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RESEARCH ARTICLE

ANXA4 promotes trophoblast invasion via the PI3K/Akt/eNOS pathway in preeclampsia

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Xu Y, Sui L, Qiu B, Yin X, Liu J, Zhang X. ANXA4 promotes trophoblast invasion via the PI3K/Akt/eNOS pathway in preeclampsia. *Am J Physiol Cell Physiol* 316: C481–C491, 2019. First published January 23, 2019; doi:10.1152/ajpcell.00404.2018.—The inadequate trophoblast invasion is associated with the development of preeclampsia (PE). Considering that annexin A4 (ANXA4) enhances tumor invasion, we aimed to explore the functional role of ANXA4 in trophoblast cells and to examine the underlying mechanism. ANXA4 expression in PE placentas was analyzed using immunohistochemistry and Western blotting. Cell proliferation, invasion, and apoptosis were determined using a MTT assay, Transwell assay, and flow cytometry, respectively. The expression levels of matrix metalloproteinase (MMP)-2, MMP-9, phosphoinositide 3-kinase (PI3K), Akt, phosphorylated (p)-Akt, and phosphorylated endothelial nitric oxide synthase (p-eNOS) were detected by Western blotting. Placentas were prepared for pathological examination using hematoxylin and eosin staining and apoptosis determination using the TUNEL method. Expression of ANXA4, PI3K, p-Akt and p-eNOS was downregulated in human PE placentas and PE placenta-derived extravillous cytotrophoblasts (EVCTs). Furthermore, ANXA4 overexpression promoted cell proliferation and invasion, inhibited cell apoptosis, and upregulated protein expression of PI3K, p-Akt, and p-eNOS in human trophoblast cells HTR-8/SVneo and JEG-3. By contrast, ANXA4 knockdown exerted the opposite effects. Furthermore, inhibition of the PI3K/Akt pathway by LY294002 abrogated the ANXA4 overexpression-mediated effects on trophoblast behavior. Furthermore, eNOS knockdown abrogated the ANXA4 overexpression-induced promotion of cell invasion and MMP2/9 expression. Additionally, in *N*-nitro-L-arginine methyl ester (L-NAME)-induced PE rats, ANXA4 overexpression alleviated PE progression, accompanied by an increase in expression of PI3K, p-Akt, and p-eNOS in rat placentas. Our findings demonstrate that ANXA4 expression is downregulated in PE. ANXA4 may promote trophoblast invasion via the PI3K/Akt/eNOS pathway.

annexin A4; invasion; PI3K/Akt/eNOS pathway; preeclampsia; trophoblast

INTRODUCTION

Preeclampsia (PE) is a pregnancy-specific disorder characterized by new-onset hypertension and proteinuria after 20 wk of gestation (28). It remains a major cause of maternal and fetal morbidity and mortality (21). Studies indicate that inadequate trophoblast invasion in the uterus, poor remodeling of the spiral arteries, and excessive trophoblast apoptosis in the placenta are key pathological features of the disease (2, 9, 19, 20, 24). However, the molecular mechanism underlying the pathogenesis of PE and the regulation of trophoblast behavior remains largely elusive.

Annexin A4 (ANXA4) has been shown to participate in regulation of cellular growth, invasion, and apoptosis (26). Growing studies have indicated the role of ANXA4 in many forms of cancer (22, 26). For example, ANXA4 knockdown attenuates cell migration and invasion of tumor cells such as ovarian clear cell adenocarcinoma cells (11) and gallbladder cells (27). Our proteomic analysis has also demonstrated that ANXA4 expression was downregulated in PE placentas compared with normal placentas. However, the exact role of ANXA4 in the invasion and survival of trophoblast cells remains less understood.

ANXA4 binds to the plasma membrane in a Ca^{2+}- dependent manner and regulates its downstream signaling transduction, including phosphoinositide 3-kinase (PI3K)/Akt signaling (8, 26). The PI3K/Akt signaling pathway is involved in cell proliferation, migration, metastasis, and invasion (10, 15). A previous study has suggested that the PI3K/Akt pathway is activated in PE placentas to regulate cell proliferation (14). Literature also suggests that PI3K/Akt signaling is involved in regulating trophoblast migration and invasion (17, 18). Furthermore, placentas from PE patients exhibited reduced levels of endothelial nitric oxide synthase (eNOS) expression (3, 5, 12). It has been reported that activation of PI3K/Akt/eNOS pathways plays an important role in regulating cell migration, angiogenesis, and preventing hypertension (7, 29, 30).

Therefore, we hypothesized that ANXA4 may activate the PI3K/Akt/eNOS signaling pathway to promote cell migration and invasion of trophoblast cells, thereby attenuating PE progression. To test our hypothesis, we examined the expression of ANXA4, PI3K, phosphorylated (p)-Akt, and p-eNOS in human PE placentas and PE placenta-derived extravillous cytotrophoblasts (EVCTs). Furthermore, we investigated the effects of ANXA4 expression on the proliferation, invasion, and apoptosis of trophoblast cells HTR-8/SVneo and JEG-3 and determined the role of the PI3K/Akt/eNOS pathway in this process. We also explored the therapeutic effects of ANXA4 overexpression on PE in a rat PE model and the alteration of expression of key kinases in the PI3K/Akt/eNOS pathway in rat placentas.

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MATERIALS AND METHODS

Placental tissue samples. The placental tissues from PE patients ($n = 8$) and normal pregnant women ($n = 11$) were collected immediately after delivery and frozen in liquid nitrogen for subsequent experiments. PE was diagnosed as systolic pressure ≥140 mmHg or diastolic pressure ≥90 mmHg on two or more occasions after gestational week 20, accompanied by proteinuria (urine protein ≥0.3 g/24 h, or ≥0.2 g/l in a random urine test). For the control group, women with chronic hypertension, renal disease, cardiovascular disease, diabetes, hepatitis, any evidence of intrapartum infection or other pregnancy complications, such as fetal anomalies or chromosomal abnormalities, were excluded from the study. Collection and experimentation of human tissues was conducted in accordance with the protocol approved by the Clinical Research Ethics Committee of Peking University People's Hospital. Written informed consent was obtained from all enrolled subjects.

Western blotting. Protein extracts were prepared from placental tissues or cells using RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO). Protein concentration was measured using the bicinchoninic acid method. Equal amounts of proteins were separated by 10% SDS-PAGE and were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk for 1 h and were then incubated with the following primary antibodies: ANXA4 (1:1000, Abcam, Cambridge, MA; cat. no. ab33009), PI3K p85α (1:1,000, Abcam, cat. no. ab191606), p-Akt Ser^{473} (1:1,000, Abcam, cat. no. ab18206), Akt (1:1,000, Abcam, cat. no. ab8805), p-eNOS Ser^{1177} (1:1,000, Cell Signaling Technology, Danvers, MA; cat. no. 9571), eNOS (1:1,000, Abcam, cat. no. ab199956), matrix metalloproteinase-2 [(MMP-2) (1:1,000, Abcam, cat. no. ab37150], and MMP-9 (1:1,000, Abcam, cat. no. ab38898). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. The protein was detected using an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL) and the band intensity was quantified with Image Pro Plus 4.5 software. β-Actin (1:1,000, Abcam, cat. no. ab8227) or α-tubulin (1:1,000, Abcam, cat. no. ab52866) served as the internal control.

Isolation and identification of extravillous cytotrophoblasts. Sterile fresh placentas were obtained from PE patients and normal pregnant women. The villus tissue of maternal surface was taken, and blood clots and fetal membranes were removed. After a washing three times with sterile D-Hanks', the placentas were cut into pieces of ~1 mm^3 and digested in 0.125% trypsin at 37°C for 30 min. When the single cell formation was observed under a microscope, the supernatant was collected, filtered, and centrifuged at 250 *g* for 10 min. The resultant cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 (1:1) suspension supplemented with 10% fetal bovine serum (FBS). Cells were diluted to a concentration of 5×10^5/ml and then plated on Matrigel-coated culture dishes. Cells were maintained in DMEM (GIBCO, Thermo-Fisher Scientific, Waltham, MA) supplemented with 10% FBS, 25 mM HEPES, 2 mM glutamine, 100 g/ml streptomycin, and 100 UI/ml penicillin and incubated in humidified air with 5% CO_2 at 37°C. After 4 h, cells were washed three times to eliminate all of the debris and were incubated with Complete medium. Following incubation for 24 h, the cells were subjected to cytokeratin 7 (CK7, a specific marker of trophoblasts) immunofluorescence staining for identification of extravillous cytotrophoblasts (EVCTs). Briefly, EVCTs were fixed with 4% paraformaldehyde for 15 min and washed with PBS three times. After being blocked with 5% BSA blocking solution, EVCTs were incubated with anti-CK7 (1:100, Abcam, cat. no. ab181598) for 1 h. Red fluorescence signals indicated CK7, and the DAPI-stained nuclei were blue. Fluorescence signals were analyzed, and images were captured under a fluorescence microscope (Nikon, Tokyo, Japan).

Cell culture. Human trophoblast cell lines HTR-8/SVneo and JEG-3 were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FBS (GIBCO). Cells were maintained in a humidified incubator (5% CO_2 and 95% air) at 37°C.

Cell proliferation assay. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated into 96-well plates at a density of 1×10^4 cells/well. Following incubation at 37°C for 24 h, 20 μl MTT (5 mg/ml, Sigma-Aldrich) was added to the medium and incubated at 37°C for 4 h. The formazan was dissolved in 150 μl/well dimethyl sulfoxide (Sigma-Aldrich) for 10 min. Cellular viability was determined by measuring the optical density (OD) at 450 nm with averages from triplicate wells by a spectrophotometer (Multiskan MK3, Thermo-Fisher Scientific).

Cell apoptosis assay. Cell apoptosis was quantified by flow cytometry using a commercially available annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Trophoblasts were plated at a density of 2×10^5 cells/ml in 1,000 μl in 24-well culture plates. At the end of treatments, the trophoblasts were collected, washed, and resuspended in binding buffer provided in the kit at a density of 1×10^6 cells/ml. The cells were then washed twice and resuspended in binding buffer containing annexin V solution and propidium iodide. Samples were incubated in the dark for 30 min, and the percentage of annexin V-positive cells was determined using the FACScan flow cytometry (BD Biosciences).

Matrigel-based invasion assay. Cell invasion was examined by the ability of the cells to cross the 8-mm pores of polycarbonate membranes (8-mm pore size; Corning Costar, Corning, NY). Briefly, cells (1.0×10^5 cells/well) were plated in the upper chambers precoated with Matrigel (BD Biosciences) membrane, and RPMI 1640 medium (600 μl) containing 10% FBS was added to the lower chamber. Following incubation for 24 h, the cells on the upper surface of the base membrane were removed with a sterile cotton swab. The cells that had transferred to the lower surface of the base membrane were stained with hematoxylin and eosin (H&E, Sigma-Aldrich). The invaded cells were observed under a microscope (Nikon E100; Nikon, Tokyo, Japan), and the number of invaded cells was counted and averaged from five randomly selected fields.

Cell transfection. To knockdown ANXA4 or eNOS, ANXA4 siRNA (si-ANXA4), si-eNOS, and their corresponding scramble siRNAs were constructed. The primers of si-ANXA4 and si-eNOS were as follows: si-ANXA4: sense, 5′-CAAAGGAGGUACUGUCAAATT-3′, antisense, 5′- UUUGACAGUACCUCCUUUGGT-3′; si-eNOS: sense, 5′-UGUGUUACUGGACUCCUUCCUCUUC-3′, antisense, 5′-GAAGAGGAAGGAGUCCAGUAACACA-3′. Cells were transfected with si-ANXA4, si-eNOS, or their scramble siRNAs using Lipofectamine RNAiMAX Transfection Reagent Lipofectamine 2000 (Invitrogen, Thermo-Fisher Scientific) according to the manufacturer's instructions. To overexpress ANXA4, the ANXA4 cDNA fragment was cloned into pcDNA3.1 vector (Invitrogen), generating pcDNA3.1-ANXA4. Cells were transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. All transfections are transient.

Animals and experimental groups. Sprague-Dawley (SD) rats (5–7 wk old, weighing 210–250 g) were provided by Chinese Slaccas (Shanghai, China). All experimental procedures were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Research Ethics Committee of Peking University People's Hospital.

These female rats were copulated with weight-matched male rats at a female: male ratio of 2:1 during estrus, and vaginal secretions were microscopically examined every morning. Gestational day 0 was confirmed by the presence of sperm.

Twenty-four pregnant female SD rats were divided into four groups ($n = 6$/group). *1*) Rats in the PE model group received a subcutaneous injection of *N*-nitro-L-arginine methyl ester (L-NAME, Sigma) at a concentration of 125 $mg \cdot kg^{-1} \cdot day^{-1}$ from the 12th gestational day for 7 days; *2*) rats in the control group received subcutaneous injection of

normal saline with equal volume of L-NAME on the 12th gestational day for 7 days; *3*) rats in the ANXA4 group received the following treatment: on the 12th gestational day, the abdomens of the rats were dissected under anesthesia, the uterus was exposed, and the 30-μl ANXA4 cDNA solution (100 ng/μl in sterilized distilled water) was injected into the maternal surface of the placentas, and then the abdomen was sutured and warmed. Meanwhile, L-NAME was subcutaneously injected as the PE model group on the 12th gestational day for 7 days. ANXA4 cDNA solution was prepared as previously described (13). Four rats in the vector group received the same treatment as rats in the ANXA4 group except that ANXA4 cDNA was replaced by empty vector.

Blood pressure measurement and urine analysis. The systolic blood pressure (SBP) of each rat was determined using the noninvasive tail cuff method and the BP-2000 Blood Pressure Analysis System (Visitech Systems, Apex, NC). Each rat was preheated for 5 min to 38 °C before each measurement was taken. The 24-h urine output of each animal was collected. Proteinuria was detected using CBB kits (Jiancheng Institute of Biotechnology, Nanjing, China).

Histopathology. The separated human or rat placentas were fixed in 10% paraformaldehyde for 24 h after a washing with PBS three times. Next, tissues were dehydrated with a graded series of ethanol, infiltrated with xylene, and then embedded in paraffin before being cut into 5-μm-thick sections. The sections were stained with H&E according to the routine staining procedure.

In situ detection of apoptotic cells. Apoptotic cells in the placentas were detected by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining according to the manufacturer's instructions for the In Situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany).

Immunohistochemistry. Immunohistochemistry was used to detect the expression of CK7 and ANXA4 in placentas. The sections were dewaxed and rehydrated and then incubated for 15 min at room temperature in 3% H_2O_2 to quench endogenous peroxidase activity. Sections were blocked with normal goat serum for 15 min at room

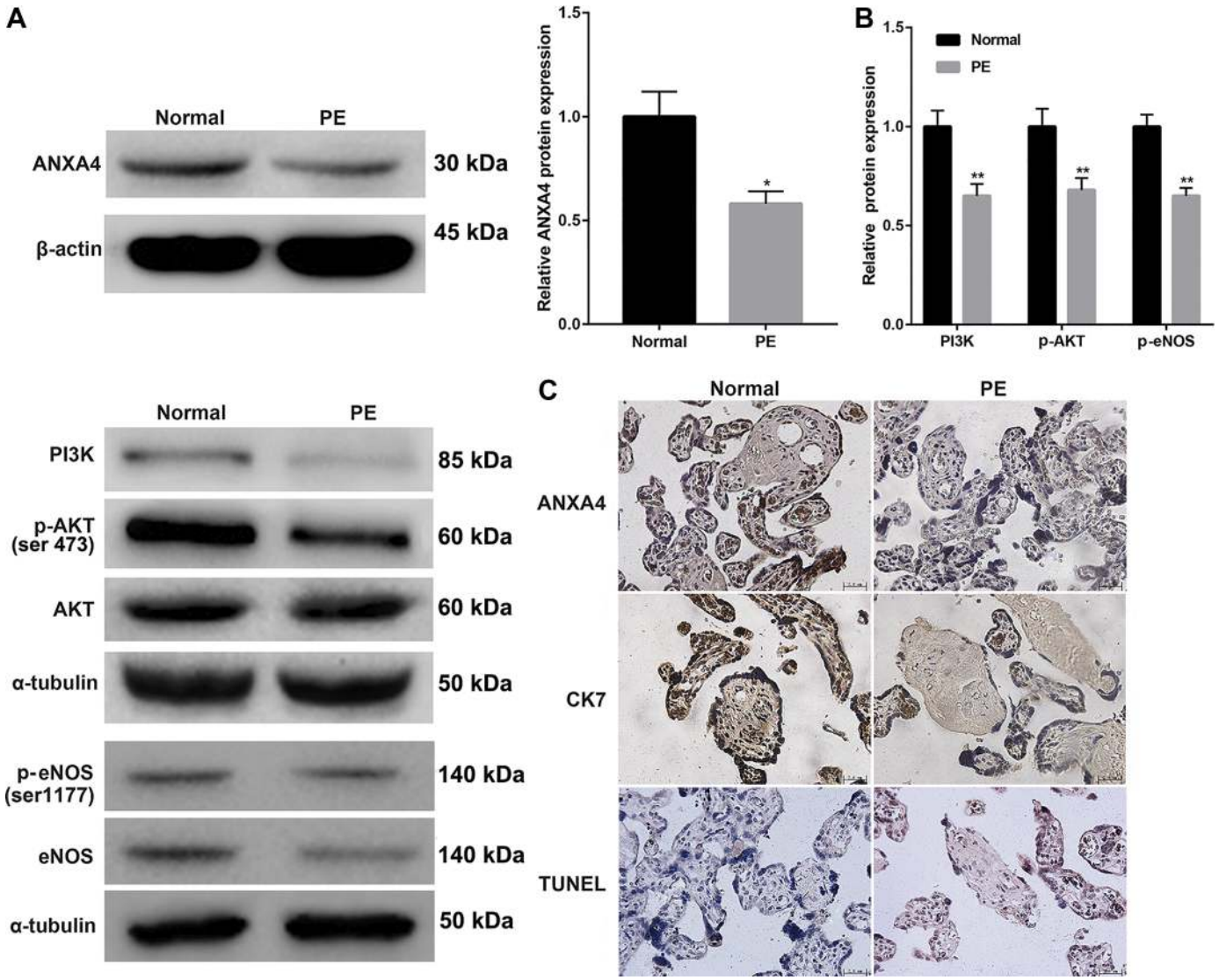


Fig. 1. Expression of annexin A4 (ANXA4), phosphoinositide 3-kinase (PI3K), phosphorylated (p-)Akt, and phosphorylated endothelial NO synthase (p-eNOS) was reduced in preeclampsia (PE) placentas. Placentas of PE patients ($n = 8$) and normal control subjects ($n = 11$) were collected immediately after delivery. Protein levels of ANXA4 (*A*), PI3K, p-Akt, Akt, p-eNOS, and eNOS (*B*) in placental tissues were determined by Western blotting. β-Actin or α-tubulin served as the internal control. *C*: immunohistochemistry was used to detect the expression of ANXA4 and cytokeratin 7 (CK7) in placentas. TUNEL staining was performed to detect cell apoptosis in placentas. Scale bar, 2.0 μm. Data are expressed as means ± SD from 3 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. Normal group.

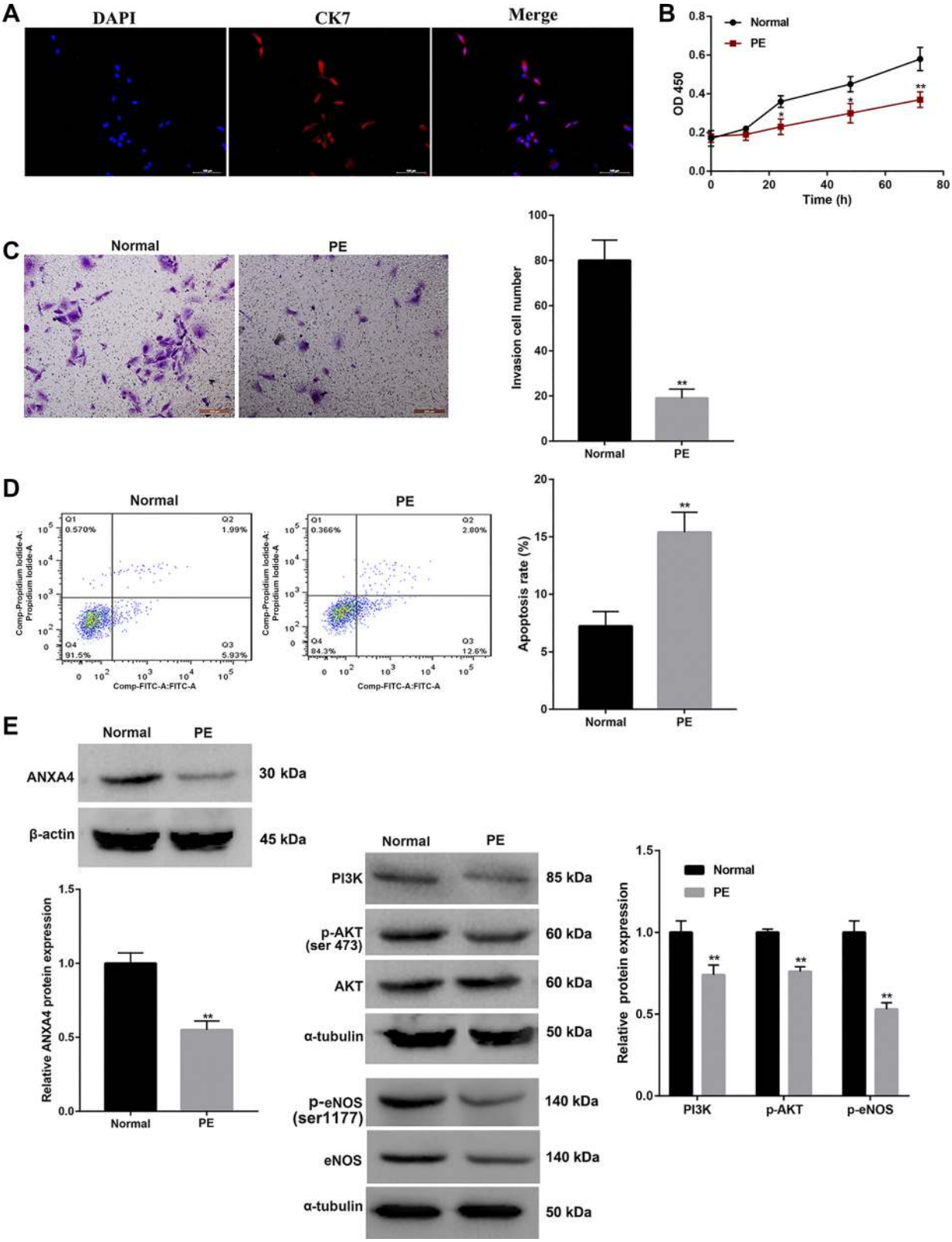


Fig. 2. Expression of annexin A4 (ANXA4), phosphoinositide 3-kinase (PI3K), phosphorylated (p-)Akt, and phosphorylated endothelial NO synthase (p-eNOS) were phosphoinositide 3-kinase (PI3K), phosphorylated (p-)Akt, and phosphorylated endothelial NO synthase (p-eNOS) reduced in extravillous cytotrophoblasts (EVCTs) from preeclampsia placentas. EVCTs were separated and cultured for 72 h. *A*: immunofluorescence staining of cytokeratin 7 (CK7) for EVCTs identification. Red fluorescence signals indicate CK7; DAPI-stained nuclei are blue. Scale bar, 100 μm. *B*: cell proliferation using MTT assay. *C*: cell invasion using Transwell invasion assay. Scale bar, 200 μm. The average number of invaded cells from 5 randomly selected fields is shown. *D*: cell apoptosis using flow cytometry and percentage of annexin V-positive cells. *E*: protein expression of ANXA4, PI3K, p-Akt, p-eNOS, and eNOS using Western blotting. Data are expressed as means ± SD from 3 independent experiments. $*P < 0.05$, $**P < 0.01$ vs. Normal group.

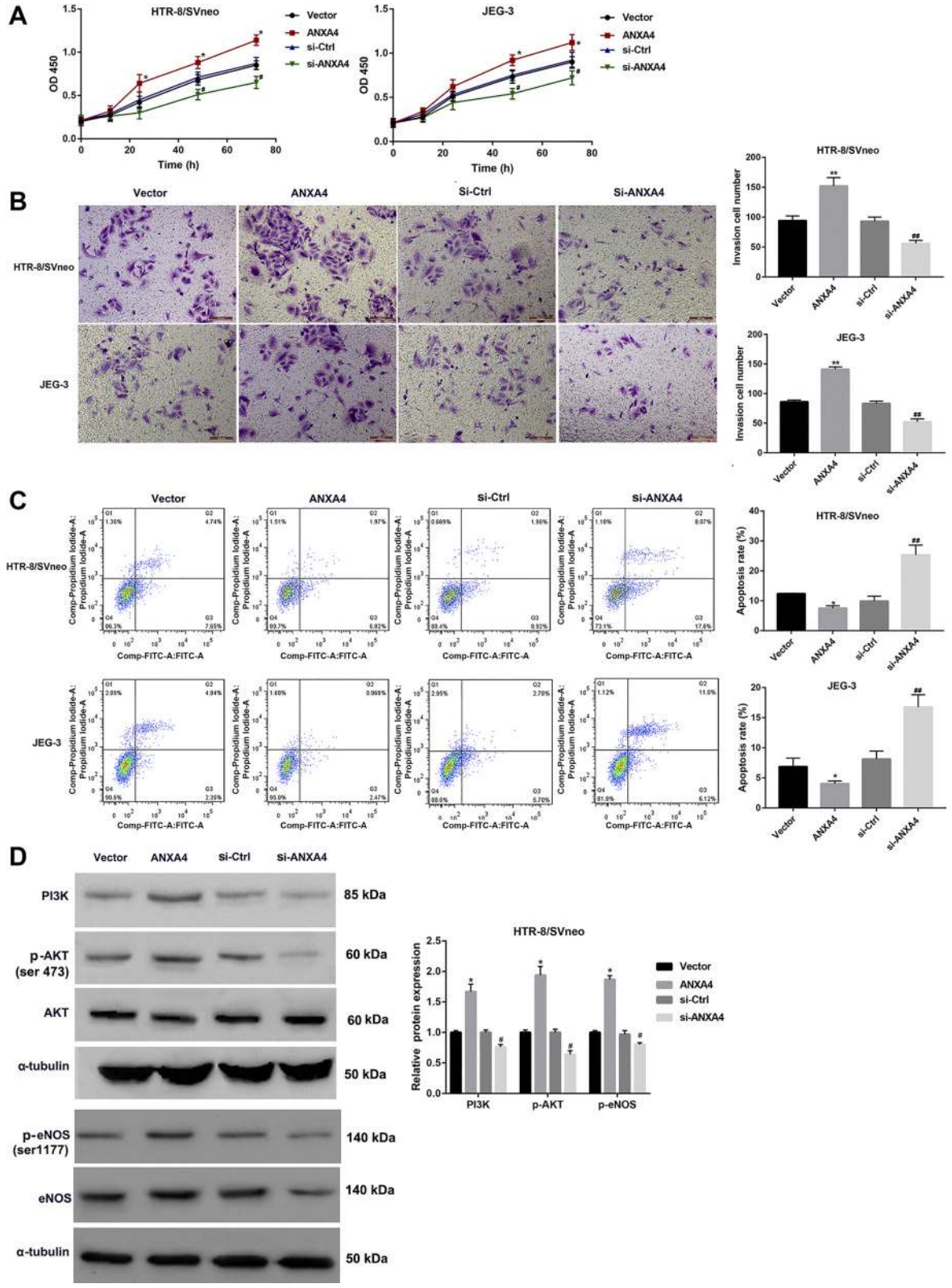


Fig. 3. Cytokeratin 7 (CK7) promoted trophoblast cell proliferation and invasion, inhibited cell apoptosis, and activated the phosphoinositide 3-kinase (PI3K)/Akt/endothelial NO synthase (eNOS) pathway. Human trophoblast cells HTR-8/SVneo and JEG-3 were transfected with pcDNA3.1-annexin A4 (ANXA4) or small interfering (si-)ANXA4, or their corresponding controls. *A*: cell proliferation using MTT assay. *B*: cell invasion using Transwell invasion assay. Scale bar, 200 μm. *C*: cell apoptosis using flow cytometry. *D*: protein expression of ANXA4, PI3K, p-Akt, p-eNOS, and eNOS using Western blotting. Data are expressed as means ± SD from 3 independent experiments. $*P < 0.05$, $**P < 0.01$ vs. vector group; $\#P < 0.05$, $\#\#P < 0.01$ vs. si-Ctrl group.

temperature and were then incubated with a primary antibody against ANXA4 (1:1,000, Abcam, cat. no. ab33009) or CK7 (1:1,000, Abcam, cat. no. ab9021) overnight at 4°C, followed by a biotinylated secondary antibody for 30 min at room temperature. Then samples were stained with diaminobenzidine, counterstained with H&E, dehydrated, and then embedded in paraffin. The sections were analyzed using a microscope.

Statistical analysis. SPSS 16.0 (SPSS, Chicago, IL) was used for data analysis. The data are presented as means ± SD from three independent experiments. The unpaired Student's *t*-test was used to analyze differences between two groups. One-way analysis of variance (ANOVA) was used to analyze differences among multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Decreased ANXA4, PI3K, p-Akt, and p-eNOS expression in PE placentas. Western blot analysis revealed that ANXA4 protein expression in the placental tissues from PE patients were significantly downregulated compared with the normal placentas (Fig. 1*A*), indicating that the reduced level of ANXA4 in the placenta may be involved in the PE pathogenesis. Furthermore, a significant reduction in protein expression of PI3K, p-Akt, and p-eNOS was also observed in PE placentas compared with the normal placentas (Fig. 1*B*). Immunohistochemistry analysis also demonstrated that expression of both CK7 and ANXA4 in PE placentas was reduced compared with

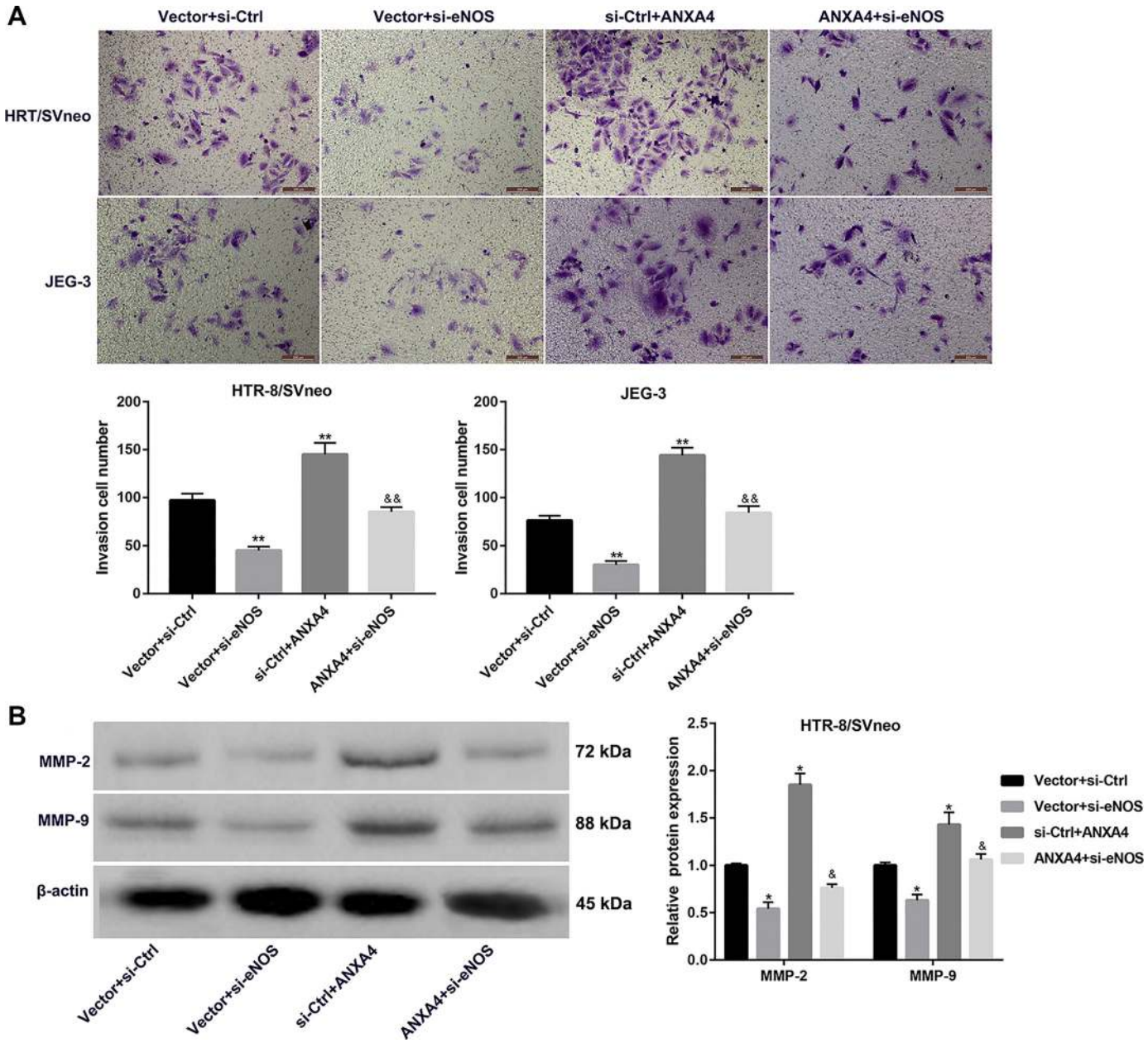


Fig. 4. Knockdown of endothelial NO synthase (eNOS) abrogated annexin A4 (ANXA4) overexpression-mediated promotion of trophoblast invasion and matrix metalloproteinase-2/9 (MMP2/9) expression. HTR-8/SVneo and JEG-3 cells were cotransfected with pcDNA3.1-ANXA4, small interfering (si-)eNOS, or their corresponding controls. *A*: cell invasion using Transwell invasion assay. Scale bar, 200 μm. *B*: protein expression of MMP-2 and MMP-9 in HTR-8/SVneo cells using Western blotting. Data are expressed as means ± SD from 3 independent experiments. *$P < 0.05$, **$P < 0.01$ vs. vector+si-Ctrl group; &$P < 0.05$, &&$P < 0.01$ vs. si-Ctrl+ANXA4 group.

the normal placentas (Fig. 1*C*). Moreover, the PE placentas exhibited increased TUNEL-positive cells compared with the normal controls, indicating enhanced apoptosis in PE placentas (Fig. 1*C*).

Decreased ANXA4, PI3K, p-Akt, and p-eNOS expression in EVCTs from PE placentas. EVCTs were isolated and cultured for 72 h. Immunofluorescence analysis revealed that the percentage of CK7-positive cells reached more than 90% (Fig. 2*A*). Furthermore, EVCTs isolated from PE placentas displayed reduced cell proliferation and cell invasion and increased cell apoptosis, as demonstrated by MTT assay (Fig. 2*B*), Transwell invasion assay (Fig. 2*C*), and flow cytometry, respectively (Fig. 2*D*). Moreover, Western blot analysis revealed that protein expression of ANXA4, PI3K, p-Akt and p-eNOS in EVCTs from PE placentas was significantly downregulated compared with the EVCTs from normal placentas (Fig. 2*E*).

ANXA4 promoted trophoblast cell proliferation and invasion, inhibited cell apoptosis, and activated PI3K/Akt/eNOS pathway. To explore the functional role of ANXA4 in trophoblast cell proliferation, invasion, and apoptosis, we transfected human trophoblast cells HTR-8/SVneo and JEG-3 with pcDNA3.1-ANXA4 to overexpress ANXA4, or si-ANXA4 to knockdown ANXA4. The data revealed that ANXA4 overexpres-

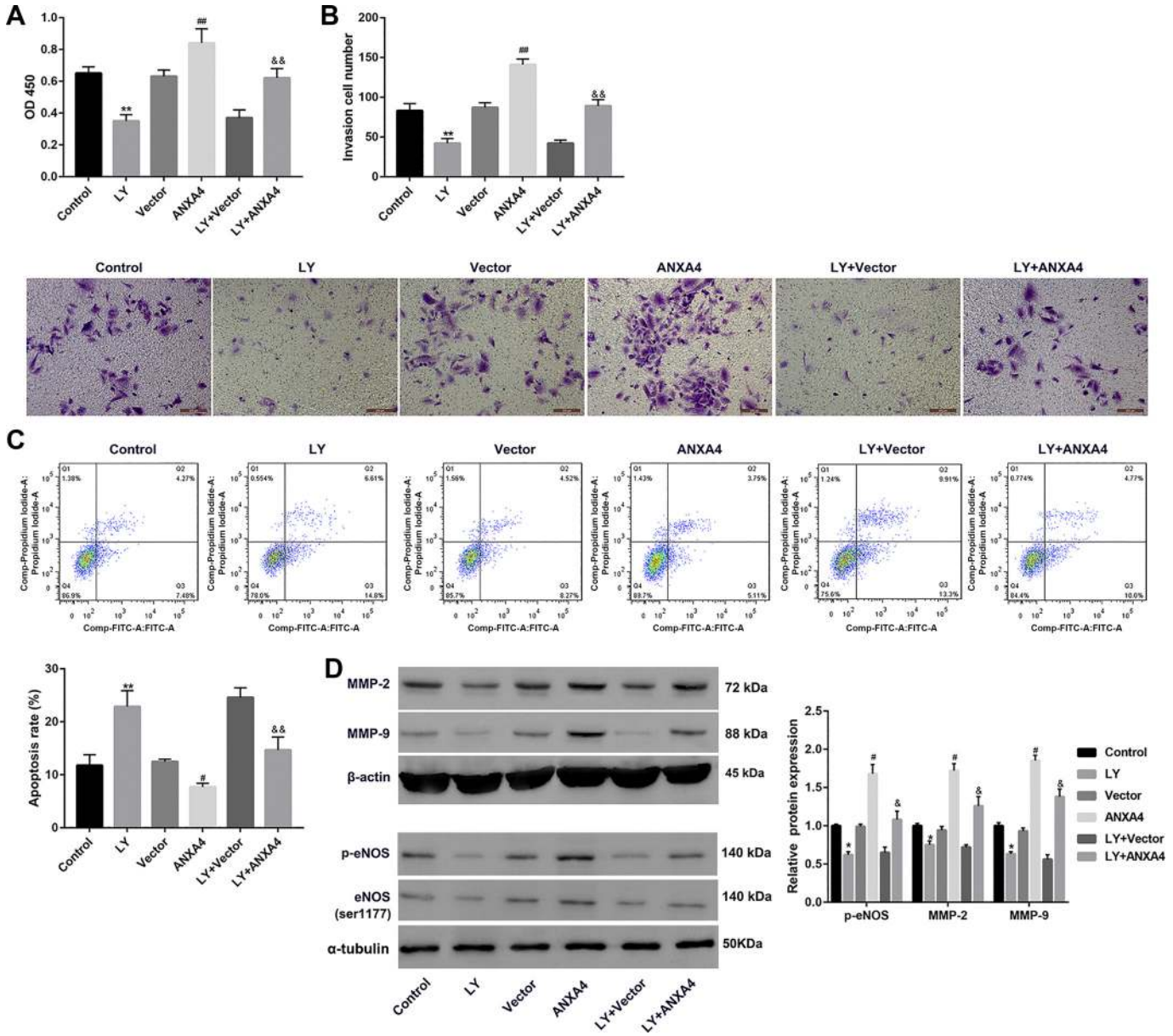


Fig. 5. Inhibition of the phosphoinositide 3-kinase (PI3K)/Akt pathway abrogated annexin A4 (ANXA4) overexpression-mediated effects on trophoblast behavior. HTR-8/SVneo cells were transfected with pcDNA3.1-ANXA4 or its empty vector controls, followed by treatment with LY294002 (LY; an inhibitor for the PI3K/Akt pathway, 24 μM). *A*: cell proliferation using MTT assay. *B*: cell invasion using Transwell invasion assay. Scale bar, 200 μm. *C*: cell apoptosis using flow cytometry. *D*: protein expression of matrix metalloproteinase-2/9 (MMP2/9), phosphorylated endothelial NO synthase (p-eNOS), and eNOS using Western blotting. Data are expressed as means ± SD from 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group; $\#P < 0.05$, $\#\#P < 0.01$ vs. vector group; $\&P < 0.05$, $\&\&P < 0.01$ vs. ANXA4 group.

sion notably promoted cell proliferation and invasion and inhibited cell apoptosis in both HTR-8/SVneo and JEG-3 cells compared with the empty vector group (Fig. 3, *A–C*). Furthermore, ANXA4 overexpression significantly upregulated protein expression of PI3K, p-Akt, and p-eNOS (Fig. 3*D*). By contrast, ANXA4 knockdown exhibited the reversed effects (Fig. 3, *A–D*). These results indicated that activation of the PI3K/Akt/eNOS signaling pathway might be involved in the effects of ANXA4 on trophoblast cell behavior.

Knockdown of eNOS abrogated ANXA4 overexpression-mediated promotion of trophoblast invasion and MMP2/9 expression. To explore whether activation of the PI3K/Akt/eNOS signaling pathway might be involved in the ANXA4 overexpression-mediated promotion of cell invasion, we cotransfected HTR-8/SVneo and JEG-3 cells with pcDNA3.1-ANXA4, si-eNOS, or their corresponding controls. The data revealed that knockdown of eNOS significantly abrogated the ANXA4 overexpression-mediated promotion of cell invasion in both HTR-8/SVneo and JEG-3 cells (Fig. 4*A*). Furthermore, MMP-2 and MMP-9 have been shown to be critical in trophoblast invasion by degrading the extracellular matrix (30). Our results showed that ANXA4 overexpression significantly increased the protein expression of MMP-2 and MMP-9 in HTR-8/SVneo cells (Fig. 4*B*). Importantly, eNOS knockdown significantly abrogated the ANXA4 overexpression-mediated increase in MMP2/9 expression (Fig. 4, *A* and *B*).

Inhibition of the PI3K/Akt pathway abrogated ANXA4 overexpression-mediated effects on trophoblast behavior. Next, we transfected HTR-8/SVneo cells with pcDNA3.1-ANXA4 or its empty vector controls, followed by treatment with LY294002 (an inhibitor of the PI3K/Akt pathway). The data revealed that LY294002 treatment significantly inhibited cell proliferation and invasion and promoted cell apoptosis (Fig. 5, *A–C*). Furthermore, LY294002 treatment significantly downregulated of MMP-2/9 protein expression and eNOS phosphorylation compared with the control group (Fig. 5*D*). ANXA4 overexpression significantly promoted cell proliferation and invasion, inhibited cell apoptosis, and increased protein expression of MMP-2/9 and eNOS phosphorylation. Of note, LY294002 treatment significantly abrogated the ANXA4 overexpression-mediated effects on trophoblast behavior, MMP-2/9 expression, and eNOS phosphorylation (Fig. 5, *A–D*).

ANXA4 overexpression alleviated rat PE progression. Next, we explored the therapeutic effects of ANXA4 overexpression on PE in a rat PE model. The results showed that PE rats exhibited increased SBP and 24-h urine protein compared with the control rats (Fig. 6, *A* and *B*). H&E staining of placentas in the PE model group showed villous cellulose-like necrosis, increased trophoblastic nodules, vascular membrane loss, and narrowing of the vascular lumen compared with the control placentas (Fig. 6*C*). Furthermore, consistent with expression in human placentas, rat PE placentas showed downregulated ANXA4 expression, increased apoptosis, and decreased protein expression of PI3K, p-Akt, and p-eNOS compared with the rat control placentas, as demonstrated by immunohistochemistry (Fig. 6*D*), TUNEL staining (Fig. 6*E*), and Western blot analysis (Fig. 6*F*), respectively. More importantly, ANXA4 overexpression significantly decreased SBP and 24-h urine protein, alleviated PE-associated histological injury, and decreased TUNEL-positive cells, accompanied by a significant increase in expression of ANXA4, PI3K, p-Akt, and p-eNOS in rat PE placentas (Fig. 6).

DISCUSSION

To the best of our knowledge, the present study is the first to demonstrate the expression and function of ANXA4 in placental tissues. Our results demonstrated that ANXA4 expression was downregulated in PE. ANXA4 may promote trophoblast invasion via the PI3K/Akt/eNOS pathway.

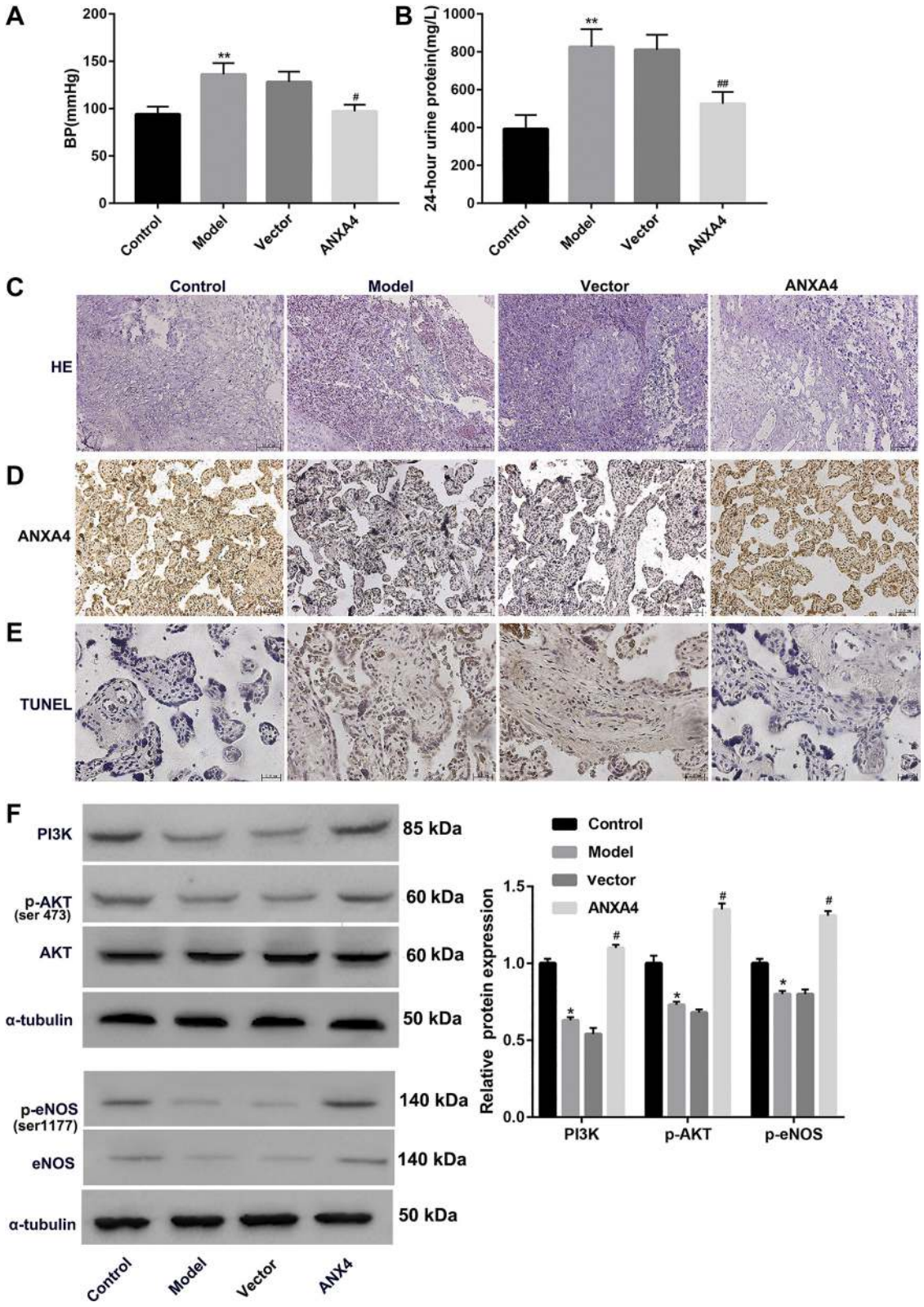
In normal and uncomplicated pregnancies, EVCTs invade through the uterine interstitium (interstitial trophoblasts) into spiral arteries and subsequently line and remodel them (endovascular trophoblasts), as a result of which, trophoblast invasion is responsible for successful establishment of maternal blood flow toward the placenta (23). Increasing studies suggest that the deficient migration and invasion of trophoblasts may lead to PE (16). Notably, trophoblast migration and invasion are often compared with tumor metastasis, for they share many of the same molecular mechanisms (6). Increasing evidence has indicated that ANXA4 knockdown attenuates tumor cell migration and invasion (11, 27). Together, these studies implicate the possible potential role of ANXA4 in PE. In the present study, the expression and role of ANXA4 in the progression of preeclampsia were investigated, and it was observed that ANXA4 expression was downregulated in human PE placentas and EVCTs from PE placentas, illustrating the possible relationship between ANXA4 expression and PE progression. Furthermore, ANXA4 overexpression promoted cell proliferation and invasion and inhibited cell apoptosis in HTR-8/SVneo and JEG-3 ANXA4 cells. By contrast, ANXA4 knockdown exerted the opposite effects. These results are in accordance with the previous studies indicating the role of ANXA4 in promoting cell invasion in human malignancies (11, 27) and suggest that downregulated expression of ANXA4 may be important in the PE progression.

Matrix metalloproteinases (MMPs) have have been shown to exhibit proteolytic activity involved in the efficiency of trophoblast invasion to the uterine wall. Furthermore, a dysregulation of these enzymes is correlated with PE. A recent study suggests that decreased MMP-2 and MMP-9 interfere with the

Fig. 6. Annexin A4 (ANXA4)overexpression alleviated preeclampsia (PE) progression and activated the phosphoinositide 3-kinase (PI3K)/Akt/endothelial NO synthase (eNOS) pathway in PE model rats. Twenty-four pregnant female Sprague-Dawley rats were divided into 4 groups ($n = 6$/group): the control group, the PE model group, the ANXA4 overexpression group, and the control vector group. After designated treatment (see MATERIALS AND METHODS) on the 20th day, systolic blood pressure (BP) of rat tail artery (*A*) and 24-h urine protein of pregnant rats (*B*) were measured. Rats were euthanized after delivery, and placentas were taken. *C*: histopathological changes in placentas were evaluated by hematoxylin-eosin (HE) staining. Scale bar, 10 μm. *D*: expression of ANXA4 protein in placentas was detected by immunohistochemistry. Scale bar, 2.0 μm. *E*: cell apoptosis in placentas was detected using TUNEL staining. Scale bar, 2.0 μm. *F*: protein expression of ANXA4, PI3K, phosphorylated (p-)Akt, p-eNOS, and eNOS protein in placentas was measured by Western blotting. Data are expressed as means ± SD from 3 independent experiments. $*P < 0.05$, $**P < 0.01$ vs. control group; $\#P < 0.05$, $\#\#P < 0.01$ vs. vector group.

normal remodeling of spiral arteries at early pregnancy stages, resulting in the initial pathophysiological changes observed in PE (4). Therefore, in this study the increased HTR8/SVneo cell invasion by ANXA4 overexpression may be due to ANXA4 overexpression-induced increased MMP2/9 expression.

The PI3K/Akt signaling pathway plays a critical role in mediating the growth-factor-dependent regulation of trophoblast growth and invasion. A previous study has stated that migration and invasion of trophoblast cells are suppressed through inhibition of the PI3K/Akt pathway (30). PI3K and



Akt are involved in regulating trophoblast invasion through the upregulation of MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression by epidermal growth factor (18). The literature also suggests that human chorionic gonadotropin stimulates trophoblast migration and invasion through PI3K/Akt signaling involving their downstream effector MMP-2 (17). In addition, studies indicate that eNOS is one of the downstream substrates of Akt that induces eNOS phosphorylation. It has been shown that placentas from PE patients exhibited reduced levels of eNOS (3, 5, 12). Studies indicate that eNOS produces nitric oxide (NO) and plays an important role in promoting vascular remodeling and trophoblast invasion in PE (5). A previous study showed that treatment with eNOS inhibitor L-NAME or PI3K/Akt inhibitor LY294002 abrogated the adrenomedullin-enhanced migration and invasion of myelomonocytic cells (25). These observations indicated the crucial role of the activated PI3K/Akt/eNOS pathway in promoting trophoblast cell migration and invasion.

In this study, the results showed that protein expression of PI3K, p-Akt, and p-eNOS was downregulated in human PE placentas and EVCTs from PE placentas, suggesting the possible relationship between the PI3K/Akt/eNOS pathway and PE progression. Furthermore, the PI3K/Akt/eNOS signaling pathway was activated by ANXA4 overexpression but was inhibited by ANXA4 knockdown. Given the role of the PI3K/Akt/eNOS pathway in promoting trophoblast cell invasion, we next verified whether the PI3K/Akt/eNOS pathway was involved in ANXA4 overexpression-mediated promotion of trophoblast invasion. More importantly, our results showed that inhibition of the PI3K/Akt pathway by LY294002 abrogated the ANXA4 overexpression-mediated promotion of cell proliferation and invasion, inhibition of cell apoptosis, and increase in MMP-2/9 protein expression and eNOS phosphorylation in HTR8/SVneo cells. Moreover, eNOS knockdown alone significantly abrogated the ANXA4 overexpression-induced promotion of trophoblast invasion and MMP2/9 expression, interestingly, even more so than LY294002 for MMP-2. MMP-2, along with MMP-9, is capable of degrading type IV collagen and is essential for invasion and metastasis. It can be speculated that the different degrees of effect of eNOS knockdown and LY294002 on MMP-2 expression may be related to our experimental conditions, such as antibody reactivity, cell type, LY294002 concentration, eNOS knockdown efficiency, etc. Taken together, these results indicated that ANXA4 overexpression promoted trophoblast invasion via the PI3K/Akt/eNOS pathway.

Our further in vivo assay revealed that ANXA4 overexpression alleviated PE-associated maternal clinical manifestations and histological injury along with an increase in protein expression of PI3K, p-Akt, and p-eNOS in placentas from L-NAME-induced PE rats. These results further supported the notion that activation of PI3K/Akt/eNOS pathway was involved in the ANXA4 overexpression-mediated alleviation of PE progression.

Although studies indicate the role of ANXA4 in promoting tumor cell migration and invasion in several human malignancies (11, 27), unlike cancer cells, trophoblastic invasion during placentation is controlled stringently in both space and time (1). Both initiation and the extent of trophoblast invasion are tightly modulated by feto-maternal cross-talk, which leads to a wide range of abnormalities when perturbed. From this perspective, more investigation concerning the mechanism by which ANXA4 promotes trophoblast invasion is required.

In conclusion, our findings demonstrate that ANXA4 expression is downregulated in human placentas in PE. ANXA4 overexpression may promote trophoblast cell invasion via the PI3K/Akt/eNOS pathway. Furthermore, ANXA4 overexpression alleviated PE progression in PE rats, associated with activation of the PI3K/Akt/eNOS pathway. This study provides a theoretical basis for PE pathogenesis, aiding to the identification of novel therapeutic strategies for PE.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.X. and X.Z. conceived and designed research; Y.X., L.S., B.Q., X.Y., J.L., and X.Z. performed experiments; Y.X., L.S., B.Q., X.Y., J.L., and X.Z. analyzed data; Y.X., L.S., B.Q., X.Y., J.L., and X.Z. interpreted results of experiments; L.S., J.L., and X.Z. prepared figures; J.L. and X.Z. drafted manuscript; Y.X., L.S., B.Q., X.Y., J.L., and X.Z. edited and revised manuscript; Y.X., L.S., B.Q., X.Y., J.L., and X.Z. approved final version of manuscript.

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