Exosomal microRNA-302a promotes trophoblast migration and proliferation, and represses angiogenesis by regulating the expression levels of VEGFA in preeclampsia

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Received May 13, 2020; Accepted June 10, 2021

DOI: 10.3892/mmr.2021.12504

Abstract. The global morbidity rate of preeclampsia (PE) is 3-7, and 10-20% of maternal deaths are associated with PE. However, the mechanism of its pathogenesis remains unknown. The aim of the present study was to examine the relationship between microRNA-302a (miR-302a) and PE. Firstly, the relative expression levels of miR-302a in placental tissues from patients with PE and normal controls were analyzed using reverse transcription-quantitative PCR. miR-302a expression was upregulated in PE tissues, particularly in severe PE. Subsequently, HTR-8/SVneo cells were transfected with miR-302a vectors to overexpress miR-302a. The overexpression of miR-302a markedly promoted cell proliferation, colony formation, migration and invasion *in vitro*. Subsequently, the present study examined the function of exosomes secreted by HTR-8/SVneo cells transfected with miR-302a vectors. Compared with the negative control vector group, miR-302a expression was markedly increased in exosomes in the miR-302a overexpression group. Additionally, exosomes with miR-302a overexpression had repressed HUVEC invasion and ring formation. The luciferase reporter assay indicated that VEGFA was a direct target of miR-302a, and miR-302a expression was negatively correlated with VEGFA expression. In conclusion, the present results demonstrated that upregulation of miR-302a may promote HTR-8/SVneo cell proliferation, migration and invasion, and repress angiogenesis by targeting VEGFA, indicating that miR-302a may be a potential target for the development of PE therapies.

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Key words: preeclampsia, microRNA-302, exosomes, angiogenesis, VEGFA

Introduction

The global morbidity rate of preeclampsia (PE) is 3-7%. According to statistics, 10-20% of maternal deaths are associated with PE, particularly early onset PE that occurs before 34 weeks of pregnancy, which is a serious threat to maternal and child health (1). The etiology and pathogenesis of PE have always been important research topics in obstetrics. Over the years, through several studies (2-5) on PE, several pathogenesis hypotheses have been formulated, including: Vascular endothelial cell damage, insufficient remodeling of spiral uterine arteries, excessive activation of inflammatory immunity, genetic factors and insulin resistance. However, the mechanism of its pathogenesis is yet to be completely understood.

The invasion of trophoblasts is regulated by various intracellular and extracellular signaling pathways involving multiple processes, such as recognition and digestion of the extracellular matrix, and directional migration (6). Cytoskeleton dynamic remodeling is an important target for regulating the invasion and migration of trophoblasts (7).

The formation of placental blood vessels ensures that the placenta has sufficient blood perfusion and meets the growth requirements of the fetus. Abnormal formation of placental blood vessels can lead to a decrease in placental blood perfusion, ischemia and hypoxia in the uterine placenta, causing PE and growth restriction (2,8). Therefore, the successful completion of the placental vascular development process is the key to maintaining the pregnancy process and pregnancy outcome. During a normal pregnancy, the balance between pro-angiogenic factors and anti-angiogenic factors is maintained to promote placental vascularization and development (9). Moreover, during the process of normal placental angiogenesis, the formation of blood vessel branches gradually increases; however, in patients with PE, capillary network formation is impaired (10).

MicroRNAs (miRNAs/miRs) are a class of small non-coding RNAs with a length of ~21-25 nucleotides, that regulate target gene expression by complementary binding to specific sites in the

3' untranslated region (3'UTR) of target gene transcripts, which is an important mechanism of epigenetic regulation. It is now known that the placenta has its specific miRNA family cluster, and these miRNAs are involved in the functional regulation of the placenta (11,12). Furthermore, the dysregulation of miRNAs in the placenta of patients with PE during pregnancy is closely associated with the occurrence and development of this disease (13-15). Additionally, a variety of miRNAs (such as miR-125a-5p and miR-215-5p) are expressed in trophoblasts, and regulate cell proliferation, differentiation, invasion and apoptosis by targeting mRNAs (15-17). Numerous studies have also reported that miRNAs may be involved in vascular remodeling and immune function regulation at the maternal-fetal interface (18-21).

miR-302a is a member of the miR-302a/367 cluster, and has been investigated in various types of cancer, such as breast and cervical cancer and glioblastoma (22-27). To the best of our knowledge, however, few studies have focused on the role of miR-302a in the pathogenesis of PE. The present study aimed to assess the expression levels of miR-302a in patients with PE and matched normotensive women using reverse transcription-quantitative (RT-q)PCR. The biological function of miR-302a in the formation of PE was investigated by cell viability, colony formation, migration and invasion assays. Furthermore, the molecular regulation mechanism was also assessed by dual-luciferase reporter, RT-qPCR and western blot assay.

Materials and methods

Tissues. The placental samples from patients with PE (n=35; 16 severe and 19 non-severe) and normal pregnancy controls (n=20; age, 26-34 years) were collected from the Department of Obstetrics, Jinan City People's Hospital (Jinan, China) from January 2017 to December 2019.

Inclusion criteria were as follows: i) Patients at 28-40 weeks gestation; ii) blood pressure higher >140/90 mmHg and iii) proteinuria >0.3 g/24 h. Exclusion criteria were as follows: i) Patients in active labor and ii) patients with a history other clinical disorders, such as chronic hypertension or renal disease. Tissue blocks (~1 cm^3) were dissected from placental tissues, washed with sterile PBS and then immediately stored at -80˚C for further research. Written informed consent was obtained from all participants. The present study was approved by the Ethics Committee of Jinan City People's Hospital (approval no. KYLL-2017-276).

Cell culture and transfection. HTR-8/SVneo cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Wisent, Inc.) at 37˚C in a humidified 5% CO_2 incubator. Cells were transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 4-6 h, according to the manufacturer's protocols. Cells were transfected with 200 nM miR-302a mimic or miR-302a inhibitor, or 200 nM negative control (NC) (Shanghai GenePharma Co., Ltd.). Subsequent experiments were performed 24 h after transfection. The miRNA sequences were as follows: miR-302a mimic, 5'-UAAGUG CUUCCAUGUUUUGGUGA-3'; miR-NC 5'-UUCUCCGAA CGUGUCACGUTT-3'; miR-302a inhibitor 5'-CACCAAAAC ATGGAAGCACTT-3'; and miR inhibitor control (5'-TAA CACGTCTATACGCCCA-3').

RT-qPCR. Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The miRNA extraction procedures from exosomes were performed as described previously (28). The miRNA was reverse-transcribed into cDNA using One Step PrimeScript miRNA cDNA Synthesis kit (Takara Biotechnology Co., Ltd.) at 37˚C for 60 min followed by 5 min incubation at 85˚C. The mRNA was reverse-transcribed with PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd.) at 37˚C for 15 min and 85˚C for 5 sec. The relative expression levels of miRNAs and mRNA were assessed using the SYBR Premix Ex Taq™ II kit (Takara Biotechnology Co., Ltd.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec, 40 cycles of annealing at 95˚C for 5 sec and elongation and extension at 60˚C for 30 sec. Small nuclear RNA U6 expression was used as an endogenous control for miRNA, and GAPDH was used as an internal control for mRNAs. Relative expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (29). The primers used were as follows: VEGFA forward, 5'-AACTTTCTGCTG TCTTGGGT-3' and reverse, 5'-TCTCGATTGGATGGCAGT A-3'; GAPDH forward, 5'-GTCTCCTCTGACTTCAACAGC G-3' and reverse, 5'-ACCACCCTGTTGCTGTAGCCAA-3'; miR-302a forward, 5'-TAAGTGCTTCCATGTTTTGGTGA-3' and reverse, 5'-GAACATGTCTGCGTATCTCAGACTTC-3'; and U6 forward, 5'-GCTTCGGCAGCACATATACTAAAA T-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'.

Western blotting. Tissues and cells were homogenized in RIPA lysis bu-er (Beyotime Institute of Biotechnology) and the protein concentration was determined using a BCA protein quantitation kit. For each sample, 60 μg protein was separated by 10% SDS-PAGE and transferred to a 0.45-μm nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS for 1 h at room temperature. Following three washes with PBS, the membrane was incubated with the primary antibodies (dilution, 1:1,000-1:2,000) overnight at 4˚C. Following washing with PBS, the membranes were incubated with HRP-conjugated secondary antibodies (goat anti-rabbit IgG-HRP, 1:3,000, cat. no. M21002; goat anti-mouse IgG-HRP, 1:3,000, cat. no. M21001; both Abmart Pharmaceutical Technology Co., Ltd.) at room temperature for 1 h. The signal intensity was detected using an enhanced chemiluminescence detection system (PerkinElmer, Inc.) and Image Quant LAS 4000 (GE Healthcare Life Sciences). β-actin was used as an internal control. ImageJ 1.52a (National Institutes of Health) was used for densitometry.

The primary antibodies used were as follows: N-cadherin (cat. no. 13116; Cell Signaling Technology, Inc.), E-cadherin (cat. no. 3195; Cell Signaling Technology, Inc.), Vimentin (cat. no. 5741; Cell Signaling Technology, Inc.), snail family transcriptional repressor 1 (Snail; cat. no. 3879; Cell Signaling Technology, Inc.), β-actin (cat. no. 4970; Cell Signaling Technology, Inc.), VEGFA (cat. no. ab1316; Abcam), CD63 (cat. no. 55051; Cell Signaling Technology, Inc.), tumor susceptibility 101 (TSG101; cat. no. ab125011; Abcam), notch receptor 1 (Notch 1; cat. no. 3608; Cell Signaling Technology, Inc.), Notch 2 (cat. no. 5732; Cell Signaling Technology, Inc.), δ like canonical Notch ligand 4 (Dll4; cat. no. 2589; Cell Signaling Technology, Inc.), phosphorylated (P-)ERK1/2 (cat. no. 4370; Cell Signaling Technology, Inc.), ERK1/2

(cat. no. 4695; Cell Signaling Technology, Inc.), P-AKT (cat. no. 4060; Cell Signaling Technology, Inc.) and AKT (cat. no. 4691; Cell Signaling Technology, Inc.).

Dual-luciferase reporter assay. The TargetScan online tool (targetscan.org/vert_71/) was used to predict the potential target genes of miR-302a. The 3'UTR of VEGFA was cloned into the pGL3 vector (Promega Corporation). The mutation in the miRNA binding site was generated using RCR-based mutagenesis (Takara Biotechnology Co., Ltd.). The wild-type or mutant luciferase reporters (50 ng) and 0.5 pmol miR-302a mimics or negative control were co-transfected into HTR-8/SVneo cells using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C. Following 36 h transfection, the luciferase activities were measured using a dual-luciferase reporter system (Promega Corporation). *Renilla* luciferase activity was normalized to firefly luciferase activity.

Cell viability and colony formation assays. Cell viability was detected using a Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology) assay according to the manufacturer's instructions. Cells were seeded into 96-well plates at a density of $2x10^3$ cells/well and transfected with miR-302a mimics, inhibitor or NC for 0, 24, 48, 72 or 96 h. At the specific time points, 10 μl CCK-8 solution was added into each well and incubated for 2 h at 37˚C. Subsequently, the optical density value at 490 nm was detected using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Inc.).

For the colony formation assays, cells were seeded into 6-well plates at a density of 500 cells/well and incubated at 37˚C for 14 days. Subsequently, the cells were washed, fixed with 100% methanol at room temperature for 15 min, then stained with 1% crystal violet solution at room temperature for 15 min. Images were captured by camera without magnification.

Cell migration and invasion assays. Motility was assessed using a wound healing assay. Cells were seeded onto 12-well plates and incubated with normal medium with 10% FBS. A wound was created on the surface of the plates using a 10-100-μl sterile micropipette tip. Subsequently, cells were washed with PBS and incubated in serum-free medium at 37˚C for 48 h. Images were captured by light microscope (magnification, x200; Olympus Corporation). The distances were measured using ImageJ 1.52a software (National Institutes of Health).

Cell migratory and invasive abilities were measured using Transwell assays. Transwell chambers (8-μm pores; BD Biosciences) were used. Cells were added to the top chamber of the inserts, and 600 μl medium with 20% FBS was added to the lower chamber. For migration assay, the chambers were not coated with Matrigel (BD Biosciences). However, the chambers were coated with Matrigel at 37˚C for 30 min in invasion assays. Following incubation at 37˚C for 24 h, cells in the upper chamber were removed with a cotton swab, and cells on the lower surface of the chamber were fixed with 100% methanol at room temperature for 15 min and stained with 1% crystal violet solution at room temperature for 15 min. The cells were counted under a light microscope (magnification, x200; Olympus Corporation).

Vascular ring formation. Matrigel (BD Biosciences) was added to each well of a 24-well plate and allowed to solidify at 37˚C for 30 min. Subsequently, HUVECs (American Type Culture Collection) were transferred to the wells at a density of $1x10^5$ cells/well. Following cell incubation at 37˚C for 24 h in a humidified chamber with 5% CO_2 in air, the formation of capillary-like structures was examined under a light microscope (magnification, x200). For each well, three fields in the central area were selected randomly.

Isolation of exosomes and purification. The isolation and purification of exosomes were performed as described previously by Thery *et al* (30). Exosomes were isolated from supernatant of the HTR-8/SVneo cells via differential centrifugation. The cells were initially removed by centrifugation at 300 x g, and other debris were removed at 3,000 x g. The supernatant was centrifuged at 10,000 x g to remove shedding vesicles and other vesicles with larger sizes. Finally, the supernatant was centrifuged at 110,000 x g for 70 min. All ultracentrifugation steps were performed at 4˚C. Exosomes were collected from the pellet and resuspended in PBS. The exosomes were visualized by transmission electron microscopy (magnification, x400; JEOL, Ltd.) as previously reported (31).

Isolation of exosomes was performed using exosome isolation reagent for plasma or serum (cat. no. C10110-2; Guangzhou RiboBio Co., Ltd.), according to the manufacturer's protocol.

Chemical assays. For RNase assay, the exosome pellet was suspended with PBS (500 μl) and 10 μg/ml RNase A (Omega Bio-Tek, Inc.) was added. The mixture was incubated at 37˚C for 1 h.

The exosome inhibitor GW4869 (10 μM; Sigma-Aldrich; Merck KGaA) was used to inhibit exosome secretion. GW4869 were pre-incubated with trophoblast cells at 37˚C for 1 h before the secretion assay.

Statistical analysis. All data are presented as the mean ± SD of at least three independent experiments. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.). Differences were analyzed using a paired Student's t-test for two group comparisons. The association between miR-302a expression and VEGFA expression was analyzed via Pearson's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-302a expression is increased in patients with PE. Placental samples were collected from 35 patients with PE and 20 matched normotensive women. The basic clinical characteristics of the patients in the PE and control groups are presented in Table I. Patients with PE exhibited a significant increase in the systolic pressure and diastolic blood pressure compared with patients in the control group. Proteinuria was also identified in the PE group. Moreover, the gestational age at delivery of patients with PE was significantly lower compared with that of patients in the control group.

The relative expression levels of miR-302a in placental tissues from patients with PE (n=35) and normal controls (n=20)

Table I. Clinical characteristics of preeclamptic and normal pregnancies.

Characteristics	Normal	Preeclampsia	P-value
Maternal age, years	29±2.67	30±3.22	0.245
Systolic blood pressure, mmHg	117±10	134±13	<0.0001
Diastolic blood pressure, mmHg	68±6	89±8	<0.0001
Proteinuria	0	1.94±0.42	<0.0001
BMI, kg/m^2	29.1±4.3	29.6±5.2	0.7171
Birth weight, g	3564±537	3018±452	0.002
Gestational age at delivery, weeks	39.2±1.8	37.1±1.5	<0.0001

Data are presented as the mean ± SD.

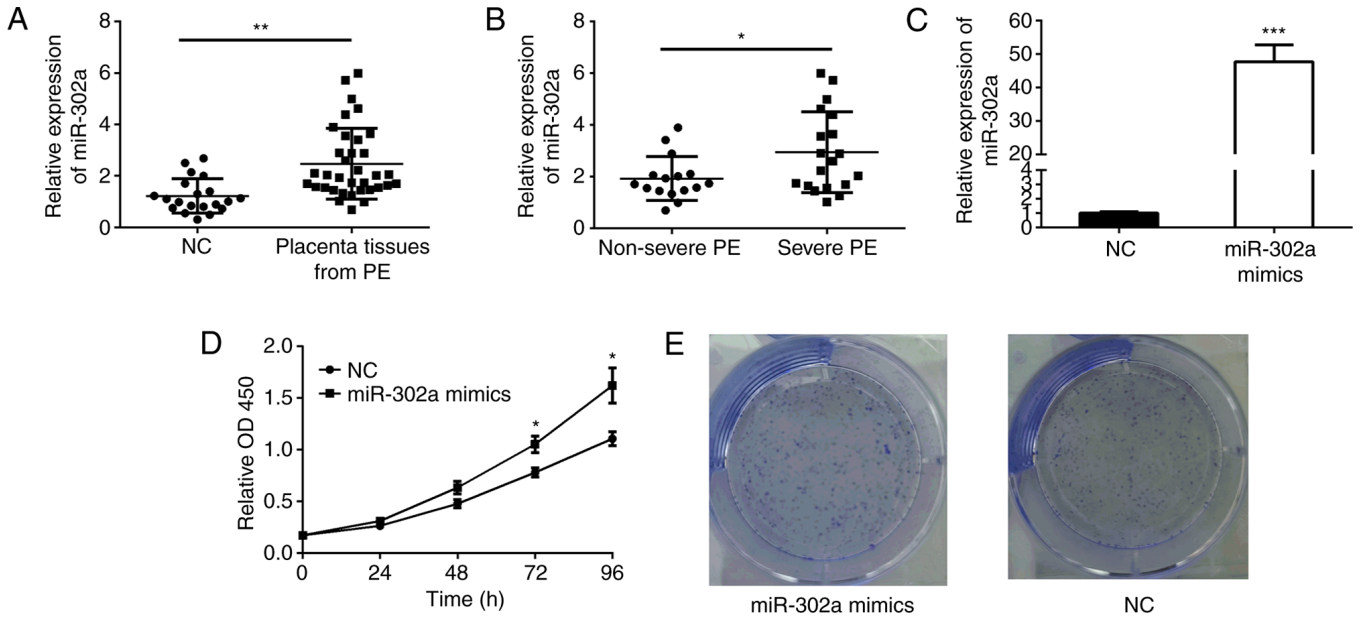

Figure 1. miR-302a expression in PE and the effect of miR-302a on cell proliferation. (A) Relative expression levels of miR-302a in the PE group (n=35) and the control group (n=20). (B) Relative expression levels of miR-302a in the severe PE group (n=16) and the non-severe PE group (n=19). (C) Relative expression levels of miR-302a in the miR-302a mimic and NC groups. (D) Cell Counting Kit-8 assay results. (E) Colony formation assay results. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. NC. miR-302a, microRNA-302a; PE, preeclampsia; OD, optical density; NC, negative control.

were detected using RT-qPCR. The results demonstrated that miR-302a expression was upregulated in PE (Fig. 1A). Subsequently, the present study compared the relative expression levels of miR-302a in placental tissues from patients with severe PE (n=16) and non-severe PE (n=19), and revealed that the expression of miR-302a was higher in patients with severe compared with non-severe PE (Fig. 1B).

miR-302a overexpression promotes cell proliferation. The miR-302a mimics were stably transfected into HTR-8/SVneo cells. The overexpression of miR-302a was confirmed by RT-qPCR, and the relative expression levels of miR-302a were increased in the miR-302a mimics group compared with in the NC group (Fig. 1C). The CCK-8 assay demonstrated that the relative cell viability in the miR-302a mimics group was higher than that in the NC group (Fig. 1D). Similarly, in a colony formation assay, the overexpression of miR-302a markedly promoted colony formation (Fig. 1E).

miR-302a overexpression promotes cell migration and invasion. A Transwell assay was used to detect the effect of miR-302a on cell migration and invasion. The results revealed that overexpression of miR-302a increased the invasive and migratory capacity compared with that in the NC group (Fig. 2A and B). A wound healing assay was used to investigate the effect of miR-302a on the motility of cells. An increased migratory rate was observed in the miR-302a overexpression group (Fig. 2C). Additionally, these results could be confirmed by investigating the expression levels of epithelial-mesenchymal transition markers. Western blot analysis results demonstrated that overexpression of miR-302a could downregulate the expression levels of the epithelial marker E-cadherin (0.602-fold change), and upregulate the expression levels of mesenchymal markers, including N-cadherin (1.675-fold change), Vimentin (1.634-fold change), Snail (1.473-fold change), p-AKT/AKT (0.868-fold change; Fig. 2D).

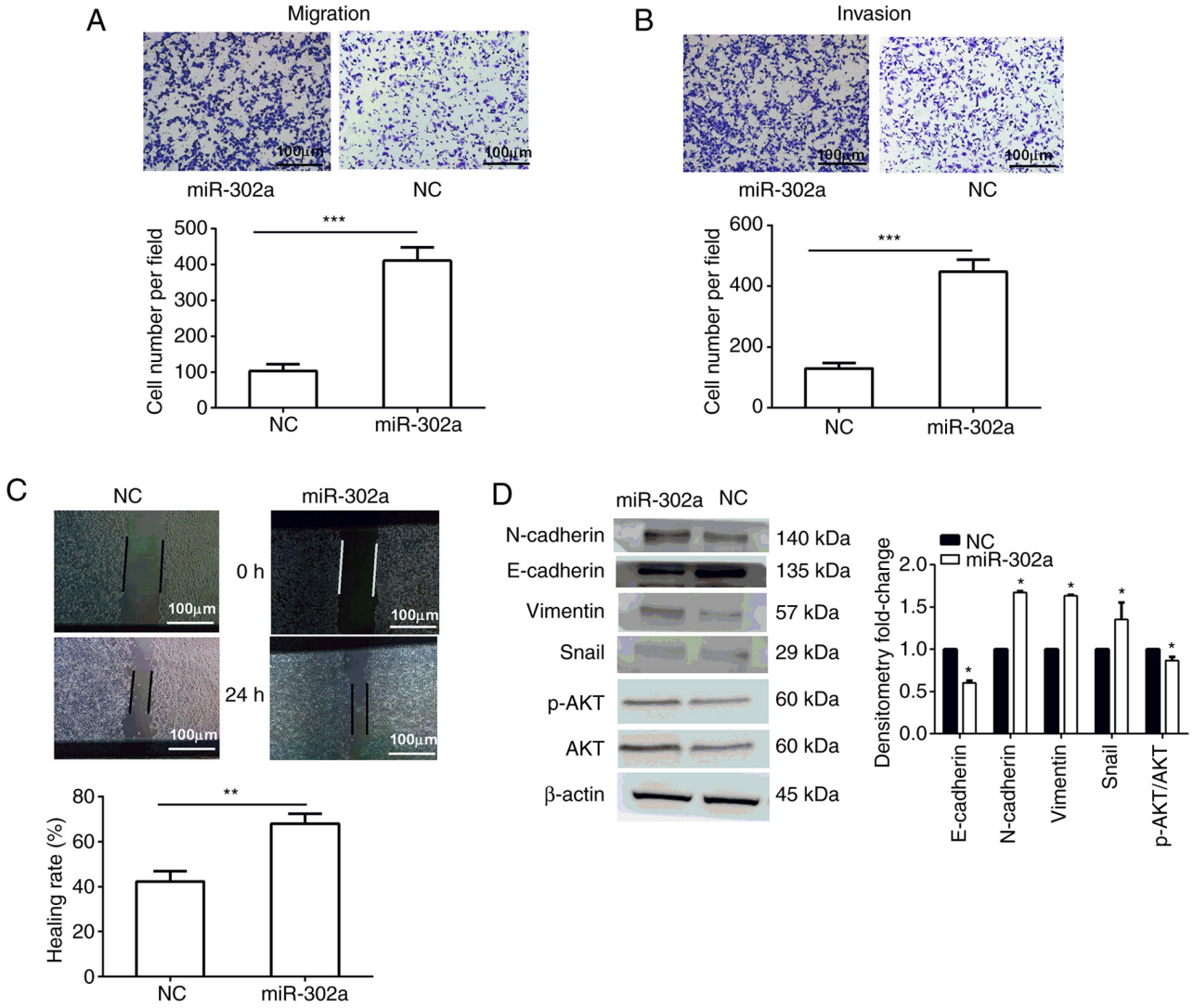

Figure 2. Role of miR-302a in cell migration and invasion. (A) Effect of miR-302a on invasion, as determined by a Transwell assay. (B) Effect of miR-302a on migration, as determined by a Transwell assay. (C) Cell healing rate detected by a wound healing assay. Magnification, x200; scale bar, 100 μm. (D) Western blot analysis of epithelial-mesenchymal transition markers and AKT signaling pathway markers. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. NC. miR-302a, microRNA-302a; NC, negative control; P-, phosphorylated; Snail, snail family transcriptional repressor 1.

miR-302a is carried by exosomes. Exosomes were isolated from serum via ultracentrifugation. The exosomes were identified by electron microscopy according to their typical morphology (Fig. 3A). Additionally, specific protein markers (TSG101 and CD63) were used to verify that these vesicles were exosomes (Fig. 3B). To determine whether miR-302a was indeed present in exosomes, RNase A (10 μg/ml) or PBS buffer was used to treat exosomes. As shown in Fig. 3C, no significant difference was identified between the RNase A and PBS groups. Subsequently, the present study detected the relative expression levels of miR-302a in exosomes. The results of RT-qPCR demonstrated that the relative expression levels of miR-302a in exosomes from the PE group were significantly higher compared with those in exosomes from the NC group (Fig. 3D).

The exosome inhibitor GW4869 (10 μM) was applied to trophoblast cells to inhibit exosome secretion. The results indicated that the expression level of miR-302a in trophoblast cells was increased, but not significantly (P>0.05). Furthermore, the migratory and proliferative abilities were not markedly promoted (Fig. S1).

Role of exosomal miR-302a in angiogenesis. Exosomes were obtained from the medium of HTR-8/SVneo cells transfected with miR-302a or control. The HUVECs were cultured using these exosomes and their biological function was evaluated. It was identified that labeled miR-302a could be transferred via exosomes to medium, and then transferred to HUVECs via exosomes (Fig. 4A). Compared with the control group, exosomes with miR-302a overexpression decreased the invasive ability of HUVECs, as evaluated by a Transwell assay (Fig. 4B), and decreased the ring formation ability, as determined by an endothelial tube formation assay (Fig. 4C). Furthermore, several protein markers of angiogenesis, including VEGFA (0.302-fold change), Notch 1 (0.693-fold change), Notch 2 (0.697-fold change), Dll4 (0.574-fold change), P-ERK1/2 (0.439-fold change) and ERK1/2 (0.51-fold change), were detected using western blotting (Fig. 4D). The results demonstrated that overexpression of miR-302a downregulated the expression levels of these protein markers. These results indicated that miR-302a carried by exosomes inhibited angiogenesis.

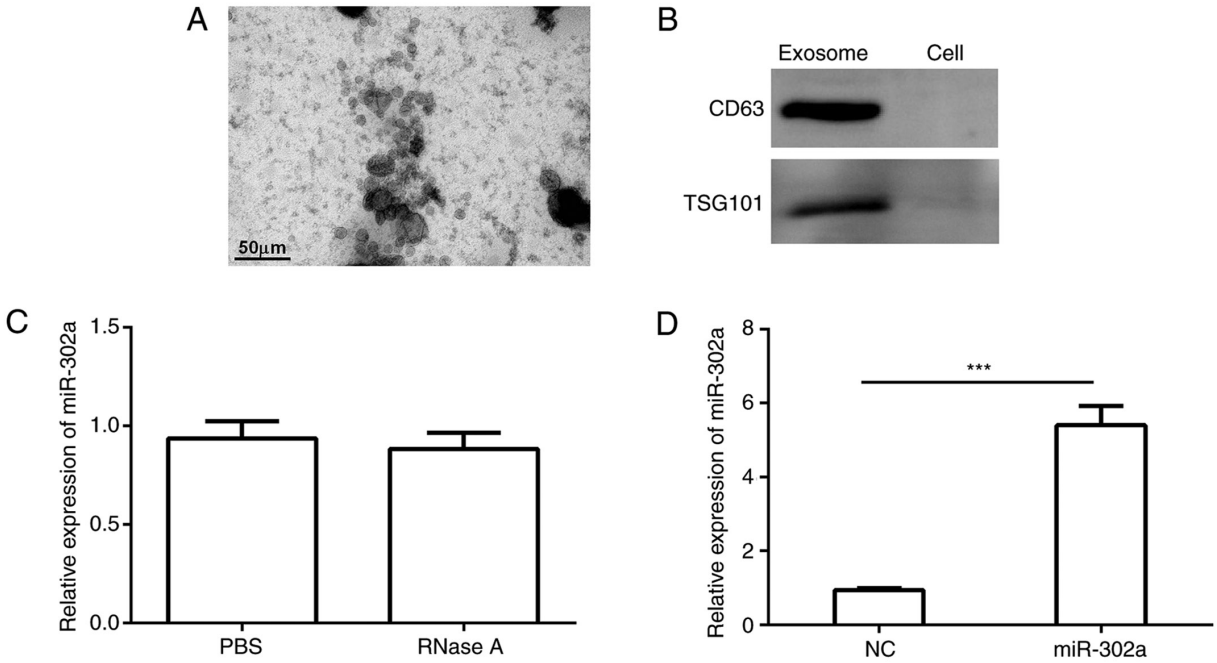

Figure 3. miR-302a is carried by exosomes. (A) Scanning electron microscopy of exosomes isolated from human serum (magnification, x400). Scale bar, 50 μm. (B) Western blot analysis of exosome markers TSG101 and CD63. (C) Relative expression levels of miR-302a in the exosomes treated with PBS or RNase A (10 μg/ml). (D) Relative expression levels of miR-302a in exosomes from the supernatant of HTR-8/SVneo cells transfected with miR mimic and NC groups. Data are presented as the mean ± SD (n=3). ***P<0.001. miR-302a, microRNA-302a; TSG101, tumor susceptibility 101; NC, negative control.

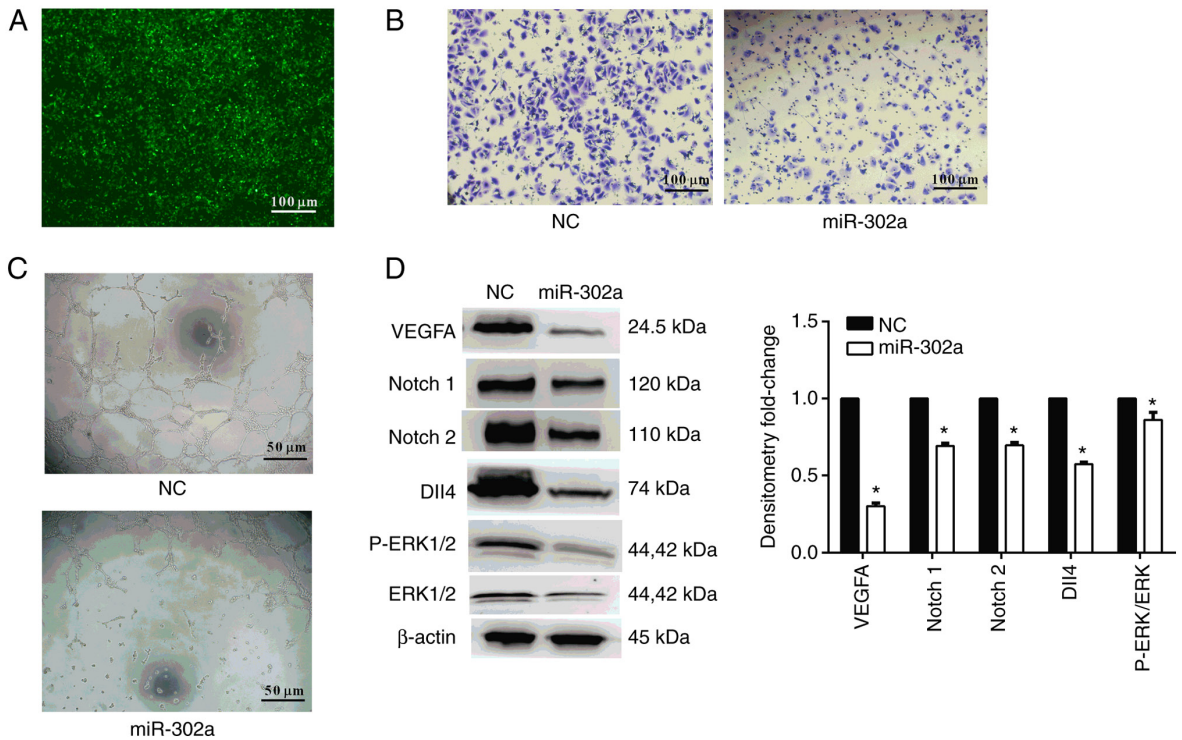

Figure 4. Effect of exosomal miR-302a on angiogenesis. (A) Fluorescence microscopy was used to detect the signals of miR-302a in HUVECs. HTR-8/SVneo cells were transfected with labeled miR-302a, and then the HUVECs were cultured with Exos isolated from HTR-8/SVneo medium (magnification, x100). Scale bar, 100 μm. (B) Effect of exosomal miR-302a on HUVEC invasion detected by a Transwell assay (magnification, x200). Scale bar, 100 μm. (C) Effect of exosomal miR-302a on angiogenesis detected by a ring formation assay (magnification, x400). Scale bar, 50 μm. (D) Western blot analysis of protein markers of angiogenesis. Data are presented as the mean ± SD (n=3). *P<0.05 vs. NC. miR-302a, microRNA-302a; NC, negative control; Notch, notch receptor; Dll4, δ like canonical Notch ligand 4; P-, phosphorylated; Exo, exosome.

miR-302a directly targets and inhibits VEGFA expression. The putative targets of miR-302a were predicted using bioinformatics tools, including TargetScan 7.1. VEGFA was found to be a putative target of miR-302a, and the binding

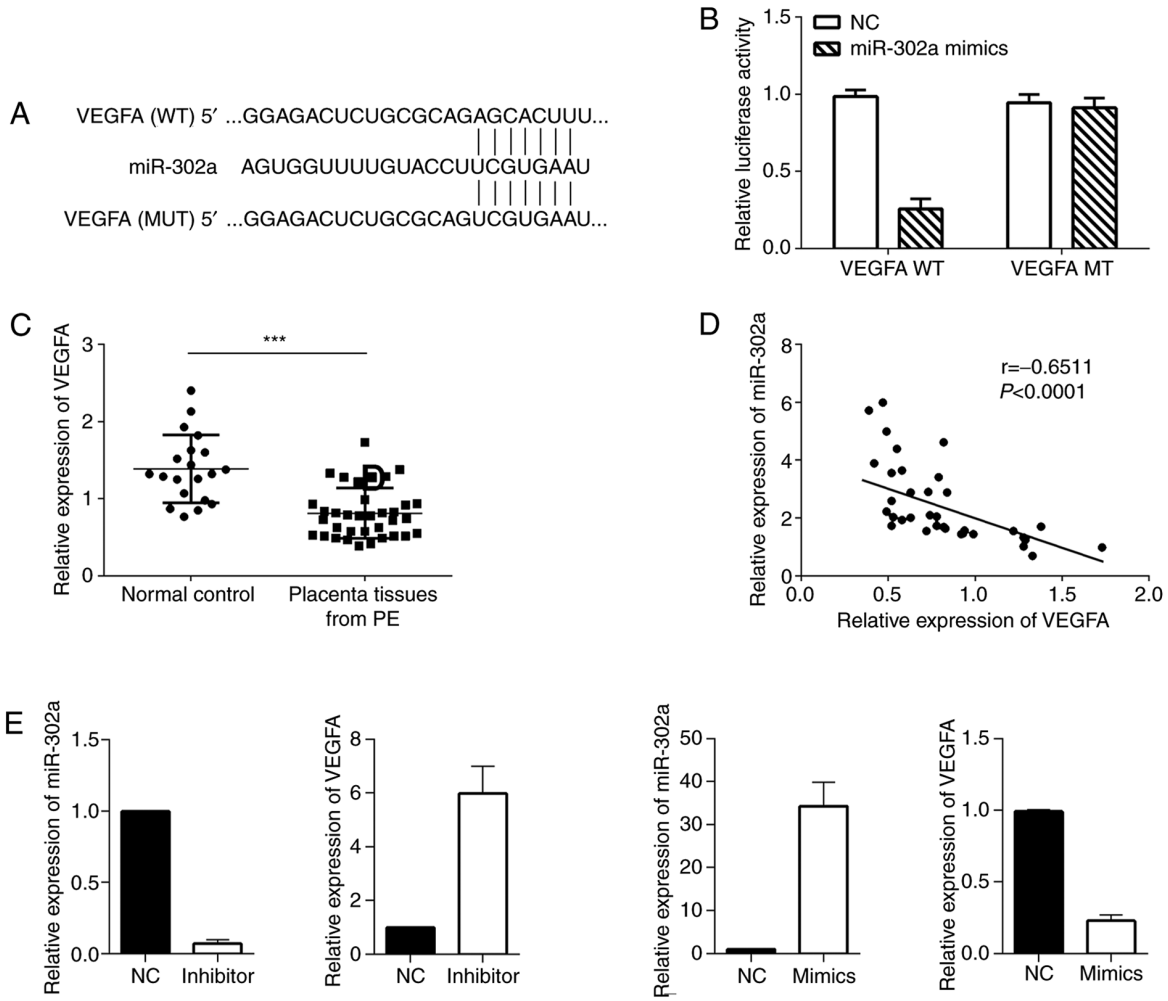

Figure 5. VEGFA is a target of miR-302a. (A) Putative binding site in VEGFA mRNA. (B) Regulatory relationship between VEGFA and miR-302a was verified using a dual-luciferase reporter assay. (C) Relative expression levels of VEGFA in placenta tissues from the PE and control groups. (D) Relative expression levels of miR-302a were negatively correlated with VEGFA expression. miR-302a, microRNA-302a; VEGFA, vascular endothelial growth factor A. (E) The relative expression levels of VEGFA were increased in the miR-302a inhibitor group and decreased in the mimics group. Data are presented as the mean ± SD (n=3). ***P<0.001. PE, preeclampsia; NC, negative control; WT, wild-type; MT, mutant; miR-302a, microRNA-302a-3p.

site was identified in the 3'UTR of VEGFA mRNA (Fig. 5A). Subsequently, a luciferase assay was used to determine the interaction between miR-302a and VEGFA 3'UTR. The 3'UTR of VEGFA or the corresponding mutant segments were cloned into pGL3 vectors and then transfected into cells to detect the differences in luciferase values. The results demonstrated that the luciferase activity was markedly reduced in cells transfected with wild-type 3'UTR of VEGFA, and no obvious reduction was observed in cells transfected with mutant 3'UTR of VEGFA (Fig. 5B). These results suggested that miR-302a could directly bind to the 3'UTR of VEGFA.

Subsequently, the relative expression levels of VEGFA were detected in placental tissues from patients with PE. Compared with that in the control group, VEGFA expression was significantly decreased in the PE group (Fig. 5C). Next, the relationship between the expression levels of VEGFA and miR-302a was analyzed. The results revealed that VEGFA expression was moderately correlated with miR-302a expression (Pearson r=-0.6511; P<0.0001; Fig. 5D). To verify the negative regulatory effect of miR-302a on VEGFA, miR-302a inhibitor or its control were co-transfected with wild-type or mutant luciferase reporters; similarly, miR-302a mimics or its control were co-transfected into cells with the wild-type or mutant luciferase reporters; It was found that VEGFA expression was increased in the miR-302a-3p inhibitor group but decreased in the miR-302a-3p mimic group (Fig. 5E).

Discussion

Placenta formation and development are important processes for maintaining normal pregnancy, and normal placental blood vessel formation is a key link between them (32). Abnormal formation of placental blood vessels can lead to a variety of pregnancy complications, including PE (33). Blood vessel formation at the maternal-fetal interface is an important process in normal pregnancy (34). The interaction of various regulatory factors and trophoblasts induces angiogenesis of the placenta, and then completes the remodeling of the uterine circumflex artery, increases placental blood flow perfusion and finally forms a highly vascularized organ to ensure the oxygen supply of the maternal fetus (35,36). The placenta is rich in angiogenesis-related factors and receptors, and the

main regulatory factors include VEGFs, fibroblast growth factors and angiopoietin, which interact with cells via paracrine and autocrine mechanisms to jointly regulate vascular development and recasting (37-39).

VEGF is a disulfide homodimeric glycoprotein, 40-45 kDa in weight, which has a certain chemotactic effect on endothelial cells, and is involved in the process of angiogenesis (40,41). Moreover, its expression is regulated by multiple cytokines, such as IFN-γ, TNF-α and IL-1β (42,43). The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor. According to alternative splicing, VEGF-A can be divided into several subtypes, such as VEGF121, VEGF165 and VEGF189 (44). VEGF is a pro-angiogenic factor that induces angiogenesis. The mechanism by which VEGF induces angiogenesis is accomplished by acting on VEGF receptors on the surface of vascular endothelial cells, promoting the proliferation and migration of endothelial cells and increasing vascular permeability (45). Therefore, VEGF is also referred to as vascular permeability factor. VEGF is highly expressed in villous trophoblasts and interstitial villi during the early stages of pregnancy, and its expression levels are relatively low in syncytiotrophoblast and no obvious expression is observed in villous endothelial cells (46,47). The receptors of VEGF include VEGFR-1, VEGFR-2 and VEGFR-3, the first two of which are tyrosine kinase receptors expressed in vascular endothelial cells and trophoblasts (48). VEGF interacts with VEGFR-1 to promote the proliferation of endothelial cells, whereas it mainly interacts with VEGFR-2 to mediate most of the other endothelial cell functions. VEGF has anti-apoptotic effects and is an important protein in VEGF signaling. VEGF promotes angiogenesis mainly by activating MEK1/2-ERK1/2 signaling pathway phosphorylation and mediating cell proliferation (49). Alterations in the levels of the angiogenesis-related factor VEGF in the maternal blood circulation lead to angiogenesis dysfunction, which is an important mechanism for the onset of PE (50).

miR-302a is a member of the miR-302a/367 cluster, which is highly expressed in embryonic stem cells (51). miR-302a has been identified as a tumor suppressor in several types of cancer, including breast cancer, glioblastoma and cervical cancer. For example, Ahmadalizadeh Khanehsar *et al* (22) reported that overexpression of the miR-302a/367 cluster inhibited the proliferation of breast cancer cells by suppressing the S-phase of the cell cycle. Yang *et al* (23) also revealed that the expression of miR-302a/367 cluster suppressed tumorigenic gene expression patterns and abolished transformation-related phenotypes in glioblastoma cells. In cervical cancer, miR-302a has been identified to inhibit cell migration and invasion by targeting defective in cullin neddylation 1 domain containing 1 (24). However, in several types of cancer, miR-302a has been identified as an oncogene. Kim *et al* (25) reported that miR-302a promotes proliferation of human mesenchymal stem cells, while Liu *et al* (52) proposed that miR-302b promoted the proliferation of gastric cancer cells by targeting CDK2, thereby inhibiting the ERK signaling pathway. In prostate cancer, miR-302a expression is upregulated, and its forced expression accelerates the proliferation of prostate cancer cells (26). In the present study, miR-302a expression was found to be upregulated in placenta cells in PE, and overexpression of miR-302a markedly promoted cell proliferation, migration and invasion.

Exosomes are a class of microvesicles, 30-150 nm in diameter, and can be involved in intercellular communication by releasing intracellular cargos, such as miRNAs, mRNAs, long non-coding RNAs and proteins, into the extracellular environment (27). Almost all cancer cells can generate exosomes (53). Emerging evidence has suggested that exosome-mediated miRNAs are involved in regulating cancer development. It has been shown that exosomal transfer of miR-126 promotes the antitumor response in malignant mesothelioma (54). In gastric cancer, exosomal transfer of miR-501 confers doxorubicin resistance and tumorigenesis via targeting of BH3-like motif containing, cell death inducer (55). Li *et al* (56) observed that seven miRNAs (including miR-153-3p and miR-325-3p) derived from exosomes were differentially expressed in women with PE. Additionally, exosomal encapsulation of miR-125a-5p has been identified to inhibit trophoblast cell migration and proliferation by regulating VEGFA expression (15). The present study identified that miR-302a overexpression in HTR-8/SVneo cells contributed to exosomal miR-302a upregulation. Additionally, exosomal miR-302a promoted the angiopoiesis of endothelial cells (HUVECs). The present study successfully constructed HTR-8/SVneo cells stably overexpressing miR-302a and obtained exosomes that were rich in miR-302a. Exosomes could be released from HTR-8/SVneo cells stably overexpressing miR-302a to serum, which further influenced the biological function of HUVECs. The present study demonstrated that exosomes with miR-302a overexpression decreased the invasive ability and inhibited angiogenesis in HUVECs.

The current study performed the aforementioned experiments only in one cell line and the findings were not verified *in vivo*. Moreover, the differential expression of miR-302a was not verified in the peripheral blood between patients with PE and normal pregnant women. Moreover, proliferation and migration belong to different biological functions compared with angiogenesis. The current study detected that miR-302a promoted the migration and proliferation of trophoblast cells but suppressed angiogenesis in HUVECs. The present study focused on the effect of VEGFA on the angiogenesis of HUVECs. Future studies will further examine the potential pathways of how miR-302 targeted VEGFA to affect PE and to identify other potential pathways via which miR-302 promotes the migration and proliferation of trophoblasts *in vitro* and *in vivo*.

In conclusion, the results of the present study demonstrated that miR-302a functioned as an oncogene. It was found that miR-302a promoted the proliferation, migration and invasion of placenta cells. Furthermore, it was demonstrated that miR-302a could be released by cells via exosomes, and exosomal miR-302a repressed the angiogenesis of HUVECs. A luciferase reporter assay indicated that VEGFA was a direct target of miR-302a. Additionally, miR-302a expression was negatively correlated with VEGFA expression. Therefore, it was suggested that miR-302a may regulate the pathogenesis of PE via VEGFA.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

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

Authors' contributions

This study was conceived and designed by LX. The data was collected, analyzed and interpreted by MW, YZ, LL and GW. MW, YZ and GW wrote the manuscript. LX and MW confirm the authenticity of all the raw data. LX inspected the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all participants. The present study was approved by the Ethics Committee of Jinan City People's Hospital (approval no. KYLL-2017-276).

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

References

1. Ding Z, Liang J, Lu Y, Yu Q, Songyang Z, Lin SY and Mills GB: A retrovirus-based protein complementation assay screen reveals functional AKT1-binding partners. Proc Natl Acad Sci USA 103: 15014-15019, 2006.
2. Peng M, Yang M, Ding Y, Yu L, Deng Y, Lai W and Hu Y: Mechanism of endogenous digitalis-like factorinduced vascular endothelial cell damage in patients with severe preeclampsia. Int J Mol Med 41: 985-994, 2018.
3. Varberg KM and Soares MJ: Paradigms for investigating invasive trophoblast cell development and contributions to uterine spiral artery remodeling. Placenta: May 3, 2021 (Epub ahead of print).
4. Miko E, Meggyes M, Bogar B, Schmitz N, Barakonyi A, Varnagy A, Farkas B, Tamas P, Bodis J, Szekeres-Bartho J, *et al*: Involvement of Galectin-9/TIM-3 pathway in the systemic inflammatory response in early-onset preeclampsia. PLoS One 8: e71811, 2013.
5. Anim-Nyame N, Gamble J, Sooranna SR, Johnson MR and Steer PJ: Relationship between insulin resistance and tissue blood flow in preeclampsia. J Hypertens 33: 1057-1063, 2015.
6. Knofler M and Pollheimer J: IFPA award in placentology lecture: Molecular regulation of human trophoblast invasion. Placenta 33 (Suppl 2): S55-S62, 2012.
7. Burke SD, Zsengeller ZK, Khankin EV, Lo AS, Rajakumar A, DuPont JJ, McCurley A, Moss ME, Zhang D, Clark CD, *et al*: Soluble fms-like tyrosine kinase 1 promotes angiotensin II sensitivity in preeclampsia. J Clin Invest 126: 2561-2574, 2016.
8. Merviel P, Carbillon L, Challier JC, Rabreau M, Beaufils M and Uzan S: Pathophysiology of preeclampsia: Links with implantation disorders. Eur J Obstet Gynecol Reprod Biol 115: 134-147, 2004.
9. Hong K, Park HJ and Cha D: Clinical implications of placenta-derived angiogenic/anti-angiogenic biomarkers in pre-eclampsia. Biomark Med 15: 523-536, 2021.
10. Grisaru-Granovsky S, Maoz M, Barzilay O, Yin YJ, Prus D and Bar-Shavit R: Protease activated receptor-1, PAR1, promotes placenta trophoblast invasion and beta-catenin stabilization. J Cell Physiol 218: 512-521, 2009.
11. Vaiman D: Genes, epigenetics and miRNA regulation in the placenta. Placenta 52: 127-133, 2017.
12. Morales-Prieto DM, Ospina-Prieto S, Chaiwangyen W, Schoenleben M and Markert UR: Pregnancy-associated miRNA-clusters. J Reprod Immunol 97: 51-61, 2013.
13. Jairajpuri DS, Malalla ZH, Mahmood N and Almawi WY: Circulating microRNA expression as predictor of preeclampsia and its severity. Gene 627: 543-548, 2017.
14. Ding J, Huang F, Wu G, Han T, Xu F, Weng D, Wu C, Zhang X, Yao Y and Zhu X: MiR-519d-3p suppresses invasion and migration of trophoblast cells via targeting MMP-2. PLoS One 10: e0120321, 2015.
15. Xueya Z, Yamei L, Sha C, Dan C, Hong S, Xingyu Y and Weiwei C: Exosomal encapsulation of miR-125a-5p inhibited trophoblast cell migration and proliferation by regulating the expression of VEGFA in preeclampsia. Biochem Biophys Res Commun 525: 646-653, 2020.
16. Brooks SA, Martin E, Smeester L, Grace MR, Boggess K and Fry RC: miRNAs as common regulators of the transforming growth factor (TGF)-β pathway in the preeclamptic placenta and cadmium-treated trophoblasts: Links between the environment, the epigenome and preeclampsia. Food Chem Toxicol 98: 50-57, 2016.
17. Yang X and Meng T: miR-215-5p decreases migration and invasion of trophoblast cells through regulating CDC6 in preeclampsia. Cell Biochem Funct 38: 472-479, 2020.
18. Tamaru S, Mizuno Y, Tochigi H, Kajihara T, Okazaki Y, Okagaki R, Kamei Y, Ishihara O and Itakura A: MicroRNA-135b suppresses extravillous trophoblast-derived HTR-8/SVneo cell invasion by directly down regulating CXCL12 under low oxygen conditions. Biochem Biophys Res Commun 461: 421-426, 2015.
19. Pankiewicz K, Fijalkowska A, Issat T and Maciejewski TM: Insight into the key points of preeclampsia pathophysiology: Uterine artery remodeling and the role of microRNAs. Int J Mol Sci 22: 3132, 2021.
20. Lv Y, Lu C, Ji X, Miao Z, Long W, Ding H and Lv M: Roles of microRNAs in preeclampsia. J Cell Physiol 234: 1052-1061, 2019.
21. Chen J, Zhao L, Wang D, Xu Y, Gao H, Tan W and Wang C: Contribution of regulatory T cells to immune tolerance and association of microRNA210 and Foxp3 in preeclampsia. Mol Med Rep 19: 1150-1158, 2019.
22. Ahmadalizadeh Khanehsar M, Hoseinbeyki M, Fakhr Taha M and Javeri A. Repression of TGF-β signaling in breast cancer cells by miR-302/367 cluster. Cell J 21: 444-450, 2020.
23. Yang CM, Chiba T, Brill B, Delis N, von Manstein V, Vafaizadeh V, Oellerich T and Groner B: Expression of the miR-302/367 cluster in glioblastoma cells suppresses tumorigenic gene expression patterns and abolishes transformation related phenotypes. Int J Cancer 137: 2296-2309, 2015.
24. Jiang Y, Hou R, Li S, Li S and Dang G: MicroRNA-302 inhibits cell migration and invasion in cervical cancer by targeting DCUN1D1. Exp Ther Med 16: 1000-1008, 2018.
25. Kim JY, Shin KK, Lee AL, Kim YS, Park HJ, Park YK, Bae YC and Jung JS: MicroRNA-302 induces proliferation and inhibits oxidant-induced cell death in human adipose tissue-derived mesenchymal stem cells. Cell Death Dis 5: e1385, 2014.
26. Guo Y, Cui J, Ji Z, Cheng C, Zhang K, Zhang C, Chu M, Zhao Q, Yu Z, Zhang Y, *et al*: miR-302/367/LATS2/YAP pathway is essential for prostate tumor-propagating cells and promotes the development of castration resistance. Oncogene 36: 6336-6347, 2017.
27. Cheng L, Sharples RA, Scicluna BJ and Hill AF: Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. J Extracell Vesicles: 3, 2014 doi: 10.3402/jev.v3.23743.
28. Melo SA, Sugimoto H, O'connell JT, Kato N, Villanueva A, Vidal A, Qiu L, Vitkin E, Perelman LT, Melo CA, *et al*: Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. Cancer Cell 26: 707-721, 2014.
29. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
30. Thery C, Amigorena S, Raposo G and Clayton A: Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 3: 22, 2006.
31. Zhu Q, Li Q, Niu X, Zhang G, Ling X, Zhang J, Wang Y and Deng Z: Extracellular vesicles secreted by human urine-derived stem cells promote ischemia repair in a mouse model of hind-limb ischemia. Cell Physiol Biochem 47: 1181-1192, 2018.

32. Liu F, Wu W, Wu K, Chen Y, Wu H, Wang H and Zhang W: MiR-203 participates in human placental angiogenesis by inhibiting VEGFA and VEGFR2 expression. Reprod Sci 25: 358-365, 2018.
33. Rada CC, Murray G and England SK: The SK3 channel promotes placental vascularization by enhancing secretion of angiogenic factors. Am J Physiol Endocrinol Metab 307: E935-E943, 2014.
34. Moser G, Guettler J, Forstner D and Gauster M: Maternal platelets-friend or foe of the human placenta? Int J Mol Sci 20: 5639, 2019.
35. Pereira RD, De Long NE, Wang RC, Yazdi FT, Holloway AC and Raha S: Angiogenesis in the placenta: The role of reactive oxygen species signaling. Biomed Res Int 2015: 814543, 2015.
36. Reynolds LP and Redmer DA: Angiogenesis in the placenta. Biol Reprod 64: 1033-1040, 2001.
37. Bogic LV, Brace RA and Cheung CY: Developmental expression of vascular endothelial growth factor (VEGF) receptors and VEGF binding in ovine placenta and fetal membranes. Placenta 22: 265-275, 2001.
38. Devi HL, Kumar S, Konyak YY, Bharati J, Bhimte A, Pandey Y, Kumar K, Paul A, Kala A, Samad HA, *et al*: Expression and functional role of fibroblast growth factors (FGF) in placenta during different stages of pregnancy in water buffalo (Bubalus bubalis). Theriogenology 143: 98-112, 2020.
39. Tian KW, Zhang YY, Jiang H and Han S: Author correction: Intravenous C16 and angiopoietin-1 improve the efficacy of placenta-derived mesenchymal stem cell therapy for EAE. Sci Rep 10: 8097, 2020.
40. Ferrara N, Gerber HP and Lecouter J: The biology of VEGF and its receptors. Nat Med 9: 669-676, 2003.
41. Jussila L and Alitalo K: Vascular growth factors and lymphangiogenesis. Physiol Rev 82: 673-700, 2002.
42. George J, Shmilovich H, Deutsch V, Miller H, Keren G and Roth A: Comparative analysis of methods for assessment of circulating endothelial progenitor cells. Tissue Eng 12: 331-335, 2006.
43. Nagineni CN, William A, Cherukuri A, Samuel W, Hooks JJ and Detrick B: Inflammatory cytokines regulate secretion of VEGF and chemokines by human conjunctival fibroblasts: Role in dysfunctional tear syndrome. Cytokine 78: 16-19, 2016.
44. Holmes DI and Zachary I: The vascular endothelial growth factor (VEGF) family: Angiogenic factors in health and disease. Genome Biol 6: 209, 2005.
45. Ferrara N: Vascular endothelial growth factor: Basic science and clinical progress. Endocr Rev 25: 581-611, 2004.
46. An H J, Kim JH, Ahn EH, Kim YR, Kim JO, Park HS, Ryu CS, Kim EG, Cho SH, Lee WS and Kim NK: 3'-UTR polymorphisms in the vascular endothelial growth factor gene (VEGF) contribute to susceptibility to recurrent pregnancy loss (RPL). Int J Mol Sci 20: 3319, 2019.
47. Coultas L, Chawengsaksophak K and Rossant J: Endothelial cells and VEGF in vascular development. Nature 438: 937-945, 2005.
48. Nevo O, Lee DK and Caniggia I: Attenuation of VEGFR-2 expression by sFlt-1 and low oxygen in human placenta. PLoS One 8: e81176, 2013.
49. Wang K, Jiang YZ, Chen DB and Zheng J: Hypoxia enhances FGF2- and VEGF-stimulated human placental artery endothelial cell proliferation: Roles of MEK1/2/ERK1/2 and PI3K/AKT1 pathways. Placenta 30: 1045-1051, 2009.
50. Song C, Xie S, Wang J, Lian J, Diao B and Tang Y: Association of angiotensinogen gene polymorphisms and angiogenic factors with preeclampsia in Chinese women. Gynecol Obstet Invest 76: 64-68, 2013.
51. Zhang Z, Hong Y, Xiang D, Zhu P, Wu E, Li W, Mosenson J and Wu WS: MicroRNA-302/367 cluster governs hESC self-renewal by dually regulating cell cycle and apoptosis pathways. Stem Cell Rep 4: 645-657, 2015.
52. Liu FY, Wang LP, Wang Q, Han P, Zhuang WP, Li MJ and Yuan H: miR-302b regulates cell cycles by targeting CDK2 via ERK signaling pathway in gastric cancer. Cancer Med 5: 2302-2313, 2016.
53. Thery C, Zitvogel L and Amigorena S: Exosomes: Composition, biogenesis and function. Nat Rev Immunol 2: 569-579, 2002.
54. Monaco F, Gaetani S, Alessandrini F, Tagliabracci A, Bracci M, Valentino M, Neuzil J, Amati M, Bovenzi M, Tomasetti M and Santarelli L: Exosomal transfer of miR-126 promotes the anti-tumour response in malignant mesothelioma: Role of miR-126 in cancer-stroma communication. Cancer Lett 463: 27-36, 2019.
55. Liu X, Lu Y, Xu Y, Hou S, Huang J, Wang B, Zhao J, Xia S, Fan S, Yu X, *et al*: Exosomal transfer of miR-501 confers doxorubicin resistance and tumorigenesis via targeting of BLID in gastric cancer. Cancer Lett 459: 122-134, 2019.
56. Li H, Ouyang Y, Sadovsky E, Parks WT, Chu T and Sadovsky Y: Unique microRNA signals in plasma exosomes from pregnancies complicated by preeclampsia. Hypertension 75: 762-771, 2020.