miR-424-5p is downregulated in the placentas of patients with preeclampsia and affects trophoblast migration and invasion

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Received January 13, 2022; Accepted August 18, 2022

DOI: 10.3892/etm.2023.11993

Abstract. Insufficient invasion of trophoblast cells has been reported to be closely associated with the pathogenesis of preeclampsia (PE). MicroRNAs (miRs) have essential roles in the trophoblasts invasion via targeting specific genes with diverse functions. However, the underlying mechanism remains largely unclear and requires further investigation. The present study aimed to identify and evaluate the potential functions of miRs in trophoblasts invasion and to reveal the underlying mechanisms. In the present study, differentially expressed miRs that were screened based on previously published microarray data (GSE96985) and a significantly downregulated miR-424-5p (miR-424) was chosen for further investigation. Subsequently, reverse transcription-quantitative PCR, CCK-8, apoptosis, wound healing and Transwell assays were performed to determine the cell viability, apoptotic rate, cell migration and invasion of trophoblast cells. The results showed that miR-424 was decreased in placenta specimens from patients with PE. Upregulation of miR-424 promoted cell viability, suppressed cell apoptosis and improved the invasion and migration of trophoblasts, whereas inhibition of miR-424 had opposite results. Adenomatous polyposis coli (APC), a key mediator of Wnt/β-catenin signaling pathway, was identified as a functional target of miR-424 and an inverse relationship was observed between APC and miR-424 in placenta specimens. Further investigations revealed that APC overexpression efficiently suppressed the effect of miR-424 in trophoblast cells. In addition, the miR-424-mediated effects on trophoblast cells were dependent on the promotion of Wnt/β-catenin signaling pathway. The present findings revealed that miR-424 regulates the trophoblast cell invasion by regulating Wnt/β-catenin pathway through targeting APC, indicating miR-424 as a potential candidate for the treatment of PE.

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Abbreviations: PE, preeclampsia; GEO, Gene Expression Omnibus; APC, adenomatous polyposis coli; miR, microRNA; Wt, wild-type; Mut, mutant-type

Key words: preeclampsia, trophoblastic dysfunction, miR-424, adenomatous polyposis coli, Wnt/β-catenin signaling

Introduction

Preeclampsia (PE) is a pregnancy-associated disorder, characterized by elevated maternal blood pressure and proteinuria after 20 weeks of gestation (1). To date, it remains the major cause of fetal and maternal mortality and morbidity worldwide (2). Although considerable efforts in recent years have been made in the understanding of PE, little improvement in disease diagnosis and treatment for PE remains unsatisfactory (3,4). Although previous studies indicate that trophoblastic dysfunction, including insufficient trophoblast migration and invasion, is critical for the pathogenesis of PE, the underlying regulatory mechanisms of trophoblast invasion remain unclear (5,6).

MicroRNAs (miRs) are a group of non-coding small RNAs (21-23 nucleotides), which can regulate gene expression through binding with target mRNA (7). Several reports have demonstrated that miRs play the key roles in the regulation of trophoblastic invasion (8,9). For example, Liu *et al* (10) showed that the miR-142-3p expression levels is upregulated in placenta specimens obtained from patients with PE and its upregulation inhibited trophoblast cell invasion and migration through disruption of the TGF-β/SMAD3 signaling pathway. Gao *et al* (11) found that miR-299 suppresses the invasion and migration of trophoblast cells partly via targeting histone deacetylase 2. Notably, a recent study showed that low expression of miR-424 in placenta is associated with severe PE (12). Another report also found that miR-424 is downregulated in primary human trophoblasts by hypoxia and associated with hindered trophoblast differentiation (13). However, these studies did not investigate the effects and underlying regulatory mechanisms of miR-424 on trophoblastic invasion. In addition, miR-424 has been reported to serve as an oncogene in several types of human cancer and is demonstrated to contribute to the migration and invasion of tumor cells (14). miR-424 expression levels were found to be increased in laryngeal squamous cell carcinoma (LSCC) tissues and its upregulation promoted migration and invasion of LSCC

cells (15). Wu *et al* (16) demonstrated that miR-424 increases the migration and invasion in pancreatic cancer cells. Based on the evidence from these previous studies, it is reasonable that miR-424 may affect trophoblastic invasion.

In the present study, the differentially expressed miRs that were screened based on GSE96985 microarray data retrieved from Gene Expression Omnibus (GEO) and miR-424 was selected for further analysis. The regulatory mechanisms and effects of miR-424 on the invasion and migration of HTR-8/SVneo cells were further investigated via *in vitro* experiments. The present findings suggested that miR-424 may be a potential therapeutic target against PE.

Materials and methods

Clinical samples. The placenta samples were collected from 60 pregnant women undergoing cesarean section (30 patients with PE and 30 healthy pregnant women with uncomplicated pregnancies) in the Department of Gynecology and Obstetrics, the Obstetrics & Gynecology Hospital of Fudan University. Patients' main clinical parameters are reported and summarized in Table I. Hypertension (≥140/90 mmHg) and proteinuria (≥300 mg protein/24 h-urine sample) occurring after 20-week gestation were the necessary inclusion criteria for patients with PE. Patients with cardiovascular diseases, diabetes, metabolic syndrome, infections, kidney disease, congenital malformations and chromosomal anomalies (number and/or structure) were excluded. All samples were immediately frozen in liquid nitrogen and stored at -80˚C until further use. The present study was approved by the Research Ethics Committee of the Obstetrics & Gynecology Hospital of Fudan University (approval no. 2018-013). Written informed consent was obtained from each participant.

Cell culture. Human trophoblast cell line HTR-8/SVneo was obtained from Cell Culture Center of the Shanghai Institute (Shanghai, China), which was shown to contain a heterogeneous population of both trophoblast and mesenchymal cells (17,18). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Sigma-Aldrich; Merck KGaA) in a humidified incubator with 5% CO_2 at 37˚C.

Immunofluorescent staining. Prior to immunofluorescent staining, the cells were fixed with 4% paraformaldehyde for 15 min at 4˚C, followed by incubation with 5% FBS containing 0.5% Triton X-100 for 5 min and finally blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) in PBS at 37˚C for 60 min. Subsequently, the cells were incubated at 4˚C overnight with primary antibodies against cytokeratin 7 (CK7; 1:4,000; cat no. ab9021; Abcam) and vimentin (1:1,000; cat no. sc7557; Santa Cruz Biotechnology, Inc.). Subsequently, the cells were washed and incubated with either Alexa Fluor 488 (1:200; cat no. ab150077; Abcam) or 568 (1:200; cat no. ab175473; Abcam) conjugated goat anti-mouse and goat anti-rabbit IgG in blocking buffer for 60 min at room temperature. The cells were mounted on slides with mounting buffer containing DAPI. Immunofluorescence was detected using a fluorescence microscope (Olympus Corporation).

miR microarray. The gene expression profiles of PE associated miRs were retrieved from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE96985, which was conducted through GPL20712 platforms (Agilent-070156 Human miRNA) and included 3 PE placenta tissues and 3 normal placenta tissue samples (19). The 'limma' package in R software (version 4.2) was applied to perform variance analysis on miRs expression in the microarray. |log Fold Change (FC)|>2 and P<0.05 were regarded as the criteria for screening the differentially expressed-miRs (DE-miRs). A heatmap of the obtained DE-miRs was constructed using the 'pheatmap' package.

Reverse transcription-quantitative PCR analysis. Total RNA was extracted from placenta tissues (100 mg) or cultured cells ($1x10^7$ cells) using a mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Inc.) as per manufacturer's instructions. The concentration and quality of total RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Reverse transcription of miRs (1 μg) was performed using the TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) with gDNA eraser kit (Takara Bio, Inc.) and miRs expression levels were measured using TaqMan MicroRNA Assay kit (Thermo Fisher Scientific, Inc.) on the ABI PRISM 7300 system (Thermo Fisher Scientific, Inc.). For detection of adenomatous polyposis coli (APC) mRNA, 1 μg RNA was reverse-transcribed into cDNA using a Reverse Transcription kit with gDNA eraser kit (Takara Bio, Inc.). Then a SYBR Premix Ex Taq II (Takara Bio, Inc.) was used for qPCR. U6 and GAPDH were used as internal controls for miR-424-5p and APC, respectively. The qPCR thermocycling conditions were: Initial denaturation at 95˚C for 30 sec; 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. The relative expression of each gene was calculated using the $2^{-\Delta\Delta Cq}$ method (20). The following primer pairs were used for the qPCR: MiR-424 (MIMAT0001341; https://www.mirbase.org/) forward, 5'-CGACCAGCAGCAATTCATGT-3' and universal anti-sense, 5'-GCAGGGTCCGAGGTATTC-3'; miR-142-3p (MI0000434) forward, 5'-GGACGTGTAGTGTTTCCTACT-3'; miR-219a (MIMAT0004567) forward, 5'-GCTAGAGTTGAGTCTGGAC-3'; miR-15a-5p (MIMAT0000068) forward, 5'-GCGACCTAGCAGCACATAATGG-3'; miR-132 (MIMAT0000426) forward, 5'-GCGATAACAGTCTACAGCCA-3'; miR-483 (MIMAT0002173) forward, 5'-ATAGTCACTCCTCTCCTCCC-3'; miR-145-5p (MIMAT0000437) forward, 5'-AGCTCGTCCAGTTTTCCCAGG-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; APC (RefSeq NM_001127511.3) forward, 5'-CGCTTCTGTACCACCCTCAG-3' and reverse, 5-ACCGCAGTTTTACTCCAGGG-3'; GAPDH (RefSeq NM_002046.3) forward, 5'-TCAACGACCCCTTCATTGACC-3' and reverse, 5'-CTTCCCGTTGATGACAAGCTTC-3'.

Cell transfection. miR-424-5p mimics (sense 5'-CAGCAGCAAUUCAUGUUUUGAA-3' and anti-sense, 5'-CAAAACAUGAAUUGCUGCUGUU-3'), miR mimics negative controls (NC; sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and anti-sense, 5'-ACGUGACACGUUCGGAGAATT-3'), miR-424-5p inhibitor (5'-UUCAAAACAUGAAUUGCUGCUG-3') and inhibitor NC

Table I. Clinical characteristics of patients with preeclampsia and normal pregnant women.

Characteristics	Control, n=30	GDM, n=30	P-value
Age, years	30.37±5.45	31.02±5.83	0.657
MAP, mm Hg	91.62±8.34	134.21±10.47	<0.0001
Urinary protein, g/24 h	-	2.4±0.2	<0.0001
Uric acid. μmol/l	251.46±34.63	396.76±39.03	<0.0001
Pregnancy, weeks	38.34±1.73	37.82±1.14	0.175
Birth weight, kg	3.41±0.37	2.83±0.43	<0.0001
BMI, kg/m^2	23.12±1.43	22.54±1.66	0.153

Data are presented as means ± standard error of the mean. MAP, mean artery pressure; BMI, body mass index.

(5'-CAGUACUUUUGUGUAGUACAA-3') were synthesized by Shanghai GenePharma Co. Ltd.. To overexpress APC, the full-length 3'UTR of human APC gene was amplified by PCR using human cDNA isolated from placenta tissues and inserted into pcDNA 3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). The APC targeted small interfering RNAs (si-APC, 5'-AGUUCUUCUAAAUAUCCAGUA-3') and si-Scramble (5'-CCAAUUGGUUACUAUAUAUCA-3') were synthesized and purified by Guangzhou RiboBio Co., Ltd. Cells (1.0x10^6/well) were seeded and grown overnight in six-well plates. When the cell confluence reached 80%, Lipofectamine® 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transient transfection of the cells with miR-424-5p mimics (50 nM), miR-424-5p inhibitor (100 nM), NC (100 nM), 2 μg plasmids or 200 nM si-RNA at 37˚C according to the manufacturer's guidelines, and cell samples were collected 48 h later for subsequent experimental studies, such as western blot and RT-qPCR assays.

Cell viability. Transfected HTR-8/SVneo cells were trypsinized to prepare a single cell suspension, which was seeded in a 96-well plate at a density of 5x10^3 cells per well. After 48 h incubation at 37˚C, 10 μl CCK-8 solutions (CCK-8; Dojindo Laboratories, Inc.), were added to each well and the cells were incubated for further 2 h at 37˚C, then the absorbance was detected at 450 nm using a microplate reader (Model 680; Bio-Rad Laboratories, Inc.).

The activity of caspase 3. Caspase-3 activity was measured using a Caspase-3 Activity kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The optical density at 405 nm was then detected using a microplate reader (Model 680; Bio-Rad Laboratories, Inc.).

Transwell assay. Cell invasion was measured using a Transwell assay (Costar; Corning, Inc.). In brief, 24 h after transfection, 100 μl of serum-free medium containing 2x10^4 transfected HTR-8/SVneo cells were added into the upper chamber precoated with Matrigel (Becton, Dickinson and Company) at 37˚C for 24 h, while the complete medium was added to the lower well at 37˚C. After 24 h incubation at 37˚C, the cells at the bottom were fixed in 4% formaldehyde solution for 15 min at room temperature and then stained with 0.1% crystal violet for another 20 min at room temperature. The cell numbers were counted from six random fields in each well at magnification x400 under an inverted microscope (Ts2r-FL; Nikon Corporation).

Wound healing assay. When transfected HTR-8/SVneo cells grown up to ~100% confluence, the cells were starved for 6 h and wounded with a sterile 100-μl pipette tip and then cells were cultured in serum-free medium. Images were taken after 24 h of scratching at magnification x200 under an inverted microscope (Ts2r-FL; Nikon Corporation) and the scratch width was calculated using ImageJ software (National Institutes of Health). The equation for calculation of the percentage of wound closure is given below.

Wound closure (%)=((W0-Wt)/W0) x100, where W0 represents the wound area at 0 h and Wt represents the wound area at 24 h (21).

Luciferase reporter assay. Luciferase reporters were generated based on the Firefly luciferase expressing vector (pmirGLO; Promega Corporation). The 3'-untranslated region (3'-UTR) of the human APC gene and its mutant of the theoretical miR-424 binding site were cloned into the pmirGLO vector to form the reporter vector, named wild-type (Wt) and mutant-type (Mut) of APC 3'UTR, respectively. To construct pmirGLO-APC-3'-UTR, a partial 3'-UTR of the APC segment of human APC mRNA containing the putative miR-424 binding sites was amplified and cloned into the vector pmirGLO. The human APC mRNA was extracted from placenta tissues and reverse-transcribed to cDNA using a Reverse Transcription Kit with gDNA eraser kit (Takara Bio, Inc.). Mutations within potential miR-424 binding sites were introduced using the QuikChange Site-Directed Mutagenesis kit (Life Technologies; Thermo Fisher Scientific, Inc.). When the HTR-8/SVneo cells grew to 60-70% confluency, the cells were co-transfected with 100 ng luciferase plasmid and 50 ng *Renilla* luciferase plasmid along with 100 nM miR-424 mimics/inhibitor or miR-NC using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection at 37˚C for 48 h, the firefly luciferase activity was measured by dual-luciferase assays kit (Promega Corporation) according to the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Western blot. Total proteins were extracted from placental tissues (100 mg) and cultured cells (1x10^7 cells) using ice

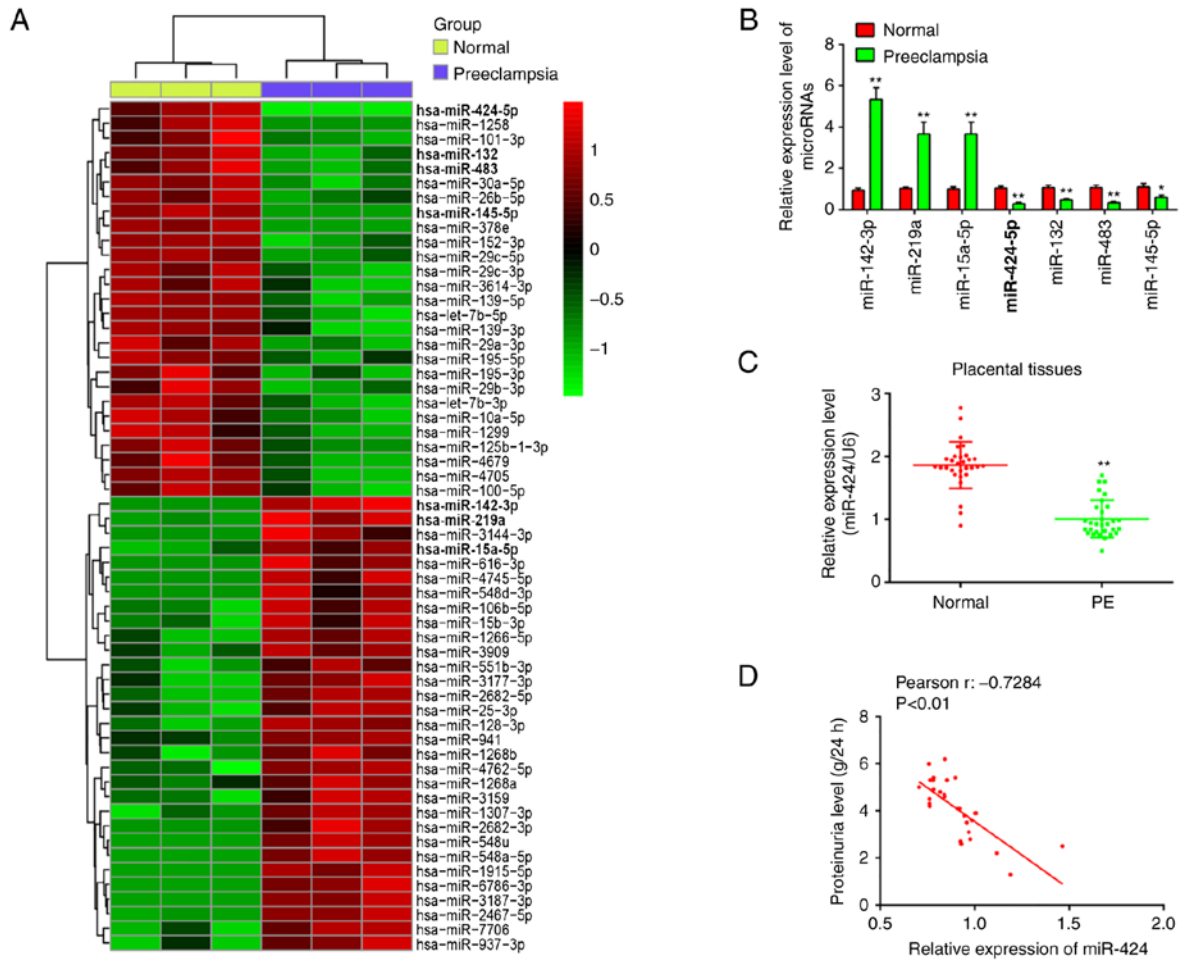

Figure 1. miR-424 is significantly downregulated in placental tissues. (A) Differentially expressed miRs between PE and normal group. Data are retrieved from Gene Expression Omnibus dataset, accession no. GSE96985. The color code in the heat map is linear and the upregulated miRs are shown in green to red, whereas the downregulated miRs are shown from red to green. (B) miR-142-3p, miR-219a, miR-15a-5p, miR-424, miR-132, miR-483 and miR-145-5p were further analyzed using RT-qPCR. (C) miR-424 expression was determined in placental tissues from PE patients (n=30) and healthy pregnant women (n=30) using RT-qPCR. (D) The positive correlation between miR-424 expression and proteinuria level in PE pregnancies (r=-0.7284; P<0.01). Data are presented as means ± standard deviation of three individual experiments. *P<0.05 and **P<0.01 vs. control. miRs, microRNA; RT-qPCR, reverse transcription-quantitative PCR; PE, preeclampsia.

cold RIPA lysis buffer (Beyotime Institute of Biotechnology) with a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) and the protein concentration was measured using a BCA kit (Beyotime Institute of Biotechnology). Proteins (40 μg/lane) were separated via SDS-PAGE on a 10% gels (Beyotime Institute of Biotechnology) and transferred onto a PVDF membrane (MilliporeSigma). The PVDF membrane was blocked with 1% BSA (Sigma-Aldrich; Merck KGaA) for 2 h at room temperature, followed by incubation overnight at 4˚C with primary antibodies including rabbit monoclonal anti-human APC (1:1,000; cat. no. 2504; Cell Signaling Technology, Inc.), rabbit monoclonal anti-human β-catenin (1:1,000; cat. no. 8480; Cell Signaling Technology), rabbit monoclonal anti-human MMP-9 (1:1,000; cat. no. 13667; Cell Signaling Technology), rabbit monoclonal anti-human MMP2 (1:1,000; cat. no. 40994; Cell Signaling Technology), the antibodies against rabbit polyclonal anti-human Wnt1 (1:1,000; cat. no. ab15251; Abcam) and rabbit polyclonal anti-human β-actin (1:2,000; cat. no. ab8227; Abcam). Following primary antibody incubation, the membrane was incubated with goat polyclonal secondary antibody to rabbit IgG-H&L Alexa Fluor® 488 (1:2,000; cat. no. ab150081; Abcam) for 1 h at room temperature.

Protein bands were visualized using a chemiluminescence detection system (MilliporeSigma) and protein expression was quantified using Quantity One software (version 4.6.6, Bio-Rad Laboratories, Inc.). All experiments were conducted in triplicate.

Statistical analysis. All statistical data were analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc.). All experiments were performed in triplicate and data are expressed as the mean ± standard deviation. Statistical analysis was performed using unpaired Student's t-test or one-way ANOVA followed by Tukey's post hoc test. Pearson's method was used in correlation test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-424 is downregulated in placenta specimens from patients with PE. To identify unique miRs involved in PE, the microarray data of GSE96985 downloaded from the GEO

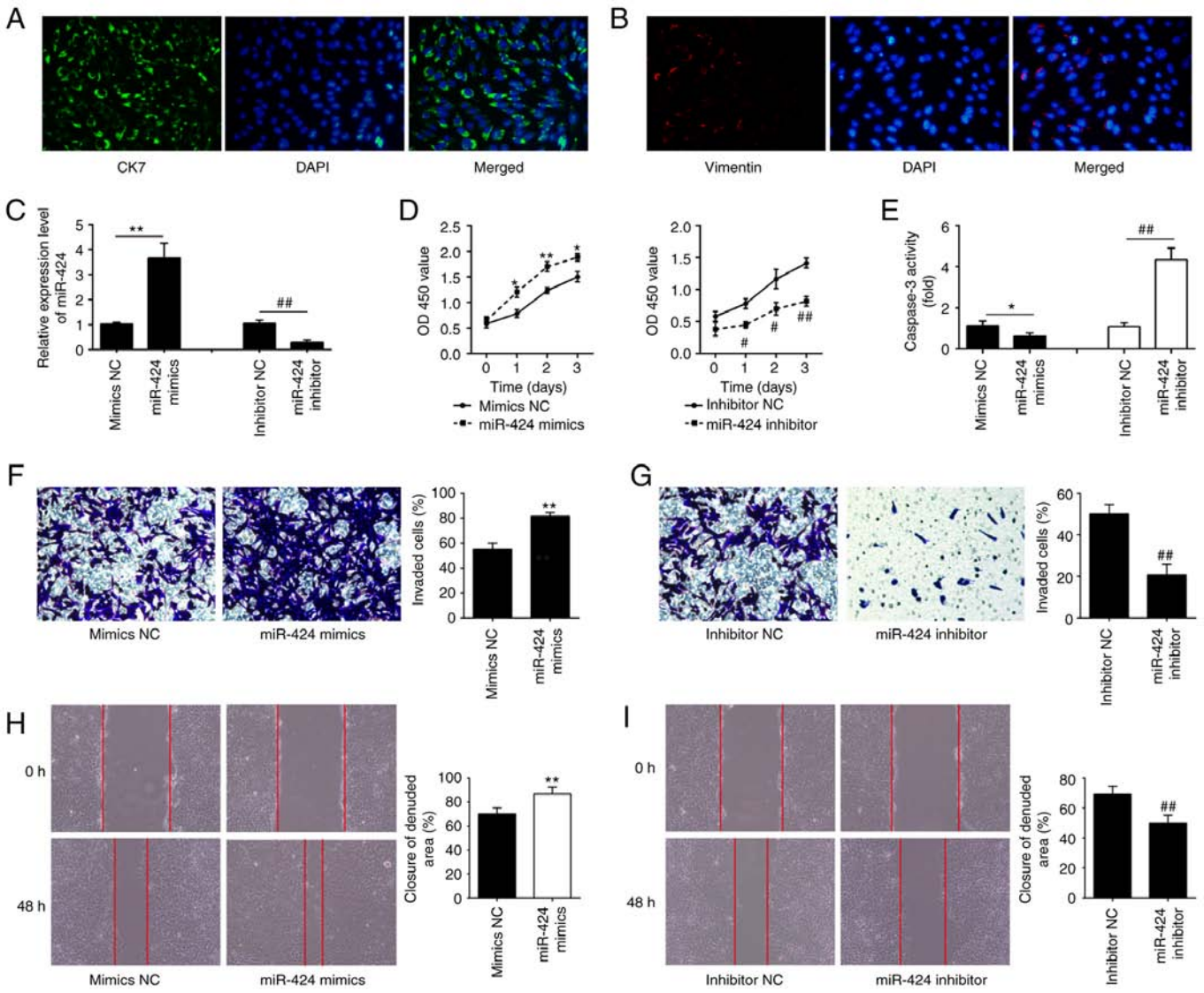

Figure 2. miR-424 improves the migratory and invasive abilities of HTR-8/SVneo cells. The HTR-8/SVneo cells were transfected with miR-424 mimics/inhibitor or corresponding NC for 48 h and then cells were harvested for subsequent experiments. (A) The levels of CK7 and (B) vimentin were measured via immunofluorescence analysis. Magnification, 200x. (C) miR-424 expression was detected using reverse transcription-quantitative PCR. (D) Cell viability was measured using CCK-8 assay. (E) The activity of caspase 3 was measured using a Caspase-3 Activity kit. (F and G) Cell invasion was assessed using Transwell invasion assay. Magnification, x400. (H and I) Cell migration was measured through wound healing assay. Magnification, x200. Data are presented as means ± standard deviation of three individual experiments. *P<0.05 and **P<0.01 vs. mimics NC. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. inhibitor NC. miRs, microRNA; NC, negative control, CK7, cytokeratin 7.

was first analyzed and the DE-miRs were screened using R language software (version 4.2; https://www.r-project.org/). In total, there were 58 differentially expressed miRs, including 31 upregulated and 27 downregulated in PE group in comparison with the normal group (Fig. 1A). Subsequently, three upregulated (miR-142-3p, miR-219a and miR-15a-5p) and four downregulated miRs (miR-424, miR-132, miR-483 and miR-145-5p) were selected to verify the results of this microarray data. miR-142-3p, miR-219a and miR-15a-5p were significantly upregulated, while miR-424, miR-132, miR-483 and miR-145-5p were downregulated in PE group compared with normal group (Fig. 1B). These results are in line with previous reports (12,22-27), suggesting the experimental reliability of this microarray results. miR-424 has previously reported to be lowly expressed in the placenta of patients with severe PE and closely associated with the severity of PE (12). Another report also found that hypoxia downregulates miR-424 in primary human trophoblasts and miR-424 is associated with hindered trophoblast differentiation (13). However, the function and molecular mechanisms of miR-424 in regulating the migration and invasion of trophoblasts remain unclear. Therefore, miR-424 was selected for further investigation.

To further verify the dysregulation of miR-424, RT-qPCR analysis was performed based on 60 placenta samples obtained from 30 patients with PE and 30 healthy pregnant women with uncomplicated pregnancies. The result showed that miR-424 expression levels were much lower in patients with PE than that in healthy controls (Fig. 1C). In addition, by analyzing the correlation between miR-424 and proteinuria levels, it was found that the expression of miR-424 was negatively correlated with the proteinuria levels in PE pregnancies (r=-0.7284;

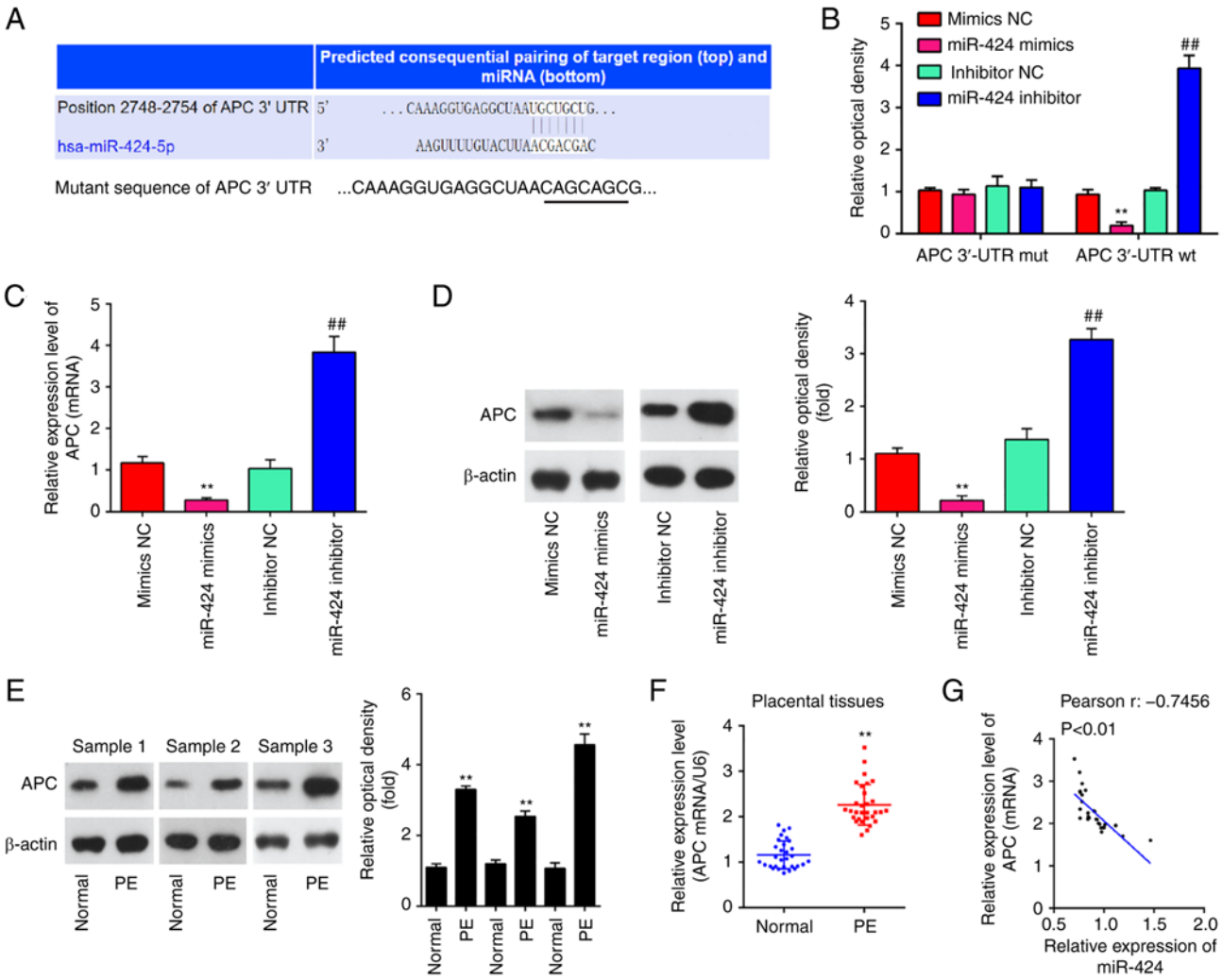

Figure 3. APC is a target of miR-424 in HTR-8/SVneo cells. (A) Predicted complementary sequences for miR-424 in the 3'-UTR of APC and mutations in the seed region of miR-424. (B) Relative luciferase activity of HTR-8/SVneo cells co-transfected with either pmirGLO-APC-3'-UTR or pmirGLO-APC-Mut-3'-UTR and miR-424 mimic/inhibitor or corresponding NC. **P< 0.01 vs mimic NC. $^{\#\#}$P<0.01 vs. inhibitor NC. (C) APC mRNA and (D) protein levels in HTR-8/SVneo cells transfected with miR-424 mimic/inhibitor or corresponding NC (E) Protein levels of APC in placenta samples from three patients with PE and three healthy pregnant women. (F) APC mRNA levels in the placental tissues from normal pregnant women and women with PE (n=30). **P<0.01 vs. normal group. (G) Negative correlation between APC and miR-424 levels in the in the placental tissues from women with PE (r=-0.7456; P<0.01). Data are presented as means ± standard deviation of three individual experiments. APC, adenomatous polyposis coli; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; PE, preeclampsia; Wt, wild-type; Mut, mutant type; 3'-UTR, 3'-untranslated region; NC, negative control.

Fig. 1D). Collectively, these data indicated that miR-424 may be a novel target for the diagnosis of PE.

miR-424 promotes the migratory and invasive abilities of HTR-8/SVneo cells. A previous study has shown that HTR-8/SV40neo cells contain a heterogeneous population of both trophoblast and mesenchymal cells (17). To validate the proportion of trophoblast and mesenchymal cells in this cell lines, CK7 epithelial and vimentin mesenchymal markers were measured via immunofluorescence analysis. The majority of cells were positive to CK7, while the percentage of vimentin-positive cells was low, confirming a prevalence of placental trophoblast cells (Fig. 2A and B). Given that this cell line is largely used to study trophoblast invasion/migration (28), the same was chosen for subsequent experiments. To further evaluate the effects of miR-424 on HTR-8/SVneo cells invasion and migration *in vitro*, miR-424 was overexpressed or knocked down in HTR-8/SVneo cells using miR-424 mimics or inhibitor. miR-424 mimics caused a significant upregulation (**P<0.01; Fig. 2C) and miR-424 inhibitor resulted in a significant downregulation of miR-424 in HTR-8/SVneo cells ($^{\#\#}$P<0.01; Fig. 2C). Reduced activity of trophoblast cells including decreased cell viability and increased apoptosis is well-recognized to lead to PE progression (29). CCK-8 and caspase 3 activity assays were performed to measure the cell viability and apoptosis of HTR-8/SVneo cells, respectively. Overexpression of miR-424 significantly enhanced the viability of HTR-8/SVneo cells compared with that in the mimics NC-transfected cells (Fig. 2D) and a remarkable reduction of caspase 3 activity was observed in HTR-8/SVneo cells (*P<0.05; Fig. 2E), while miR-424 knockdown had opposite effects (Fig. 2D and E). Moreover, Transwell invasion and wound healing assays were performed to measure the invasiveness of HTR-8/SVneo cells. Overexpression of miR-424 significantly promoted

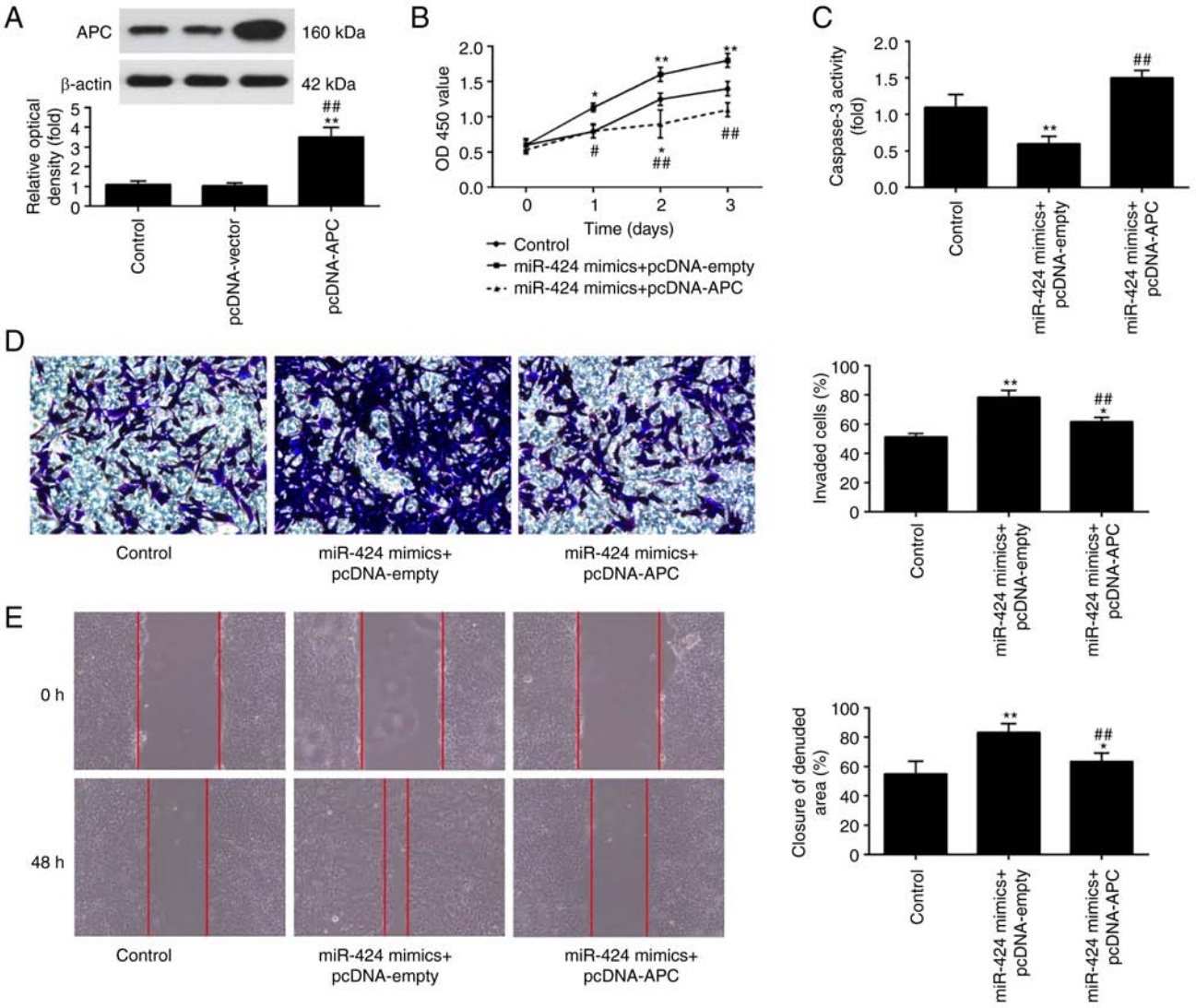

Figure 4. miR-424 suppresses the invasion and migration of HTR-8/SVneo cells by targeting APC. (A) Expression levels of APC protein measured via western blot assay in HTR-8/SVneo cells transfected with pcDNA-empty or pcDNA-APC. (B) Cell viability, (C) activity of caspase 3, (D) cell invasion (magnification, x400) and (E) cell migration (magnification, x200) in HTR-8/SVneo cells co-transfected with miR424 mimics + pcDNA-empty or miR424 mimics + pcDNA-APC. Data are presented as means ± standard deviation of three individual experiments. *P<0.05 and **P<0.01 vs. control. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. miR-424 mimics + pcDNA-vector. APC, adenomatous polyposis coli; miR, microRNA.

the cell invasion compared with mimics NC group (**P<0.01; Fig. 2F), while knockdown of miR-424 had opposite effects ($^{\#\#}$P<0.01; Fig. 2G). miR-424 overexpression significantly increased the migratory activities of HTR-8/SVneo cells compared with mimics NC group (**P<0.01; Fig. 2H), whereas miR-424 knockdown led to a marked reduction of the migratory activities of HTR-8/SVneo cells compared with the inhibitor NC group ($^{\#\#}$P<0.01; Fig. 2I). These results indicated that miR-424 may be involved in the pathogenesis of PE through regulating the migration and invasion of HTR-8/SVneo cell.

APC was a target of miR-424 in HTR-8/SVneo cells. To examine the molecular mechanisms by which miR-424 promoted the migration and invasion of HTR-8/SVneo cells, TargetScan was used to predict the target genes of miR-424. Among several candidates (https://www.targetscan.org/cgi-bin/targetscan/vert_80/targetscan.cgi?species=Human&gid=&mirsc=&mirc=&mirnc=&mirvnc=&mirg=hsa-miR-424-5p), APC was chosen because it is a well-known negative regulator of the Wnt/β-catenin signaling pathway that has been previously reported to be associated with PE (30). One potential binding site for miR-424 was found in the 3'-UTR region of APC mRNA (Fig. 3A). To test whether miR-424 targets APC, dual luciferase reporter assay was performed. The miR-424 mimics significantly decreased the luciferase activity, while miR-424 inhibitor increased the luciferase activity of constructs containing the Wt APC 3'-UTR. These effects were all lost if the miR-424 binding sites were mutated (Fig. 3B). Subsequently, the effect of miR-424 on the expression of APC was measured at the mRNA and protein levels in HTR-8/SVneo cells using RT-qPCR and western blot analysis. The mRNA and protein (**P<0.01 for both; Fig. 3C and D) levels of APC were significantly decreased following the overexpression of miR-424, whereas was upregulated following knockdown of miR-424 ($^{\#\#}$P<0.01 for both; Fig. 3C and D). In addition, the protein levels of APC in placenta samples from 3 PE patients and 3 healthy pregnant women were measured

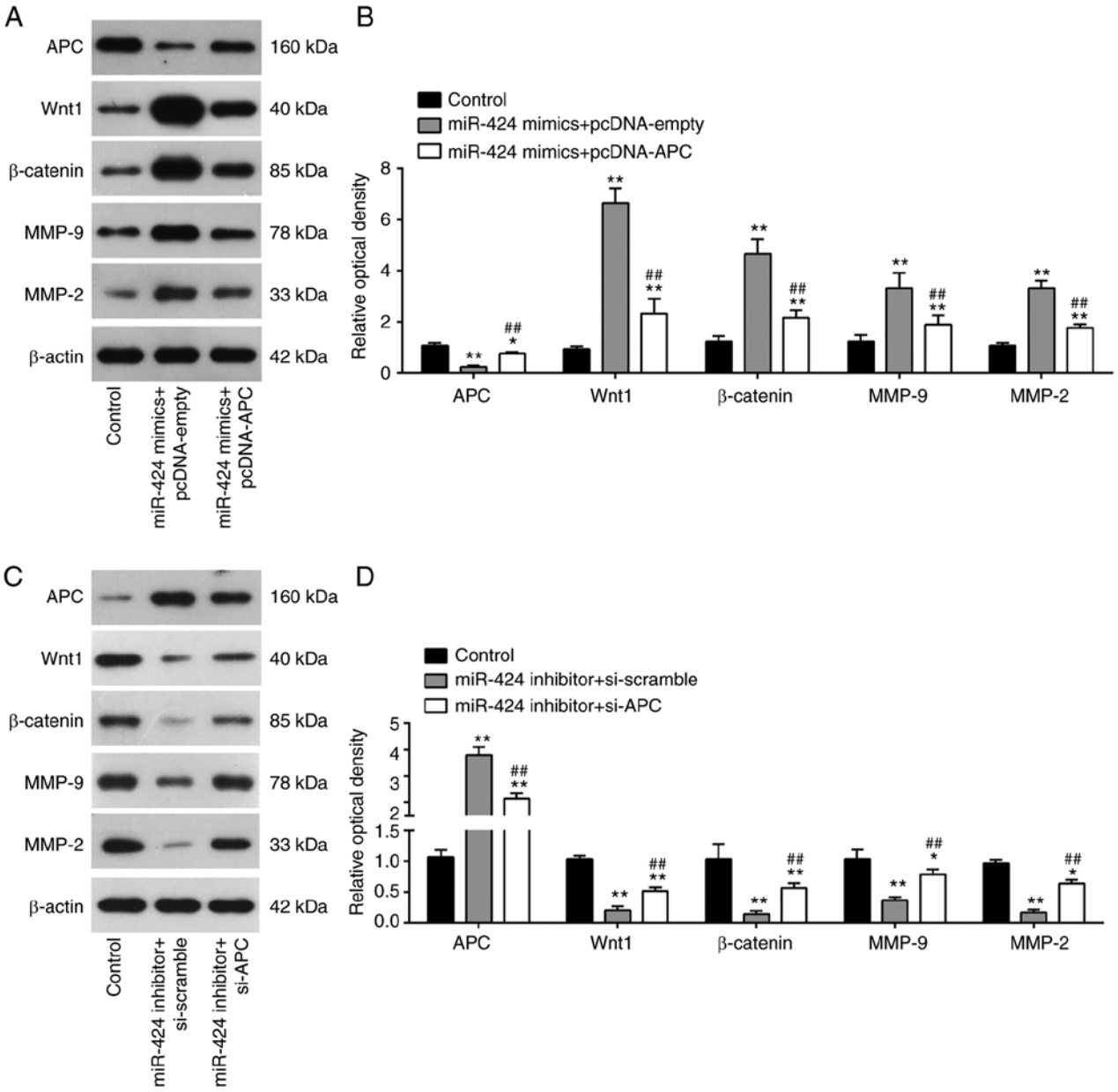

Figure 5. miR-424 activates Wnt/β-catenin signaling pathway by directly targeting APC. (A) Wester blots and (B) protein levels of APC, Wnt1, β-catenin, MMP-9 and MMP2 in HTR-8/SVneo cells transfected with miR-424 mimics + pcDNA-empty or miR-424 mimics + pcDNA-APC. (C) Western blots and (D) protein levels of APC, Wnt1, β-catenin, MMP-9 and MMP2 in HTR-8/SVneo cells transfected with miR-424 inhibitor + si-scramble or miR-424 inhibitor + si-APC. Data are presented as means ± standard deviation of three individual experiments. *P<0.05 and **P<0.01 vs. control. $^{\#\#}$P<0.01 vs. miR-424 mimics + pcDNA-vector or miR-424 inhibitor + si-scramble. APC, adenomatous polyposis coli; miR, microRNA; si, small interfering.

via western blot analysis. It was shown that APC protein levels were significantly increased in patients with PE compared with those in healthy controls (**P<0.01 for all; Fig. 3E). APC mRNA expression was significantly increased in patients with PE compared with that in healthy controls (n=30; **P<0.01; Fig. 3F). Furthermore, a negative correlation between the levels of expression of APC and miR-424 was observed in placenta samples from PE patients (r=-0.7456; P<0.01; Fig. 3G). These findings indicate that APC is a functional target of miR-424 in HTR-8/SVneo cells.

miR-424 suppresses the invasion and migration of HTR-8/SVneo cells by targeting APC. To examine whether APC mediates the positive effects of miR-424 upregulation on HTR-8/SVneo cells, miR-424 mimics and pcDNA-APC were co-transfected into HTR-8/SVneo cells. APC protein expression levels were significantly increased after pcDNA-APC transfection in HTR-8/SVneo cells (**P<0.01 vs. control; $^{\#\#}$P<0.01 vs. cpDNA-vector; Fig. 4A). Furthermore, it was shown that miR-424 overexpression resulted in a significant increase in cell viability, while the same was decreased following APC upregulation (Fig. 4B). Meanwhile, APC upregulation reversed the suppressive effect of miR-424 mimics on the activity of caspase 3 (**P<0.01 vs. control; $^{\#\#}$P<0.01 vs. miR-424 mimics + pcDNA-empty; Fig. 4C). In addition, the findings of Transwell and wound healing assays indicated that APC upregulation attenuated the promoting effect of miR-424 mimics on the invasion

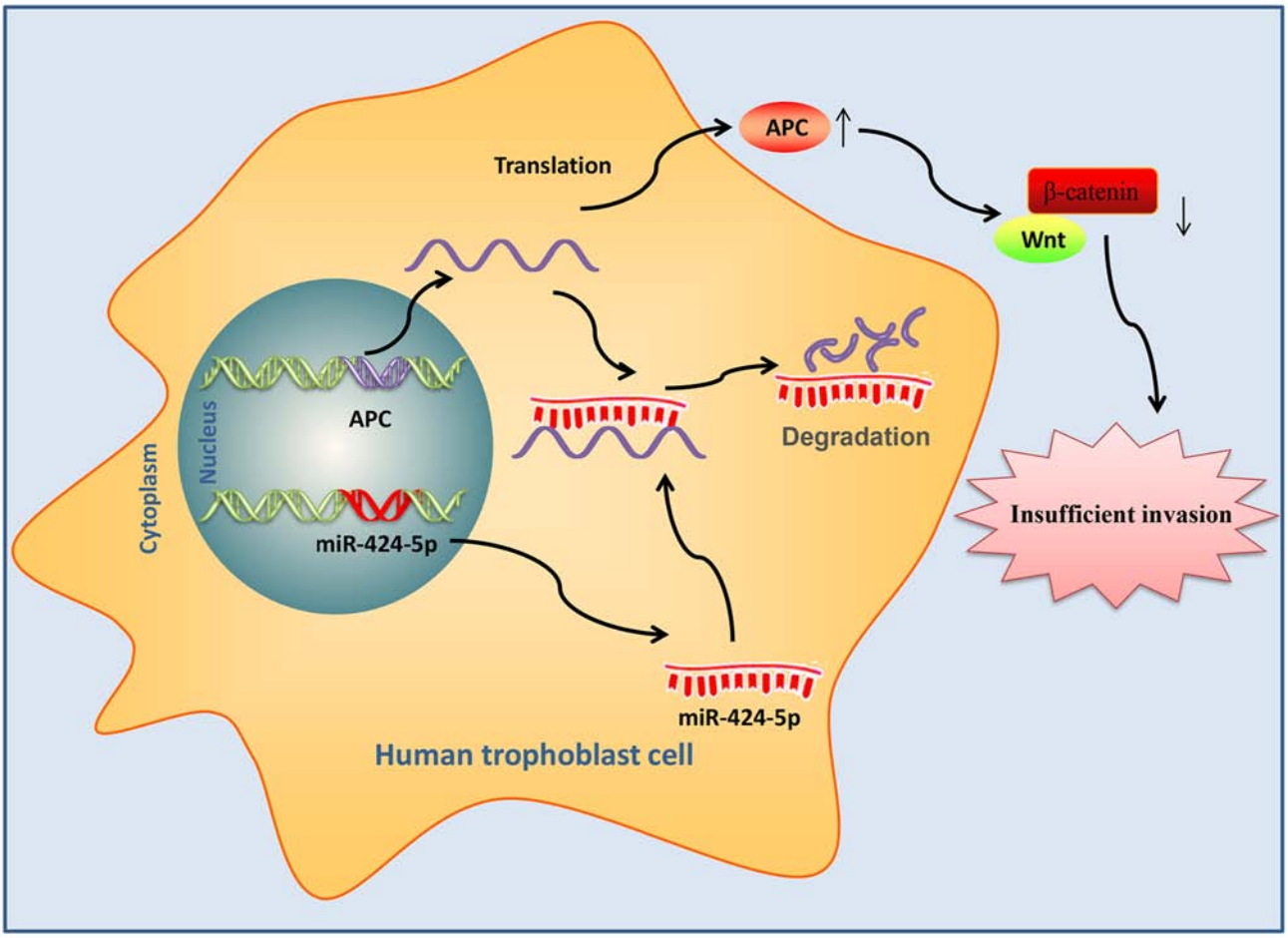

Figure 6. Schematic diagram of the molecular mechanism underlying the miR-424 promotion of human placental trophoblasts invasion and migration. APC, adenomatous polyposis coli; miR, microRNA.

and migration of HTR-8/SVneo cells (*P<0.05 and **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. miR-424 mimics + pcDNA-empry; Fig. 4D and E). These findings demonstrated that upregulation of miR-424 may promote trophoblast cell invasion and migration by downregulating APC expression. However, the underlying molecular mechanism remains unclear and need to be further invetigated.

miR-424 directly targets APC-mediated Wnt/β-catenin signaling pathway. Since APC is a key transcriptional modulator of Wnt/β-catenin signaling pathway, which play a critical role in the regulation of trophoblasts migration and invasion (31-33), western blot analysis was performed to examine if miR-424 affects APC mediated Wnt/β-catenin signaling pathway in HTR-8/SVneo cells. miR-424 overexpression significantly increased the expression levels of Wnt1, β-catenin, MMP-9 and MMP-2, while APC upregulation attenuated the promoting effects of miR-424 mimics on these proteins (Fig. 5A and B). In the contrast, it was also found that miR-424 knockdown markedly decreased the expression levels of Wnt1, β-catenin, MMP-9 and MMP-2, while APC inhibition reversed the inhibitory effects of miR-424 knockdown on these proteins (Fig. 5C and D). Thus, these findings indicated that miR-424 could activate Wnt/β-catenin signaling pathway by directly targeting APC (Fig. 6).

Discussion

In the present study, miR-424 was downregulated in placenta samples obtained from patients with PE and negatively correlated with proteinuria levels. Moreover, miR-424 overexpression enhanced cell viability, suppressed cell apoptosis and promoted the migration and invasion of HTR-8/SVneo cells by targeting APC-mediated Wnt/β-catenin signaling pathway. The present findings suggested that miR-424/APC axis may serve as a novel therapeutic target for patients with PE.

It is well-known that shallow trophoblast invasion is closely associated with the pathogenesis of PE (34). Therefore, in-depth analysis of the mechanisms of inadequate trophoblast invasion is crucial in order to improve PE. Several studies have indicated the involvement of miRs during pregnancy in the regulation of trophoblast invasion (35,36). Jiang *et al* (35) showed that miR-520g upregulation inhibits the invasion of HTR-8/SVneo cells via inhibition of MMP2. Xiao *et al* (36) reported that miR-144 overexpression promotes the trophoblastic cell migration and invasion through targeting PTEN in PE. A recent study showed that the miR-424 expression levels were decreased in placentas of patients with severe PE, suggesting that miR-424 may play an important role in the development of PE (12). Thus, miR-424-5p was selected in this study for further investigation.

A number of studies have previously focused on the roles of miR-424 in the tumor metastasis in various types of human cancers, such as hepatocellular carcinoma and gastric cancer (14,37). Liu *et al* (38) demonstrated that miR-424-5p is increased in thyroid cancer cells and miR-424-5p overexpression enhances the invasion and migration of thyroid cancer cells by inactivating Hippo signaling pathway. In addition, miR-424 has also been found to be downregulated and act as a tumor suppressor to repress the migration and invasion in basal-like breast cancer cells (39). It is generally considered that trophoblast cells are invasive and share behavioral characteristics with tumor cells (34). Therefore, we hypothesize that miR-424-5p may have similar role in the trophoblast invasion and migration. In the present study, *in vitro* assays confirmed that miR-424 overexpression enhances cell viability, suppresses cell apoptosis and promotes the invasion and migration of HTR-8/SVneo cells, while miR-424 knockdown exerts opposite effects. This is in agreement with the findings of Li and Li (40) where miR-424-5p was found to regulate the proliferation, migration and invasion of trophoblast cells in PE. However, the exact molecular mechanism by which miR-424 affects trophoblast invasion and migration has not been studied in depth.

Previous studies have demonstrated that the importance of WNT signaling pathway in implantation and placental development (41,42). Moreover, aberrant Wnt/β-catenin signaling pathway has been reported to be engaged in the pathogenesis of PE and blocking the Wnt/β-catenin pathway results in dysregulation of trophoblast cell proliferation and invasion (43). A previous study has demonstrated that stathmin-1 can inhibit the activation of Wnt/β-catenin signaling pathway and in turn downregulates MMPs expressions, leading to abnormal trophoblast invasion (44). The abnormal activation of Wnt signaling may contribute to the process of PE through regulation of trophoblast cells. The present study revealed that miR-424 can target APC, a well-known negative regulator of the Wnt/β-catenin signaling pathway (45). The regulatory relationship between miR-424 and APC in PE was further investigated and the results showed that miR-424 negatively regulates the expression levels of APC in HTR-8/SVneo cells and that there is an inverse association between the expression levels of miR-424 and APC in placenta tissues. To the best of our knowledge, the present results showed for the first time that the overexpression of APC effectively attenuates the promoting functions of miR-424 on trophoblast migration and invasion. Moreover, the present data showed that miR-424 upregulation raises the levels of Wnt/β-catenin signaling pathway proteins by suppressing APC. MMPs are proved to be involved in extracellular matrix remodeling and can degrade extracellular matrix (ECM) through the function of protease, which in turn affects placental vascular remodeling and trophoblast cell invasion (46). Notably, MMP-2 and MMP-9 are gelatinase proteins in the MMPs family (47). The effects of miR-424 on the expression of MMP-2 and MMP-9 was also evaluated in the present study. The miR-424 upregulation increased the levels of MMP-2 and MMP-9 by targeting APC. The present results suggest that miR-424 activates APC-mediated Wnt/β-catenin signaling pathway to promote trophoblast cells migration and invasion.

Previous studies have shown that the approach for miR-424 to exert its biological functions is mainly through binding mRNA to regulate downstream pathways (48,49). In the present study, the targets of miR-424 were predicted using miR target prediction tools like TargetScan and microRNA.org. Among these targets, miR-424 regulates estrogen-related receptor γ to suppress trophoblast proliferation and invasion (50). Moreover, miR-424 targeted Wnt3a to influence Wnt/β-catenin signaling pathway and then affected the proliferation, apoptosis, migration and invasion of trophoblast cell line HTR-8/SVneo (51). In addition to Wnt/catenin signaling pathway, miR-424 also wields its function by regulating other pathways, such as PI3K/AKT signaling pathway in PE (40). In the future, it would be interesting to clarify if miR-424 plays its role via these mechanisms in PE.

Although some interesting results were found in the present study, there were still some limitations. First, this study only used HTR-8/SVneo cell line, which is the most common cells used to study trophoblastic dysfunction, therefore key experiments need to be further performed in different cell lines, such as TEV-1, ACH-3P, SGHPL-5 and HIPEC65, to corroborate the findings published in the present study. Additionally, although several differently expressed miRs were screened based on previously published microarray data retrieved from GEO database (GSE96985), only miR-424 was investigated in the present study. Further studies are required to investigate the functions of other miRs. Third, PE is a complex pathological process involving several miRs and target genes (52). The present study focused exclusively on the miR-424 and APC/Wnt/β-catenin signaling pathway. The underlying relationship between this pathway and other related miRs needs further investigations.

In conclusion, the present study demonstrated that miR-424 promotes trophoblast migration and invasion by targeting APC and activating Wnt/β-catenin signaling. These findings suggest that miR-424 upregulation may serve as a potential diagnostic target for patients with PE. In future, *in vivo* studies and clinical trial data are required to validate the preliminary *in vitro* results obtained in this study.

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

WZ performed the experiments, contributed to data analysis and wrote the paper. XC analysed the data. XC conceptualized the study design, contributed to data analysis and experimental materials. WZ and XC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the Obstetrics & Gynecology Hospital of Fudan University (approval no. 2018-013). All individuals provided written informed consent for the use of human specimens for clinical research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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