mimics and expression was increased in the anti-miR-203 group. A luciferase reporter assay confirmed the interaction between the 3'-UTR of VEGFA and miR-203. Taken together, it can be speculated that miR-203 participates in PE through the downregulation of VEGFA expression. Nevertheless, it is still necessary to delineate the numerous pathways that miR-203 may affect and to investigate these pathways to development possible therapeutic targets for the treatment of PE.

In summary, the results of the present study suggest that miR-203 suppressed trophoblast cell proliferation, migration and invasion through the downregulation of VEGFA expression in the placenta samples. A correlation between miR-203 and VEGFA was identified, which may contribute to the onset and/or progression of PE. The miR-203/VEGFA pathway may be an effective biomarker or a viable drug target for therapeutic intervention in PE.

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