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ARTICLE

The potential role of pregnancy-associated plasma protein-A2 in angiogenesis and development of preeclampsia

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Abstract

Compromised placentation strongly predisposes to preeclampsia (PE) which is a severe complication of pregnancy. Pregnancy-associated plasma protein-A2 (PAPP-A2) has higher expression in the placenta than in any other tissues. However, the possible role of PAPP-A2 in placental development and in the pathogenesis of PE remains unclear. In this study, we aimed at exploring placental expression of PAPP-A2 in early- and late-onset of severe PE and its role in the mechanism inducing the development of PE. We found that expression of PAPP-A2 mRNA and protein was elevated in placentas from women with severe PE compared to control placentas and was localized to differentiated trophoblasts; higher in early-onset PE than that in late-onset PE. PAPP-A2 was expressed in the cytoplasm of both primary trophoblasts and HTR-8/SVneo cells. Elevated PAPP-A2 attenuated migration, invasion, explant outgrowth and network formation of trophoblast cells in vitro without affecting cell proliferation and apoptosis. PAPP-A2 attenuated trophoblast invasion and migration by restraining epithelial–mesenchymal translations via downregulation of the Hedgehog signaling pathway. Overall, the increased expression of placental PAPP-A2 is specific to different period of PE onset and PAPP-A2 may contribute to poor placentation and inadequate angiogenesis thereby leading to the development of preeclampsia.

Introduction

Preeclampsia (PE) is a severe disorder unique to human gestation that affects 2–8% of pregnancies and carries significant maternal–fetal morbidity and mortality [1, 2]. It is characterized by new onset of maternal hypertension and proteinuria after 20 weeks of gestation [3]. The pathogenesis of PE remains unclear, but it has been widely acknowledged that the condition originates from within the placenta [4]. While poor initial placentation strongly predisposes to early-onset preeclampsia (EOPE, <34 weeks' gestation), late-onset preeclampsia (LOPE, 34 weeks' onwards) is the condition largely associated with maternal cardiovascular or metabolic disorders [2, 5, 6]. Furthermore, delivery of the placenta (and fetus) remains the only curative recourse. Normal implantation involves invasion of trophoblasts into the endometrium. Inadequate trophoblast invasion into the maternal decidua coinciding with impaired spiral artery remodeling appears to be an early indication to a later progression in the development of PE [7].

Pregnancy-associated plasma protein-A2 (PAPP-A2, also known as pappalysin-2) was first identified in 2001 [8]. Its sequence of amino acid residues is 45% homologous with pregnancy-associated plasma protein-A (PAPP-A), and it is the only homolog of PAPP-A in the vertebrate genome [8, 9]. Together, these proteins make up the human pappalysin family [10]. Both are metzincin metalloproteases that specifically cleave insulin-like growth factor binding proteins (IGFBPs), resulting in local activation of insulin-like growth factors (IGFs) [11]. PAPP-A has been extensively studied for its biomarker roles in Down's syndrome [12], yet the physiological and pathological functions of PAPP-A2 are largely unknown.

Placental and serum PAPP-A2 is significantly increased in preeclampsia, as well as in HELLP syndrome and fetal

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growth restriction [13–16]. However, there is not much information about the expression pattern of PAPP-A2 in different stages of preeclampsia. PAPP-A2 is highly associated with the IGF signaling pathway that appears involved in the etiology of PE [11, 17]. IGFBPs, along with the enzymatic modifiers, PAPPA, PAPP-A2 or ADAM (a disintegrin and metalloprotease), may contribute to the regulation of placental differentiation and growth via the IGF pathway [13]. PAPP-A2 is also indispensable for postnatal growth and bone development via its involvement to the IGF pathway [18–21]. In zebrafish embryos, PAPP-A2 modulates bone morphogenetic protein (Bmp) and notch signaling by independent mechanisms [9]. However, the mechanism for the effects of PAPP-A2 on extravillous trophoblast (EVT) invasion remains unclear.

In this study, we hypothesized that PAPP-A2 might play an important role during placentation. To date, the physiological and potential pathological functions of PAPP-A2 in normal and severe PE have not been clarified. Therefore, the aim of this study was to investigate the expression pattern of PAPPA2 in the placentas of EOPE and LOPE and to investigate the potential role of PAPP-A2 in angiogenesis and placentation and the underlying mechanism in PE.

Materials and methods

Participants and tissue collection

A total of 36 women with singleton pregnancies complicated by severe PE (18 EOPE and 18 LOPE) were enrolled. Gestational age-matched controls were 18 pregnant women with uncomplicated preterm deliveries for the EOPE group, and 18 healthy pregnancies without complications for the LOPE group. Severe PE was defined as systolic pressure >160 mmHg and/or diastolic blood pressure >110 mmHg on at least two occasions, with proteinuria (>5 g/24 h), oliguria <500 mL/24 h or multiorgan involvement such as pulmonary edema, abnormal liver function, right upper quadrant pain, thrombocytopenia or fetal growth restriction [22–25]. EOPE was defined as onset of PE at <34 weeks' gestation, and LOPE was from 34 weeks' onwards [26]. Placental tissue samples of PE and controls were obtained from each participant immediately after delivery and stored at −80 ℃ until processed. Placental tissue samples from early pregnancy (5–12 weeks' gestation) were obtained from pregnancy termination and primary culture was performed immediately.

This study has been approved by the ethics committee at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Each participant provided written informed consent.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The cDNA was synthesized from total RNA using a reverse transcription kit (TaKaRa, Japan) and then stored at −20 °C until use. Quantification of transcripts was performed by a real-time PCR (Bio-Rad CFX96 system) using the SYBR Premix ExTaq Kit (TaKaRa, Japan). The PCR primers used were listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the data. All experiments were performed in triplicate.

Western blot assay

Cells and tissues were lysed in RIPA lysis buffer (Beyotiem, Shanghai, China) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Protein samples were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated sequentially with primary antibody (PAPP-A2, R&D Systems, Minneapolis, MN, USA; E-cadherin/ZO-1/ Vimentin/Snail/Slug, Cell Signaling Technology, Danvers, MA, USA; N-cadherin/GAPDH, Proteintech, Wuhan, China; Gli1/Gli2, Abcam,

Table 1 Sequences of the primers

Primer name	Sequences (5′-3′)
PAPP-A2-forward	TGTGCTTGTGAACTGTGAGC
PAPP-A2-reverse	CTCCGTCGTCAAAGTCGTTC
E-cadherin-forward	TTCTTCGGAGGAGAGCGG
E-cadherin-reverse	CAATTTCATCGGGATTGGC
ZO-1-forward	CAAGCCTGCAGAGTCCAAGC
ZO-1-reverse	TGAAGGTATCAGCGGAGGGA
Vimentin-forward	AGGCAAAGCAGGAGTCCACT
Vimentin-reverse	CGTTCCAGGGACTCATTGGT
N-cadherin-forward	GCAACGACGGGTTAGTCACC
N-cadherin-reverse	GACACGGTTGCAGTTGACTGAG
Gli-1-forward	GTCCCACACCGGTACCACTG
Gli-1-reverse	AAGCATATCTTGCCCGAAGC
Gli-2-forward	CACCAGAATCGCACCCACTC
Gli-2-reverse	CATTCTCTTTGAGCAGCGGTG
Snail-forward	TGCCCTCAAGATGCACATCC
Snail-reverse	GCCGGACTCTTGGTGCTTG
Slug-forward	AGACCCTGGTTGCTTCAAGG
Slug-reverse	CAGCCAGATTCCTCATGTTTGT
GAPDH-forward	GCAGGGGG GAGCCAAAAGGGT
GAPDH-reverse	TGGGTGGCAGTGATGGCATGG

Cambridge, UK) and then horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were detected by an enhanced chemiluminescent detection kit (Amersham, Biosciences, Uppsala, Sweden), and the image was captured using VisionWorksLS image acquisition and analysis software, and the EC3 imaging system (UVP LLC, Upland, CA, USA).

Immunohistochemistry (IHC)

IHC was performed as previously described [27]. In brief, paraffin-embedded tissues were deparaffinized and rehydrated in xylene and gradients of ethanol. After boiling in citrate buffer at 92–98 ℃ for 15 min to retrieve antigen, the slides were incubated with 3% H_2O_2 to quench endogenous peroxidase and were blocked with bovine serum for 30 min. The slides were then incubated with primary antibodies against PAPP-A2 (Novus, Littleton, CO, USA) overnight at 4 ℃. Controls were processed by omitting primary antibodies. The following day, the slides were incubated with biotinylated secondary antibody for 30 min. After incubation, the slides were stained with 3,3′-Diaminobenzidine (DAB) working reagent and counterstained with hematoxylin. Immunostaining scoring of PAPPA2 in the tissues was evaluated by three independent observers based on staining intensity [28].

Primary trophoblast and cell culture

Trophoblasts were obtained from placental villous tissues of early pregnancy as previously described [29]. Placental villous tissues were dissected and digested with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA). Trophoblasts were purified by Percoll (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation and then collected at density between 1.048 and 1.062 g/mL. Cell viability which was identified by trypan blue was over 95%. Cell identity was confirmed via staining with cytokeratin 7 (CK7, Abcam, Cambridge, UK). Primary trophoblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM)/high glucose (GIBCO, Waltham, MA, USA) medium with 15% fetal bovine serum (FBS, GIBCO, Waltham, MA, USA).

The human HTR-8/SVneo cell line was purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China) [27]. It was derived from invasive EVT transfected with simian virus 40 large T antigen48 and used as a common model for EVT migration and invasion. HTR-8/SVneo cells were maintained in DMEM/high glucose medium supplemented with 10% FBS. The cells were cultured at 37 ℃ in a 5% CO_2 humidified incubator. The small interfering RNA (siRNA) for silencing PAPP-A2 and a scrambled siRNA control were purchased from Genomeditech (Shanghai, China). The target sequence for PAPP-A2 was as follows: forward primer, 5′-GCUUAUCUCGG CAAUCAAAtt-3′; reverse primer, 5′-UUUGAUUGCCGA GAUAAGCgt-3′. Transfection was performed using lipofectamine 3000 regent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48–72 h, transfection efficiency was determined by mRNA and protein expression analysis.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 15 min, and then washed in phosphate buffered saline (PBS) and blocked with 10% goat serum for 1 h. Cells were subsequently incubated with the primary antibodies against PAPP-A2, CK7 or negative control (NC) overnight at 4 ℃. Cells were subsequently incubated with secondary Cy5 conjugated goat anti-rabbit antibody or FITC conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA, USA) at room temperature for 1 h. All slides were counterstained with DAPI for nucleus staining and analyzed under a fluorescent microscope (Olympus IX73, Tokyo, Japan).

Cell proliferation assay

HTR-8/SVneo cells were placed in 96-well plates in DMEM/high glucose medium containing 10% FBS until 60–65% confluence. The cells were then incubated with recombinant human PAPP-A2 (rPAPP-A2) (R&D, Minneapolis, MN, USA) at concentrations of 0, 1.5, 3, 7.5 or 15 nM. After incubating for 24–72 h, the medium was removed and replaced with 100 μL of fresh serum-free culture medium. Cell proliferation was determined by the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) as per the manufacturer's instructions. The absorbance was read at 450 nm. All procedures were conducted in sexplicates at least three times.

Cell apoptosis assay

HTR-8/SVneo cells were seeded in 12-well plates in a medium containing 1.5 nM rPAPP-A2, or PBS as the control. After 48 h, the cells were digested with 0.25% trypsin and re-suspended in binding buffer before incubation with fluorescein isothiocyanate (FITC)-Annexin V solution and propidium iodide (PI) as per the manufacturer's instructions (BD Biosciences, New York, NY, USA).

Migration and invasion assays

The effect of PAPP-A2 on migration of HTR-8/SVneo cells was assessed by the wound-healing assay. Cells were pretreated with 1.5 nM rPAPP-A2 for 48 h and then seeded in 6-well plates until 80% confluence. A sterile 20 μL tip was used to generate the wound. The wound was photographed at 24 h and the affected area assessed by Image J software, and later

compared to the initial wound area. For invasive assays, transwell inserts containing polycarbonate filters with 8 μm pores were coated with 50 μL matrigel matrix (1×10^4 mg/mL). After 48 h pretreatment with 1.5 nM of PAPP-A2, the HTR-8/SVneo cells were seeded into the upper chamber at a density of 5×10^4 cells/well with 100 μL serum-free DMEM medium. DMEM medium with 10% FBS acted as a chemoattractant in the lower chamber. After 24 h, the cells on the upper side of the inserts were removed by cotton swabbing. The remaining cells were fixed and stained with 0.5% crystal violet. The number of invading cells were photographed and counted. All experiments were done in triplicate wells and at least three times.

First trimester villous explant cultures

The placental villi were fully dissected before being explanted into the Millicell-CM culture dish inserts coated with matrigel and placed into 24-well culture plates. Serum free DMEM/F12 medium was added to the upper inserts and 10% FBS was added to the lower chamber. Explants were incubated with rPAPP-A2 (1.5 nM) or an equal volume of media for 72 h. Assessment of EVT outgrowth was performed as described previously [30]. EVT migration distance was measured by SPOT software (SPOT Imaging Solutions, Sterling Heights, MI, USA) from the villous tip to the distal edge. EVT outgrowths were quantified using the Adobe Illustrator software by dividing the area for 72 h by the initial area of the explant at 0 h.

Tube formation assay

Matrigel ~60 μL was layered into wells of the 96-well plate and then 5×10^4 pretreated cells (1.5 nM PAPP-A2 treatment, 50 nM PAPP-A2 siRNA transfection or 50 nM scrambled siRNA control transfection) were added and incubated at 37 ℃ for 8 h. Observations were made using an optical microscope (Olympus IX73, Tokyo, Japan). Phase-contrast images were taken at different time points to document developmental stages. Tube length and total number of branch points were assessed in four quadrants at ×10 magnification. Quantification was measured using Image J software. The assay was done in triplicate and repeated at least three times.

Statistical analysis

Results are presented as mean value ± standard error of mean (SEM) or as percentage of the control value from at least three independent experiments. Statistical analyses were performed with Prism 6.0 GraphPad software. Student's *t*-test was used to compare between two groups and one-way analysis of variance (ANOVA) was used to compare multiple groups. A P-value < 0.05 was considered significant.

Results

Characteristics of the participants

The demographic and clinical characteristics of the participants in the four study groups are shown in Table 2. The preterm and normal pregnancy groups were matched with EOPE and LOPE, respectively, in terms of maternal age, weight, gestational age at delivery and incidence of primiparae. All participants presented with PE had proteinuria, and both PE groups had systolic and diastolic blood pressures that were significantly higher than their matched control groups.

Upregulation of PAPP-A2 mRNA and protein expression in PE

Expression of the molecules that limit cytotrophoblasts invasion and differentiation is dysregulated during

Table 2 Demographic and clinical characteristics of patients enrolled in the study ($n = 18$ in each group)

	Preterm pregnancy	EOPE	*P*-value	Normal pregnancy	LOPE	*P*-value
Maternal age, years	30.50 ± 1.18	30.17 ± 1.05	>0.05	29.22 ± 0.86	30.56 ± 1.10	>0.05
Prepregnancy weight, kg	55.11 ± 1.79	55.89 ± 1.79	>0.05	52.28 ± 1.74	51.17 ± 1.58	>0.05
Primiparae(n)	6 (18)	6 (18)	NA	11 (18)	11 (18)	NA
Gestational age at diagnosis, weeks	NA	31.09 ± 1.32	NA	NA	35.99 ± 0.94	NA
Gestational age at delivery, weeks	32.43 ± 0.38	32.09 ± 0.37	>0.05	38.02 ± 0.18	37.57 ± 0.18	>0.05
Systolic BP, mmHg	111.44 ± 2.52	168.30 ± 3.10	<0.05	117.90 ± 2.52	161.70 ± 3.90	<0.05
Diastolic BP, mmHg	71.89 ± 1.29	110.20 ± 2.78	<0.05	73.89 ± 2.93	109.40 ± 2.60	<0.05
Proteinuria	− to +	++ to +++	NA	− to +	− to +++	NA

The data are shown as mean ± SEM

BP blood pressure, *EOPE* early onset preeclampsia, *LOPE* late onset preeclampsia, *NA* not determined or not applicable

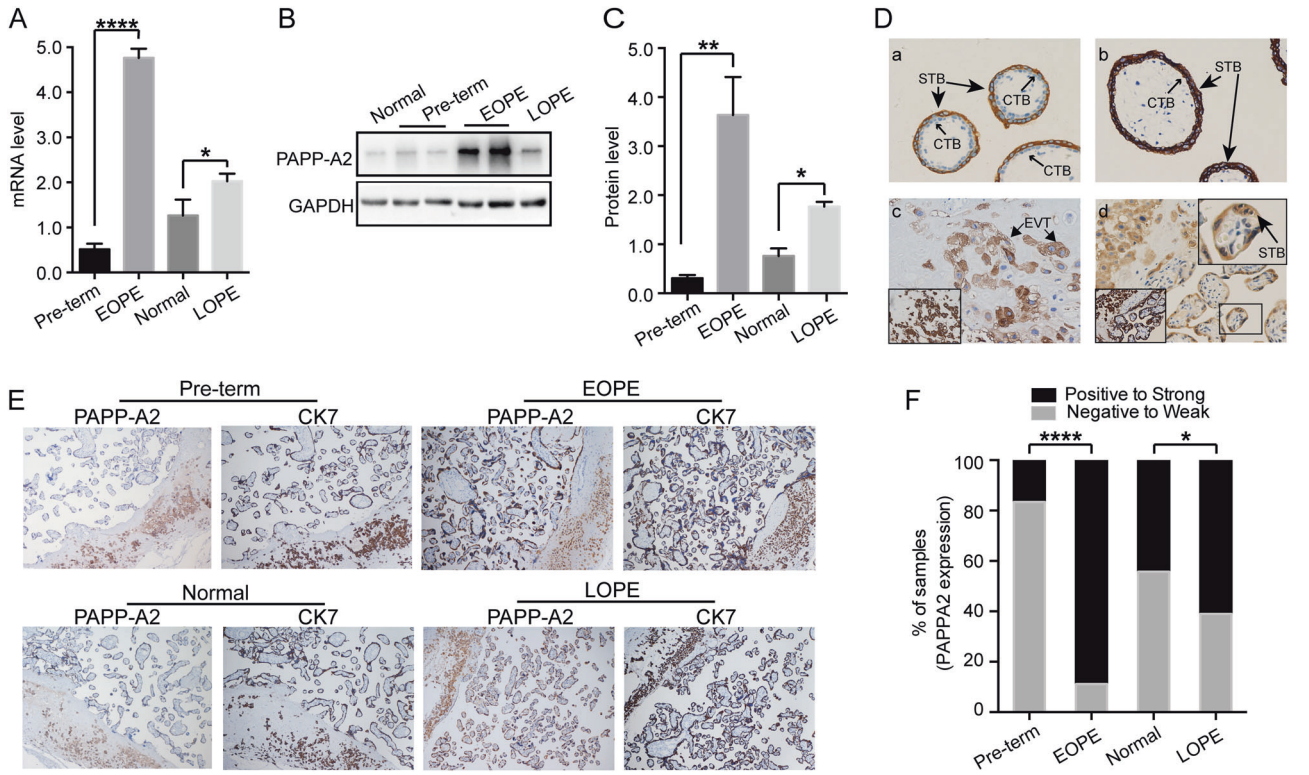

Fig. 1 PAPP-A2 (pregnancy-associated plasma protein-A2) mRNA and protein levels are elevated in preeclampsia placentas compared with gestation-matched controls. Real-time PCR (**a**), western blot (**b**, **c**), immunohistochemistry (**e**) detected the relative abundance of PAPP-A2 mRNA and protein in placental tissue lysate. Quantification analysis (**a**, **c**) and scoring of PAPP-A2 expression (**f**) showed that PAPP-A2 expression was elevated in EOPE (compared with gestation-matched preterm pregnancy) and LOPE (gestation-matched normal pregnancy). PAPP-A2 protein was localized to STBs but not in CTBs of the chorionic villi at early gestation (**d-**a). **d**-b showed CK7 staining to identify all trophoblasts. **d-**c and **d-**d demonstrated that CK7 positive (lower inserts) EVTs at BP showed prominent PAPP-A2 staining. **d-d** showed that STBs that are CK7 positive (lower inserts) stained for PAPP-A2. The upper insert is a partial enlargement of villus. EOPE severe early onset preeclampsia, LOPE severe late onset preeclampsia, STB syncytiotrophoblasts, EVT extravillous trophoblasts, BP basal plate. Data are expressed as mean ± SEM (*$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$)

development of PE. Therefore, we analyzed the levels of PAPP-A2 mRNA and protein in placental tissues from women with and without PE to evaluate if PAPP-A2 expression is affected during placental ischemia.

PAPP-A2 mRNA was detected by real-time PCR analysis of placental tissue. As shown in Fig. 1a, placental PAPP-A2 mRNA levels in the PE groups were higher than in controls. The EOPE group was significantly higher than the preterm control group, while the LOPE group was only slightly higher than the heathy control group (preterm, 0.51 ± 0.07 vs. EOPE, 4.76 ± 0.12; $p < 0.0001$; normal, 1.27 ± 0.20 vs. LOPE, 2.03 ± 0.10; $p < 0.05$). In addition, the expression of PAPP-A2 mRNA in placental tissue from women with EOPE was higher than that from those with LOPE. GAPDH was used to normalize the total RNA added during amplification. The results were expressed as the ratio of PAPP-A2 to GAPDH (Fig. 1a). Consistent with the mRNA results, PAPP-A2 protein was also present in higher levels in PE tissues than in controls (Fig. 1b, c; preterm, 0.31 ± 0.04 vs. EOPE, 3.63 ± 0.45; $p < 0.01$; normal, 0.76 ± 0.11 vs. LOPE, 1.77 ± 0.69; $p < 0.05$). Immunohistochemistry identified the level and localization of PAPP-A2 protein expression in placenta (Fig. 1d–f). PAPPA2-positive signals were observed in the syncytiotrophoblast layer of the villi and invasive extravillous trophoblasts at the basal plate zone, but not in the cytotrophoblasts at early gestation. EOPE showed stronger signal than its matched preterm group and LOPE group also had higher signal than matched normal pregnancy (Figs. 1e, f). Consistent with the above results, the intensity of the signal in EOPE group was significantly higher than that in LOPE group. These results indicated that PAPP-A2 expression is enhanced in PE.

PAPP-A2 expression in placental trophoblast cells

We conducted this experiment using primary placental trophoblasts isolated by an enzymatic digestion technique and the HTR-8/SVneo cell line. Immunofluorescent microscopy using PAPP-A2 antibody showed a positive signal in the cytoplasm of both primary trophoblasts and the immortalized trophoblast cell line (Fig. 2). Our results

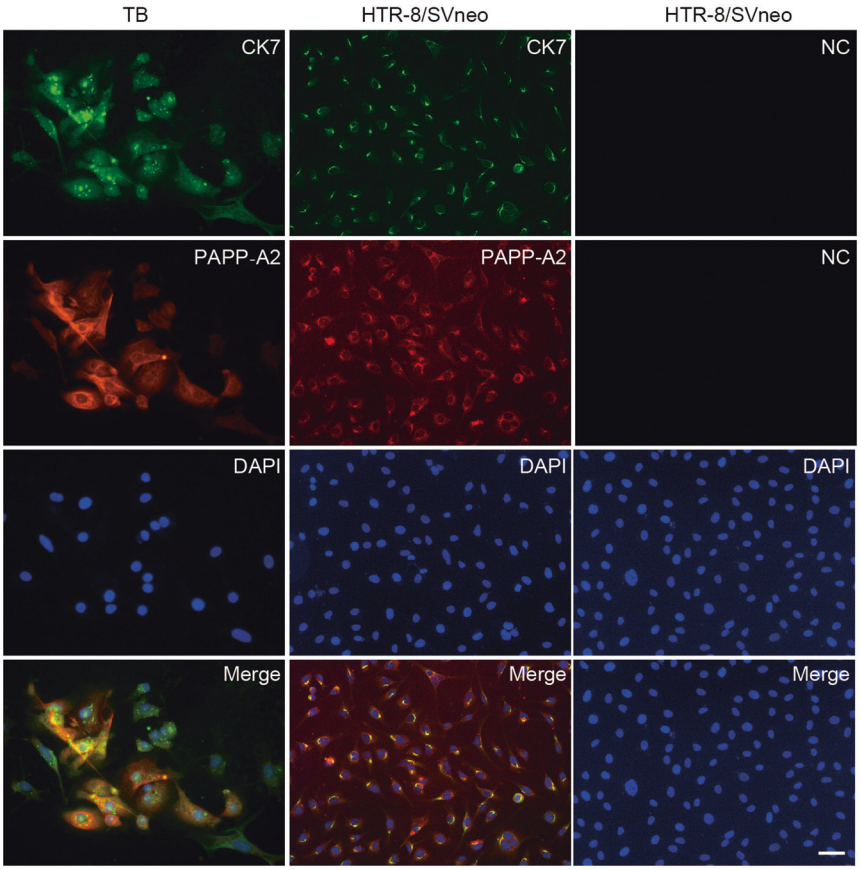

Fig. 2 Expression of PAPP-A2 (pregnancy-associated plasma protein-A2) in primary villous trophoblasts and HTR-8/SVneo cells. Immunofluorescent staining of the cells with PAPP-A2 antibody and monoclonal cytokeratin 7(CK7) antibody as shown in the photographs (bar = 50 μm). CK7 acts as the marker of placental trophoblasts

supported that PAPP-A2 protein was expressed in placental trophoblast cells.

PAPP-A2 inhibits trophoblast migration, invasion and tube formation

EVT migration and invasion are key events in human placentation. Incubation of HTR-8/SVneo cells with 0–15 nM of rPAPP-A2 for 48 h caused no significant effect on proliferation (Fig. 3a, $p > 0.05$). Furthermore, the effect of PAPP-A2 on apoptosis in HTR-8/SVneo cells was investigated via flow cytometry with FITC-Annexin V and PI staining. Cells exposed to rPAPP-A2 were not significantly different to controls (Figs. 3b, c, $p > 0.05$).

To determine the effect of rPAPP-A2 on EVT migration, we performed the wound-healing assay with HTR-8/SVneo cells. In the absence of rPAPP-A2 (control), HTR-8/SVneo cells migrated to the middle of the area at 24 h. However, after exposure to rPAPP-A2, the migration velocity of HTR-8/SVneo cells slowed significantly (Fig. 4a, b; 88.98 ± 1.74 vs. 42.40 ± 1.46). The first trimester human placental villous explants were cultured on matrigel to model villous outgrowth and invasion (Fig. 4c). Compared to the control group, the migration distance and area of outgrown cells treated with rPAPP-A2 were substantially decreased (Fig. 4d; 100 ± 0.00 vs. 57.40 ± 3.28).

The effect of PAPP-A2 on invasion of HTR-8/SVneo cells was studied using the transwell assay. Incubation of HTR-8/SVneo cells with rPAPP-A2 resulted in a significant decrease in cell invasion (Fig. 5d, e; control, 262.50 ± 5.14; rPAPP-A2, 120 ± 6.75; $p < 0.0001$). However, PAPP-A2 did not affect the proliferation and apoptosis of EVT within 72 h of observation. The suppressive effects of PAPP-A2 were detected in migration and invasion assays but did not impair cell proliferation and apoptosis.

To ascertain these findings on PAPP-A2, we performed siRNA-mediated PAPP-A2 knockdown. Transfection of PAPP-A2 siRNA into cells resulted in a significant reduction in PAPP-A2 mRNA and protein expression in HTR-8/SVneo cells compared with transfection of the control scramble siRNA (Fig. 5a–c; mRNA, 0.600 ± 0.058, 0.077 ± 0.011; protein, 0.4616 ± 0.047, 0.099 ± 0.025). However, knockdown of PAPP-A2 expression in HTR-8/SVneo cells did not affect invasion (Fig. 5d, e). The results indicated that only abnormally elevated PAPP-A2 inhibited the invasion of trophoblasts and knockdown of PAPP-A2 did not cause any effect.

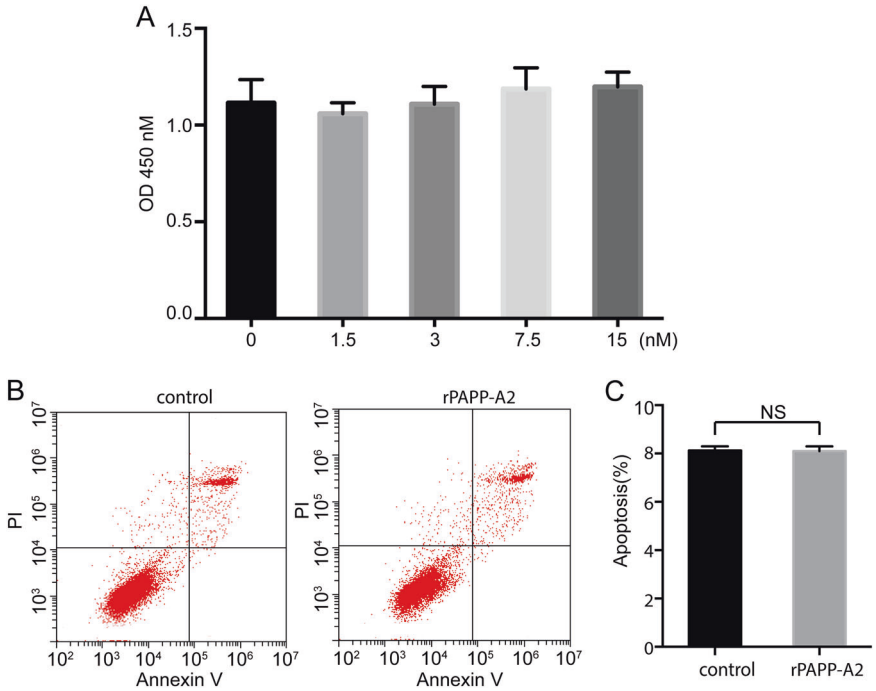

Fig. 3 PAPP-A2 (pregnancy-associated plasma protein-A2) shows no effect on proliferation and apoptosis of HTR-8/SVneo cells. **a** Proliferation of HTR-8/SVneo cells after treatment with recombinant PAPP-A2 (rPAPP-A2, 0–15 nM) for 48 h. The statistical significance of the differences between groups was determined by analysis of variance (ANOVA). **b**, **c** HTR-8/SVneo cells were harvested with recombinant PAPP-A2 (rPAPP-A2, 1.5 nM) for analysis with Annexin V staining. (NS means not significant)

PAPP-A2 suppresses tube formation of EVT

Remodeling of maternal spiral arteries increases placental blood flow thereby ensuring sufficient oxygen and nutrient supply to the fetus. As this process is associated with trophoblast invasion we examined tube formation in HTR-8/SVneo cells. These cells were pretreated with 1.5 nM of rPAPP-A2 for 24 h and then seeded into a 96-well plate. The rate of formation of classical tube-like structures was decreased in PAPP-A2 treated cells compared to controls (Fig. 5f, g; 69 ± 5.05 vs. 34.25 ± 3.88; $p < 0.01$). In accord with the above findings, knockdown of PAPP-A2 expression in HTR-8/SVneo cells did not affect tube formation of trophoblasts.

PAPPA2 suppresses trophoblast invasion by inhibiting EMT via the Hedgehog signaling pathway

In order to explore whether PAPPA2 inhibited invasion of trophoblasts by mediating epithelial–mesenchymal translations (EMT), we performed a series of RT-qPCR and western blot assays to detect EMT markers. We have found that elevated PAPP-A2 decreased the expression of Vimentin and N-cadherin (the mesenchymal markers) whereas E-cadherin, ZO-1 (the epithelial markers) were upregulated in trophoblast cells (Fig. 6a–c). These results demonstrated that PAPPA2 suppresses trophoblast invasion by restraining EMT. To further investigate the molecular mechanisms of PAPP-A2 in regulating EMT, we analyzed the Hedgehog signaling pathway, including Gli1, Gli2, Snail and Slug. The mRNA and protein levels of Gli1, Gli2, Snail and Slug were significantly decreased in PAPP-A2-treated HTR8/SVneo cells, compared with normal controls. (Fig. 6d–f).

Discussion

In this present work, we have analyzed the expression of PAPP-A2 in placenta under different pathological and physiological conditions and also questioned and investigated the role of PAPP-A2 in placental development and angiogenesis in vitro. Evidence for the functional role of PAPP-A2 in placentation was deficient and controversial in some aspects. To our knowledge, it is the first time that PAPP-A2 is shown to have no effect on trophoblast proliferation and apoptosis but can suppress trophoblast migration, invasion by inducing EMT which can be related with the pathogenesis of PE.

PE arises subsequent to impaired spiral artery remodeling which results in reduced placental perfusion. Poor placentation ensues, causing systemic pathophysiological changes in the maternal circulation [31]. It is becoming clear that the etiology of EOPE and LOPE, as well as intrauterine growth restriction may be caused by overlapping yet distinct underlying reasons. The pathogenesis of PE is established prior to the appearance of clinical symptoms. LOPE accounts for ~80% of PE cases worldwide with normal placental morphology and umbilical artery Doppler profiles while EOPE tends to be more severe with abnormal

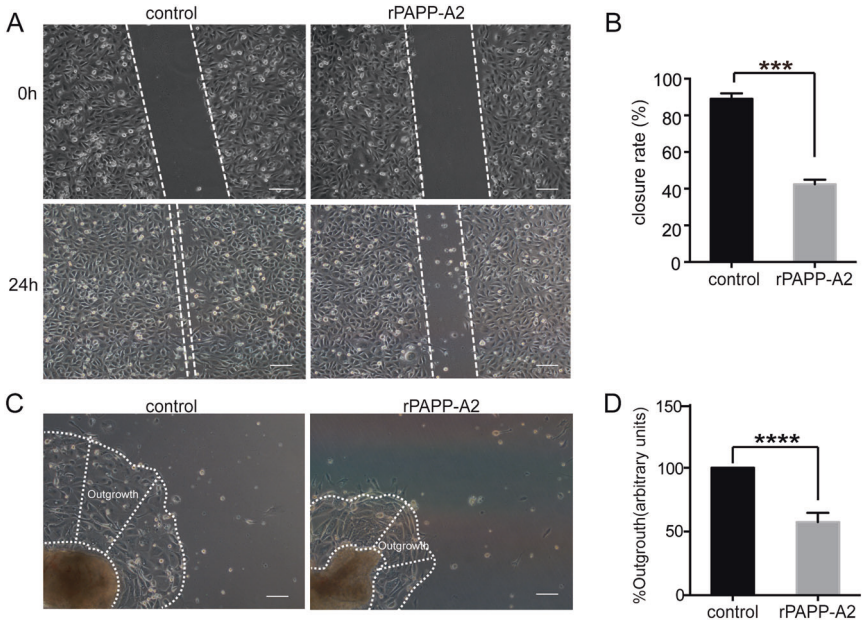

Fig. 4 PAPP-A2 (pregnancy-associated plasma protein-A2) suppresses the migration of trophoblast cells and the outgrowth of EVTs from human placental explant. **a**, **b** Migration of HTR-8/SVneo cells after treatment with recombinant PAPP-A2 (rPAPP-A2, 1.5 nM) for 0 and 24 h by wound-healing assay (Bar = 100 μm). **c**, **d** The outgrowth area of the placental explants treated with 1.5 nM rPAPP-A2 was significantly lowered compared with that of explants treated with an equal volume of media as a control for 72 h (Bar = 100 μm). Data are expressed as mean ± SEM. (***$p < 0.001$, ****$p < 0.0001$)

placental villous and vascular morphology [1]. Unlike previous studies, we have studied PAPP-A2 expression in EOPE and LOPE groups separately. PAPP-A2 synthesis was significantly higher in EOPE compared with preterm controls, whereas LOPE placentas produced slightly increased expression when compared with healthy controls. We then demonstrated differential PAPP-A2 expression in EOPE versus LOPE placentas, showing higher expression in EOPE that lead us to assume that the extent of increased PAPP-A2 expression seems to correlate with PE severity and the underlying mechanisms behind the different types of PE.

Immunohistochemistry was performed to identify specific sites and levels of PAPP-A2 expression in EOPE and LOPE placentas. Placental tissues from PE demonstrated increased levels of PAPP-A2, with higher levels in EOPE. Consistent with previous research results [13, 14], PAPP-A2 was expressed in syncytiotrophoblasts, invasive cytotrophoblasts but not in villous cytotrophoblasts. In preterm placenta, equivalent to the second trimester, a marked decrease staining was seen. PAPP-A2 is expressed in syncytiotrophoblasts throughout pregnancy and EVT in the basal plate showed intense expression of PAPP-A2. We speculated that PAPP-A2 participates in the differentiation of trophoblasts and the invasive phenotype of EVT.

Adequate uterine blood flow and intervillous space perfusion play important roles in sustaining normal pregnancies and fetal growth. They are achieved by extravillous trophoblast invasion and spiral remodeling of large diameter vessels [32]. The mechanism for the inhibitory effects of PAPP-A2 on EVT invasion remains unclear. Previous studies have identified that IL-11, partly via upregulation of PAPP-A2, impairs EVT invasion into the endometrium and endovascular EVT remodeling of the maternal spiral artery [33]. It has been reported that the presence of hypoxia responsive element (HRE) and two nuclear factor (NF)-kB binding sites within the PAPP-A2 promoter region, supports a link between hypoxia and elevated PAPP-A2 expression in the severe EOPE placenta [34]. Thus, the upregulation of PAPP-A2 in PE may be due to placental ischemia and hypoxia, resulting in poor placentation. Further research is required to fully comprehend the molecular mechanisms underpinning the development of PE.

In our study, additional rPAPP-A2 suppresses trophoblast cell migration, invasion and tube formation suggesting that PAPP-A2 participates in the pathogenesis of pregnancy-associated complications. Therefore, we propose that increased expression of placental PAPP-A2 may have a negative effect on trophoblast function but does not impair cell proliferation and apoptosis. This may lead to poor remodeling of maternal spiral arteries and the abnormal uteroplacental arterial blood flow associated with PE. Previous studies [14, 35] showed that PAPP-A2 was expressed in normal pregnancy and variations over gestation suggested that PAPP-A2 may have a physiological effect in maintaining a normal pregnancy. Once a certain threshold was exceeded, pathological effects might occur which may explain why knockdown of endogenous PAPP-A2 did not affect the migration and invasion of trophoblast. Thus, PAPP-A2 has the potential to be a therapeutic target or predictor of severe PE. Previous study shows that serum PAPP-A2 has good sensitivity and specificity for PE severity [13], further supporting its potential application in PE detection.

EMT plays crucial roles in carcinoma progression and metastasis and is associated with the loss of epithelial-cell

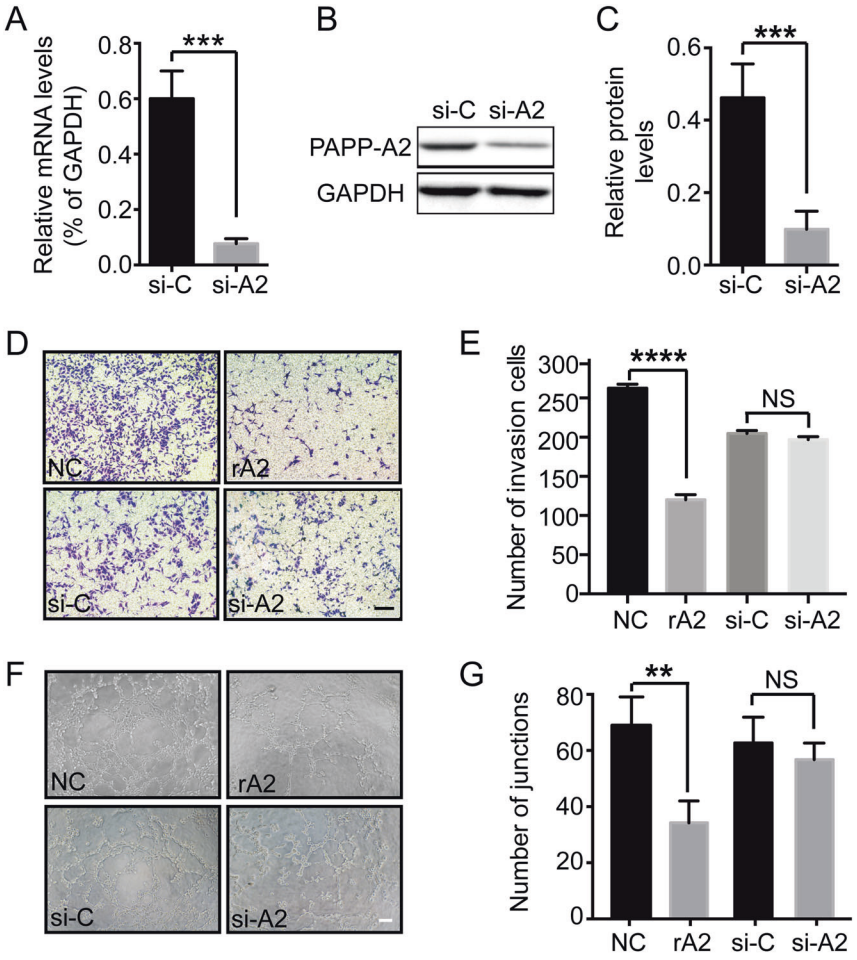

Fig. 5 PAPP-A2 (pregnancy-associated plasma protein-A2) suppresses the invasion and tube formation of trophoblast cells. Real-time PCR (**a**), western blot (**b**) and quantification of PAPP-A2 protein (**c**) demonstrated a significant reduction of PAPP-A2 mRNA and protein in HTR-8/SVneo cells transfected with siRNA specific for PAPP-A2 (50 nM, si-A2) compared with scrambled siRNA controls (si-C). **d**, **e**, The effect of treatment with 1.5 nM recombinant PAPP-A2 (rA2) or si-A2 on the invasion of HTR-8/SVneo cells by transwell assay (Bar = 20 μm). **f**, **g**, The network formation of HTR-8/SVneo cells cultured with 1.5 nM r-A2 or si-A2 assessed by the matrigel assay. (Bar = 100 μm). Data are expressed as mean ± SEM. (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, NS means not significant, NC means negative control)

adhesiveness and polarity, acquisition enhanced mobility and change to a mesenchymal phenotype [36]. Molecularly, EMT involves the decreased level of epithelial-type proteins E-cadherin and ZO-1 and the upregulation of mesenchymal markers including vimentin as well as EMT associated transcriptional factors, including Snail and Slug [37]. Remarkably, EVTs show a phenotype very similar to that of cancer cells, with regards to their capacity for invasion and migration [38]. The invasive phenotype of extravillous trophoblasts is associated with a decline in E-cadherin and increased levels of N-cadherin [39]. In our study, recombinant PAPP-A2 significantly induced mesenchymal–epithelial translations (MET) directing the downregulation of vimentin and N-cadherin expression and increased E-cadherin and ZO-1 expression. Therefore, these results indicated that PAPP-A2 was positively related to EMT markers and PAPP-A2 might suppress EVT invasion by restraining EMT process.

The Hedgehog signaling pathway plays a vital role in regulating cell differentiation during embryonic development and participates in the transformation of epithelial cells into mesenchymal cells in trophoblast cells [40, 41]. Gli1 and Gli2 are transcriptional regulator factors in the Hedgehog signaling pathway. It has been reported that Gli1 induces the expression of key EMT regulators including Twist, Snail and Slug and both Gli1 and Gli2 directly attenuates CDH1 gene encoding E-cadherin in human trophoblasts [42]. In our study, we demonstrated that PAPP-A2 downregulated the expression of Gli1 and Gli2 as well as EMT transcription factors Snail and Slug. Both Snail and Slug repress adherents (E-cadherin) and tight junction components (ZO-1) in ovarian cancer cells [43]. Through our results, we have testified that PAPP-A2 downregulated the invasive ability of trophoblasts by repressing the Hedgehog signaling pathway.

In conclusion, our study demonstrates that elevated placental expression of PAPPA2 expresses changes in placentas associated with severe PE and could regulate the biological functions of trophoblasts. The marked increase in expression in EOPE than LOPE was possibly due to prolonged placental ischemia and hypoxia. Moreover, PAPP-A2 is involved in trophoblast dysfunction via EMT by the Hedgehog signaling pathway. Our findings reveal a potential role for PAPP-A2 in regulating placental function and propose the possible underlying mechanism of PE. PAPP-A2 might be a promising predictor or therapeutic option for

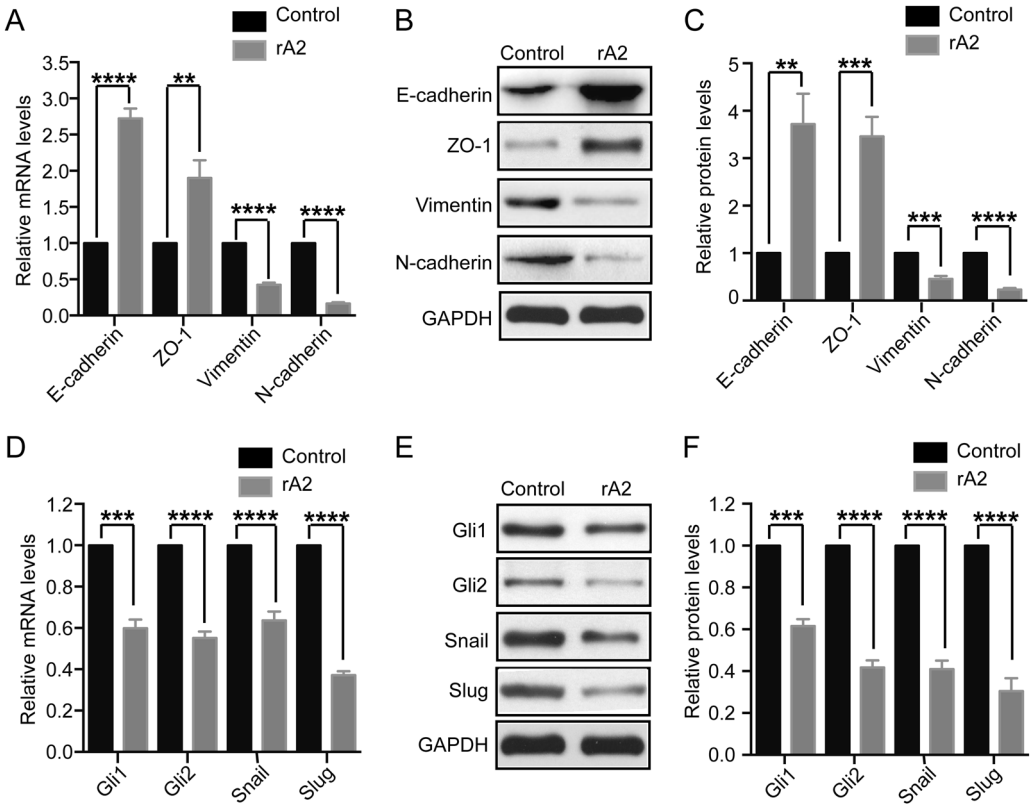

Fig. 6 PAPP-A2 (pregnancy-associated plasma protein-A2) suppresses trophoblast invasion by restraining Hedgehog signaling pathway mediated EMT. Real-time PCR (**a**) and western blot (**b**, **c**) analysis of E-cadherin, ZO-1, Vimentin and N-cadherin in HTR-8/SVneo cells treated with or without recombinant PAPP-A2 (rA2, NC). The mRNA and protein levels of Gli1, Gli2, Snail and Slug were detected by RT-qPCR (**d**) and western blot (**e**, **f**) analysis, respectively. Data are expressed as mean ± SEM. (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

severe PE progression. Our present research accentuates on the need for further research into the implications of PAPP-A2 in pregnancy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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