Placenta 87 (2019) 38–45

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Reduced ELABELA expression attenuates trophoblast invasion through the PI3K/AKT/mTOR pathway in early onset preeclampsia

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ARTICLE INFO

Keywords:
ELA
PI3K
AKT
Invasion
Preeclampsia

ABSTRACT

Introduction: Early onset preeclampsia is linked to abnormal trophoblast invasion, leading to insufficient recasting of uterine spiral arteries and shallow placental implantation. This study investigated ELABELA (ELA) expression and its involvement in the pathogenesis of early onset preeclampsia.
Methods: We used immunohistochemistry, quantitative PCR and Western blot to calculate ELA levels in the placentas. Transwell assays were utilize to assess the invasion and migration of trophoblastic Cells. Western blot was used to identify the concentrations of vital kinases in PI3K/AKT/mTOR pathways and invasion-related proteins in trophoblast cells.
Results: ELA was expressed in villous cytotrophoblasts and syncytiotrophoblasts in placental tissue. Compared with the normal pregnancies, ELA mRNA and protein expression was significantly reduced in early onset preeclampsia placentas. In the HTR-8/SVneo cells, when ELA was knocked down, the invasion and migration capability of cells decreased significantly, with MMP2 and MMP9 expression downregulated and the expression of important kinases in the PI3K/AKT/mTOR pathways being significantly decreased compared to the control group. Overexpression of ELA was on the contrary. Besides, while PI3K was blocked, the invasion and migration capability of HTR-8/SVneo cells and the expression of key kinases in PI3K/AKT/mTOR pathways were decreased significantly.
Discussion: ELA stimulates the invasion and migration of trophoblastic cells through activation of downstream PI3K/AKT/mTOR pathway and is complicit in early onset preeclampsia pathogenesis. Our research offers a potential novel treatment for PE.

1. Introduction

Preeclampsia (PE) is a serious systemic disease occurring during pregnancy in which proteinuria and hypertension are observed after 20 weeks of pregnancy. The incidence rate of preeclampsia is approximately 3%–8% annually [1–3]. It is a serious complication that endangers the health of both the mother and child [3–5]. Preeclampsia is a multifactorial disease that is caused by maternal, fetal and environmental factors without an effective treatment at present, although the pathogenesis remains unclear. Early onset preeclampsia was proposed by European and American researchers in the 1980s as a disease manifesting before 34 weeks of gestation [6,7]. This form of the disease has attracted much attention due to its potential for causing severe maternal complications and adverse perinatal consequences [8,9]. It is largely thought that insufficient infiltration of trophoblast cells and disordered remodeling of the uterine spiral artery can lead to superficial placental implantation, causing insufficient uterine and placental blood flow that leads to early onset preeclampsia [10–12]. The migration and invasion of trophoblastic cells are influenced by multiple factors and the precise molecular mechanisms underlying the pathogenesis of the disease remain unclear.

ELA, also known as Apela or Toddler, is a recently discovered 54 amino acid peptide hormone which binds and activates the APJ receptor. ELA is highly conserved among different species, suggesting that it is a key regulator of essential physiological functions [13,14]. It is known to be involved in the development of zebrafish embryos via

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https://doi.org/10.1016/j.placenta.2019.08.077
Received 9 March 2019; Received in revised form 22 July 2019; Accepted 7 August 2019
0143-4004/

promotion of APJ receptor signaling-mediated endoderm differentiation [13,14]. ELA regulates mammalian circulation and embryonic development [15]. Human ELA is detectable in adult kidneys, prostate and embryonic stem cells in addition to placental tissue [13,16]. The ELA/APJ axis can effectively protect the heart and kidneys, and induces vasodilation in adults [17–19]. ELA promotes the growth and self-renewal of human embryonic stem cells via activation of the PI3K/Akt pathway [16]. ELA has a critical role in the development of the placenta and heart and promotes angiogenesis during embryonic development [20,21]. It has been reported that Elabela-deficient mice develop preeclampsia [22], suggesting it has a role in the pathogenesis of preeclampsia, but the specific mechanism remains unknown.

ELA is highly expressed in human placental trophoblasts. The placenta lacks innervation [23]. Both ELA and APJ have been found to be simultaneously expressed in the placenta, suggestive of potential autocrine regulation of placental memory via the ELA/APJ pathway. Exogenous ELA has also been shown to significantly increase the invasiveness of trophoblasts, suggesting that ELA has a paracrine effect on trophoblast differentiation [20]. ELA can induce cell invasion through both autocrine and paracrine signaling and may promote subsequent spiral artery reconstruction that prevents the development of preeclampsia [20], although further research is required to confirm the underlying mechanisms.

Many investigations have demonstrated that the PI3K/AKT pathway is involved in the pathogenesis of preeclampsia [24,25], with abnormalities being implicated in the abnormal development of the placental labyrinth [26,27]. Downregulation of p-AKT in extravillous trophoblasts affects trophoblast proliferation and invasion [28]. Together, these observations suggest that the PI3K/AKT pathway is involved in trophoblast invasion, performing an important role in preeclampsia.

It is generally accepted that cell invasion and migration are closely related to the activity of the two proteases, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) [29–31]. Both MMP-2 and MMP-9 promote cell invasion by degrading collagen type IV [32,33], the principal component of the extracellular matrix and basal membrane of the endometrium [34,35], and are proteases closely related to trophoblast invasion [36–38].

In this study we aimed to study the hypothesis that ELA plays a role in the mechanism of trophoblast invasion and migration through the PI3K/AKT/mTOR pathway. To test this hypothesis, the expression of ELA in placental villi was measured and then ELA expression in the placentas of women with early onset preeclampsia was compared with that in healthy pregnant women. Finally, the migration and invasion of trophoblasts in response to different ELA concentrations were measured *in vitro*, in addition to assessing the levels of related signaling cascade and invasion-related proteins, with the goal of ascertaining the potential role of ELA in the pathogenesis of early onset preeclampsia.

2. Materials and methods

2.1. Patient and placenta information

Ethical approval was granted by the Qingdao Municipal Hospital and written informed consent was provided by all patients. Placentas were collected from pregnancies that fulfilled the following criteria: (1) Normal pregnancy (no complication, no proteinuria, blood pressure < 140/90 mm Hg); (2) Early onset preeclampsia (proteinuria and hypertension) occurring after 20 weeks but before 34 weeks of gestation, with systolic blood pressure ≥ 140 mm Hg or/and diastolic pressure ≥ 90 mm Hg; urine protein levels over 24 h ≥ 300 mg). All pregnant women were non-smokers, nulliparous and gave birth by cesarean section. Patients with other complications were excluded. Maternal age and body mass index were matched in both groups.

During cesarean section, placentas were collected under sterile conditions after delivery. Tissue samples were removed from the central region of the placenta and processed within 30 min. All samples were repeatedly rinsed with cold phosphate buffered saline (PBS), with one piece placed in formaldehyde for immunohistochemistry and another quickly frozen and maintained at −80 °C until required for subsequent RNA and protein extraction.

2.2. Cell culture, RNA interference and transfection with plasmids

Human HTR-8/SVneo first trimester cytotrophoblast cells [39] were acquired from the Chinese Academy of Sciences in Shanghai. Cells were cultured in DMEM (Invitrogen, USA) containing 10% FBS plus penicillin/streptomycin and maintained at 37 °C in an atmosphere containing 5% CO_2.

Small interfering RNA (siRNA) sequences used in this study were transfected into cells for 24 h, in accordance with the manufacturer's protocol (Shanghai GenePharma Co., Ltd, Shanghai, China). siRNA sequences were as follows. Targeting ELA (siRNA-ELA-sense: 5′-GUGAU UCUCGUGCCUCAACTT-3'; siRNA-ELA-antisense: 5′-GUUGAGGCACGA GAAUCACTG-3′) and control siRNA (scramble-sense; 5′-GAGUGGGUC UGGGUCUUCCCGTT-3'; scramble-antisense; 5′-CGGGAA GACCCAGAC CCACUCTG-3′).

HTR-8/SVneo cells were transfected with over-expressed ELA or blank control plasmids for 24 h following the manufacturer's protocols (Shanghai GenePharma Co., Ltd, Shanghai, China).

2.3. Histology, H&E staining and immunohistochemical analysis

Polyclonal antibodies against ELA were produced as previously described [13] by BGI, Beijing, China. The placental tissue samples were cut into 5 mm pieces, fixed in formaldehyde, paraffin embedded, dewaxed in xylene, then hydrated through a rising gradient of alcohol concentrations.

H&E staining: Some sections were stained with hematoxylin, blue color developed in ammonia water, dyed with eosin, then dehydrated, cleared, sealed with neutral gum then observed using light microscopy.

Antigens were retrieved by boiling unstained sections in citrate buffer then cooling naturally. Endogenous peroxidase activity was terminated by incubating sections at room temperature for 10 min in 3% hydrogen peroxide. A polyclonal rabbit anti-human ELA antibody (dilution 1:200) was used as a primary antibody to stain sections overnight at 4 °C. A Goat anti rabbit antibody was then added as a secondary antibody (dilution 1:10,000, Cell Signaling Technology, Danvers, MA, USA) at room temperature or 37 °C for 30 min. After rinsing in PBS, DAB (Beijing, China) was applied for color development for 5–10 min, followed by sample dehydration, clearing, sealing then examination using a light microscope.

2.4. qRT-PCR

Total placental RNA was isolated using an RNAiso Plus kit (TaKaRa, China), then cDNA generated using a 1 μL reaction system (TaKaRa, China) which was then stored at −20 °C until required for experimentation. The following primers were used: 5'-

CTGAGGTTTGTCACTAGAATGTGAA-3' (forward) and 5'-

TAAGCAATCACGCTGTTGGCATCA-3' (reverse) (360 bp) for ELA, 5′-ACTGCCACCCAGAAGACT-3' (forward) and 5′-GCTCAGTGTAGCCCA GGAT-3' (reverse) (292 bp) for GAPDH. Real time amplification was performed in a 20 μl volume by mixing 10 μl of SYBR Green Master Mix (TaKaRa, China) with 2 μl forward and reverse primers, 0.5 μl of template cDNA and 7.5 μl water. Ninety six well plates were then placed in the ABI7500 sequence detector (Applied Biosystems, CA, USA). After analysis using real-time PCR, relative expression of PCR products was calculated from the Ct values of the target gene in comparison with values for the GAPDH endogenous control using the ΔΔCt method. Melt curves were used to ascertain whether amplification had been successful.

2.5. Western blot analysis

One hundred μg placental tissue were cut into small fragments and 300 μl of protein lysis buffer (PIC/PMSF) added. Samples were then homogenized on ice, centrifuged at 12,000 g for 15 min at 4 °C and the supernatants recovered and stored at −20 °C. For the HTR8/Svneo cells, RIPA lysis buffer (Beyotime, Shanghai, China) was used to lyse the cells on ice while agitating for 30 min, followed by centrifugation at 12,000 g at 4 °C for 20 min. A Bradford assay was utilized to quantify protein concentrations in all samples. Equal quantities of each sample (20 μg of protein) were then separated on 15% SDS-PAGE gels for 30 min at 80 V, then for 1 h at 120 V on ice. Proteins were subsequently transferred onto PVDF membranes for 1 h which were then washed with PBS-Tween (PBST) and incubated with primary antibodies at 4 °C overnight. Primary antibodies used in Western blotting were: rabbit polyclonal anti-ELA, MMP2, MMP9, PI3K, phospho (p)-PI3K, AKT, pAKT, mTOR (all diluted 1:1,000, Abcam, Cambridge, MA, USA) and anti-GAPDH (diluted 1:2,000, Abcam, Cambridge, MA, USA). Membranes were then washed with PBST three times for 10 min each. HRP conjugated goat anti-rabbit secondary antibody (diluted 1:2000, Abcam) was then added to blots for 1 h, and membrane protein levels assessed using enhanced chemiluminescence (Amersham Life Science, USA) by analysis using comparative protein band densitometry as assessed using an MSF-300G scanner (Microtek Laboratory, United States).

2.6. Cell migration and invasion assays

Invasion assays were conducted using 24-well transwell plates (Costar; Corning, NY, USA). Transwell chambers were initially coated with Matrigel. HTR8/SVneo cells were then suspended in serum-free medium and 10^4 cells placed into the upper chamber and 600 μl medium containing 10% FBS into the lower chamber. After 24 h of routine culture, the non-invasive cells on the surface were gently removed with a cotton swab. The invading cells were fixed with methanol for 30 min, and the chamber dried then stained with 0.5% crystal violet for 20 min. Cells in 5 random fields (× 100) were counted. The cell migration assay was conducted using the same protocol except that the Matrigel pre-coating was omitted.

2.7. LY294002 inhibition

LY294002 (10 μmol/L) was dissolved in DMSO (0.5%) (Abmole Bioscience, USA). HTR-8/SVneo cells cultured in normal medium for 24 h were denoted the control group. LY294002 group cells were cultured in medium containing 10 μmol/L LY294002 for 24 h. LY294002 + ELA group cells were cultured in medium containing 10 μmol/L LY294002 for 24 h then transfected with over-expressed ELA plasmids for 24 h. The migration and invasion of the cells were compared across the three groups. Western blot analysis was used to determine protein expression of ELA, pAKT and mTOR in the three groups.

2.8. Statistical analysis

Each experiment was repeated three times. The data are represented as means ± SD and assessed using SPSS v22.0 statistical software. Differences between groups were calculated using a one-way analysis of variance or non-paired two-tailed *t*-test, as appropriate. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical findings

Patient clinical characteristics are displayed in Table 1. Thirty early onset preeclampsia patients displayed a significant increase in systolic and diastolic blood pressure and were administered magnesium sulfate to prevent eclampsia and dexamethasone to promote fetal lung maturation. In these patients, the incidence of placental abruption was 6.67%, fetal growth restriction 20% and premature delivery was 83.33%. No such events occurred in the normal group.

3.2. ELA expression was decreased in early onset PE placentae

H&E staining and immunohistochemistry were used to evaluate tissue sections (Fig. 1), and qRT-PCR and Western blotting (Fig. 2A and B) to locate and quantify ELA levels in placental samples. Immunohistochemistry revealed that ELA was strongly expressed in trophoblasts and syncytial trophoblasts in the placenta. The expression of ELA in placental tissue from normal full-term pregnancies was higher than that in tissue associated with early onset preeclampsia (Fig. 1). As shown in Fig. 2A, the expression of ELA in the placental tissue of patients with early onset preeclampsia (EOPE) was significantly lower than that of normal pregnant women. In addition, we measured the expression of ELA protein in placentae using Western blotting. The expression of ELA protein in placental tissue of normal full-term pregnant women was significantly greater than in the early onset preeclampsia group (Fig. 2B). These results are consistent with the immunohistochemistry findings, suggesting that ELA is significantly reduced in early onset preeclampsia patients.

3.3. Knock down of ELA inhibits cell migration and invasion

ELA expression was found to be lower in early onset preeclampsia placentae and so the biological roles of ELA in trophoblast migration and invasion were next evaluated, investigating whether it plays a crucial role in the progression of early onset preeclampsia. HTR8/SVneo cells were transfected with ELA-specific siRNA for 24 h. ELA protein expression was significantly reduced in these cells compared with cells transfected with scrambled control siRNAs, as confirmed by Western blotting (Fig. 3A). Transwell migration assays revealed that the migratory capability of the HTR8/SVneo cells was significantly suppressed after ELA knock-down (Fig. 3B). The number of ELA-silenced HTR8/SVneo cells that penetrated the membrane was significantly reduced relative to numbers of cells treated with scrambled siRNA (Fig. 3C). In summary, knock-down of ELA lessened the ability of HTR8/SVneo cells to invade and migrate.

MMP2 and MMP9 expression was found to be closely related to invasion and migration. The influence of ELA on MMP2 and MMP9 expression in HTR8/Svneo cells was measured. Western blot analysis demonstrated that knockdown of ELA significantly reduced MMP2 and MMP9 protein expression, an effect not observed in the scramble group (Fig. 3D). These observations are consistent with the results of ELA knock down in which reduced invasion and migration by HTR8/Svneo cells were observed.

3.4. ELA overexpression promotes cell migration and invasion in HTR-8/SVneo cells

To further corroborate the role of ELA in trophoblast invasion, ELA was overexpressed in HTR-8/SVneo cells. As shown in Fig. 4A, ELA protein concentration was significantly up-regulated in the ELA group compared with controls. As expected, the invasive and migratory capabilities of HTR-8/SVneo cells were significantly enhanced following ELA overexpression (Fig. 4B and C). As shown in Fig. 4D, MMP2 and MMP9 levels increased markedly in the ELA overexpressed group compared with the control group. Together these results support the hypothesis that ELA promotes invasion and migration in HTR8/Svneo cells.

Table 1
Demographic characteristics of the study population.

Characteristic	Normal (n = 30)	EOPE(n = 30)
Maternal age (years)	31.50 ± 2.76	32.47 ± 4.52 [p = 0.3221]
Gestational age at delivery (weeks)	39.40 ± 0.73	32.80 ± 3.03 [p < 0.001]
BMI (kg/m^2)	28.46 ± 3.75	30.24 ± 3.82 [p = 0.073]
Systolic blood pressure	120.30 ± 7.17	168.37 ± 17.29 [p < 0.001]
Diastolic blood pressure	76.30 ± 6.49	106.87 ± 8.0613 [p < 0.001]
Birth weight (g)	3388.33 ± 368.52	1821.00 ± 742.99 [p < 0.001]

EOPE = Early onset preeclamptic; BMI = body mass index; BP = Blood pressure.

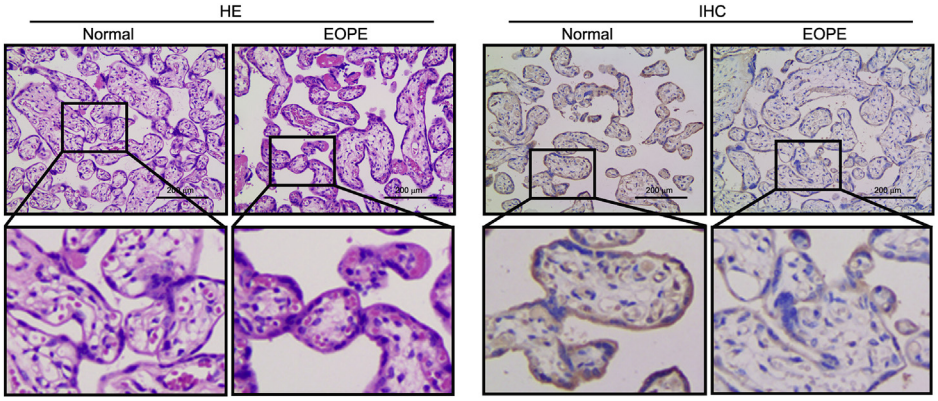

Fig. 1. ELA was expressed and localized in placenta of Normal and EOPE (Early onset preeclampsia) group by HE and immunohistochemistry (IHC) (× 200). Up, HE detected. Down, IHC detection of ELA expression. Bar = 200 μm.

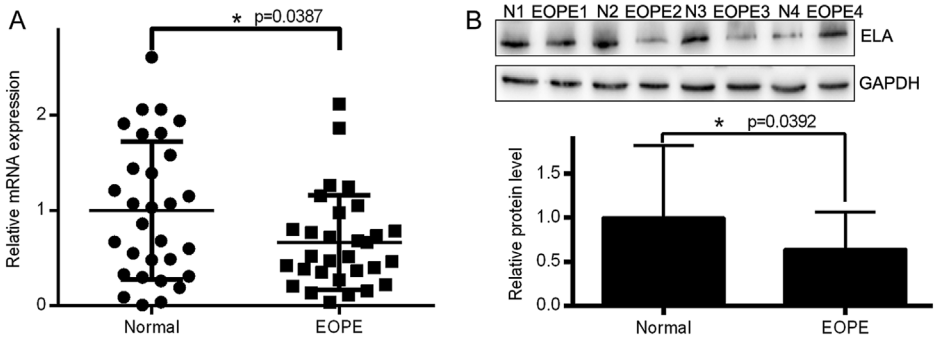

Fig. 2. ELA mRNA and protein expression in placental tissue showed significantly decreased in the early onset PE group opposed to healthy controls (P = 0.0387, < 0.05; P = 0.0392, < 0.05). A, Quantitative Real-time PCR determinated ELA mRNA in placenta, with quantifcation relative to GAPDH. B, Western blot determinated the expression of ELA protein in placenta in EOPE group and normal group with quantifcation relative to GAPDH. Relative mRNA and protein levels were calculated using the mean ± SD of three independent experiments.

3.5. ELA modulates the PI3K/AKT/mTOR signaling pathway in HTR8/SVneo cells

Various factors regulate trophoblastic invasion via the PI3K/AKT pathway [40,41]. In order to explore the mechanisms by which ELA promotes trophoblast invasion, the activation of key kinases in the PI3K/AKT/mTOR pathway was assessed in HTR8/SVneo cells. Western blot analysis was employed to establish protein expression. As shown in Fig. 5A, knockdown of ELA significantly decreased the expression of pPI3K, pAKT and mTOR as opposed to the scramble group. As shown in Fig. 5B, overexpression of ELA significantly upregulated pPI3K, pAKT and mTOR. No differences in total PI3K or AKT levels between groups were detected in the HTR8/SVneo cells. These data suggest that ELA operates through the PI3K/AKT/mTOR signaling pathway in HTR8/SVneo cells.

To further confirm that ELA affects trophoblast invasion through the PI3K/AKT/mTOR signaling pathway, HTR8/SVneo cells were treated with the PI3K inhibitor LY294002 to investigate the invasion and migration of trophoblasts and the expression of proteins downstream of PI3K. As shown in Fig. 6A and B, blockade of PI3K(LY) reduced migration and invasion compared with the control and LY + ELA group HTR8/Svneo cells. The invasion and migration behavior of control group and LY + ELA group cells was essentially the same. pAKT and mTOR protein levels were significantly reduced compared with the control and LY + ELA group HTR8/SVneo cells (Fig. 6C), while there were no significant differences in pAKT or mTOR levels in the control or LY + ELA groups. These results suggest that pAKT and mTOR are downstream effectors of the PI3K signaling pathway. These results indicate that ELA can inhibit the invasion and migration of trophoblasts through the PI3K/AKT/mTOR signaling pathway.

4. Discussion

Preeclampsia is the most common complication of pregnancy and the principal contributor to maternal mortality [42]. At present, there is no effective treatment for this condition, clinicians generally delivering the placenta, leading to a premature birth that seriously endangers the health of the newborn, especially in cases of early onset preeclampsia [43]. The specific mechanism underlying this condition remains unclear. It is generally believed that the pathogenesis of early-onset preeclampsia is linked to inadequate trophoblast invasion [44–46]. In this study, we found that ELA mRNA and protein levels decreased in early onset preeclamptic placentas compared with those that were healthy (Fig. 2). In HTR8/SVneo cells, knockdown of ELA inhibited cell

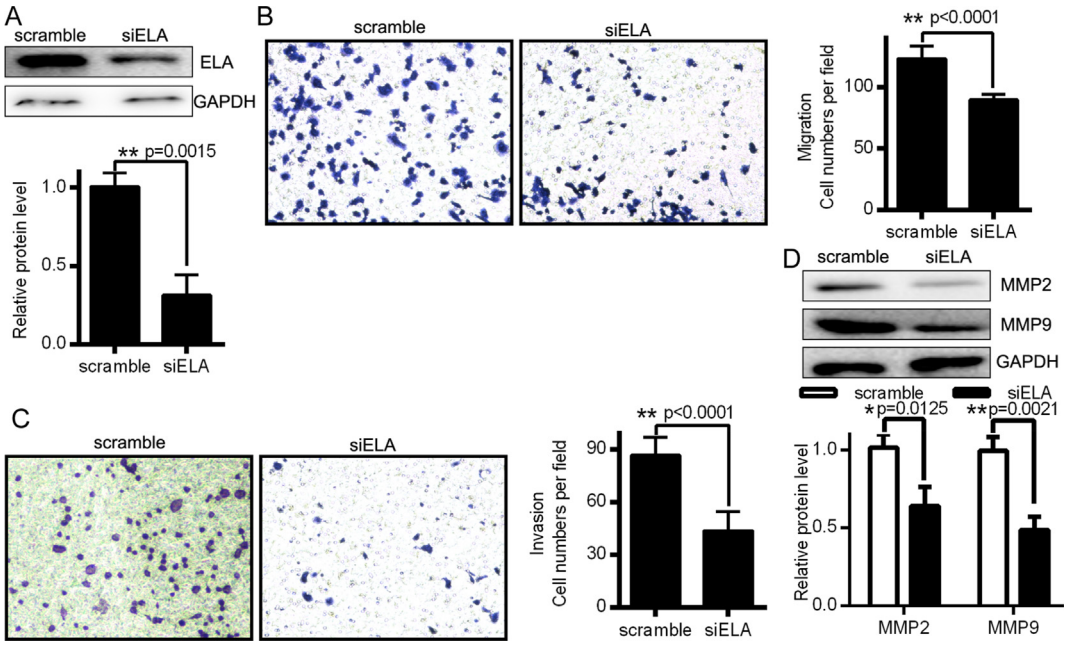

Fig. 3. Reduced expression of ELA affected the invasion and migration in HTR8/SVneo cells. A, ELA protein expression was significantly reduced in HTR8/SVneo transfected cells with si-ELA, compared to scramble transfected cells. B, In si-ELA cells, cells migration were decreased compared with scramble using the Transwell migration assay. C. Cells invasion were determined using the Matrigel invasion assay. D Knocking down ELA reduced MMP2 and MMP9 protein expression compared to the scramble group. (N = 3) (*P < 0.05, **P < 0.01).

migration and invasion (Fig. 3) and reduced the activation of PI3K and Akt (Fig. 5), while overexpression of ELA promoted cell migration and invasion (Fig. 4) and activation of PI3K and Akt (Fig. 5). In addition, following blockade of PI3K signaling, the migration and invasion capability of cells decreased significantly, in addition to the significant decrease in levels of pAKT and mTOR (Fig. 6). From these results, we infer that ELA participates in trophoblast invasion and migration via the PI3K/Akt/mTOR signaling pathway.

ELA was recently found to be the second endogenous peptide ligand for the APJ receptor [13,14]. It has been found that ELA participates in the regulation of blood pressure via the binding of APJ [47]. The expression of ELA was shown to decrease in both hypertension patients and in a rat model [48]. Animal studies have demonstrated that pregnant ELA-, but not Apelin, -knockout mice developed symptoms of preeclampsia such as proteinuria and hypertension [20]. Abnormal placental formation is closely related to the onset of preeclampsia [49].

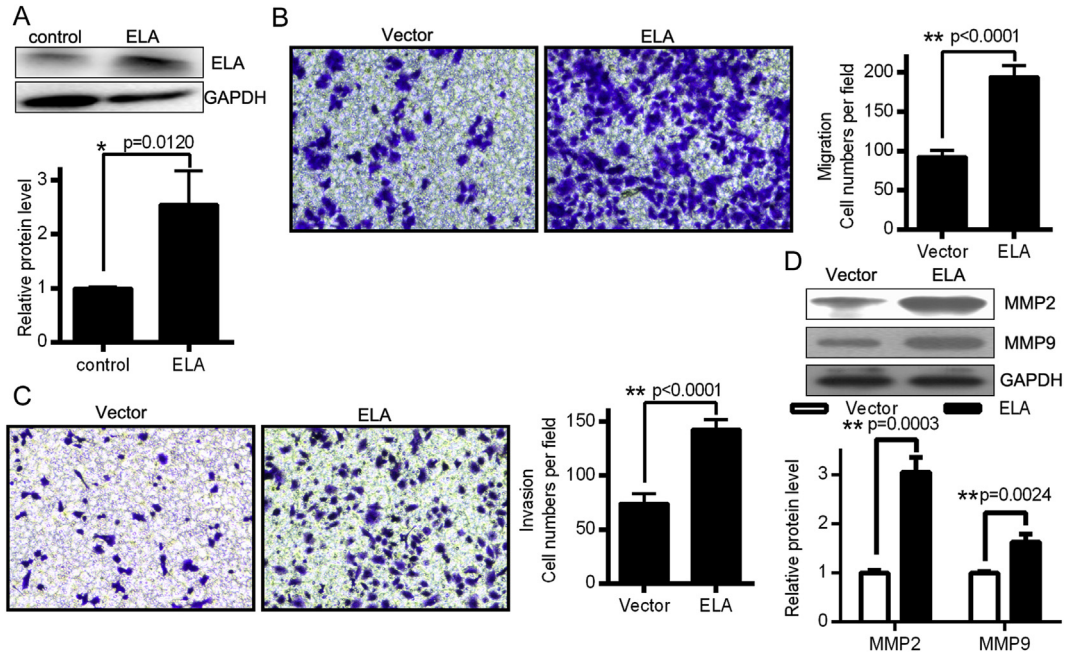

Fig. 4. Increased the expression of ELA affected the invasion and migration in HTR8/SVneo cells. A, Overexpress ELA in HTR8/SVneo cells, ELA protein increased significantly opposed to the vector group (*P < 0.05). B, While overexpress ELA, cells migratory abilities were markedly enhanced compared with vector group using the Transwell migration assay (**P < 0.01). C, Overexpress ELA, cells invasion were increased using the Matrigel invasion assay (**P < 0.01). D, Overexpress ELA, the expression of MMP2 and MMP9 protein was raised compared to the vector group (**P < 0.01). N = 3.

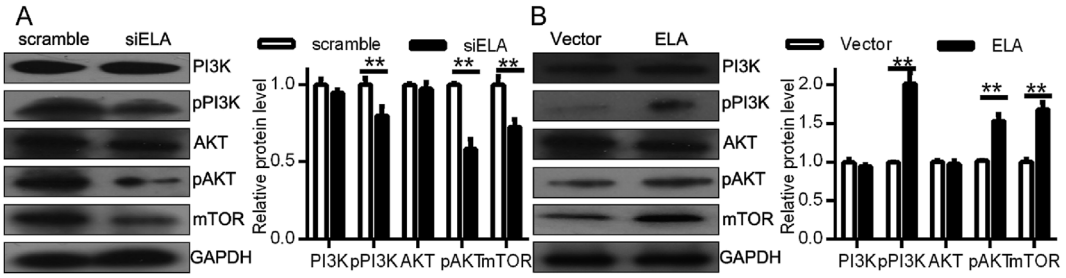

Fig. 5. Western blot was employed to assess the expression of crucial proteins complicit in the PI3K/AKT/mTOR pathway in HTR8/SVneo cells. A, The PI3K/AKT/mTOR pathways were inhibited by ELA knockdown in HTR8/SVneo cells. B, The PI3K/AKT/mTOR pathways were activated by ELA overexpression in HTR8/SVneo cells. Data are displayed as the mean ± SD of three seperate experiments. (N = 3) (*P < 0.05, **P < 0.01).

ELA deficiencies have been found to manifest as placental labyrinth and angiogenesis disorders, with decreased cell proliferation and increased apoptosis [50]. Contrary to previous opinions, a study of women with late onset preeclampsia, rather than early onset preeclampsia, found that ELA levels were significantly reduced [51], another study finding that Elabela was not significantly associated with the onset of preeclampsia [52]. These inconsistencies may be related to differences in specimens, the small numbers studied and differences in assay kits and test environment. In the present study, IHC staining indicated that ELA was expressed in placental cytotrophoblasts and syncytial trophoblasts (Fig. 2). Abnormal down-regulation of ELA mRNA and protein levels in the early-onset PE group (Fig. 2) suggested that ELA is related to early-onset preeclampsia. Very few pregnant women treated with magnesium sulfate and dexamethasone experienced no complications, all giving birth by cesarean section at about 32 weeks. Investigations of such women will continue through the collection of additional cases as they arise. In order to further study the possible mechanisms of the involvement of ELA, we measured the migration and invasion of trophoblasts after modulating the expression of ELA levels in HTR8/SVneo cells.

Previous studies have found that ELA activates the APJ receptor and promotes cell movement during zebrafish gastrulation, regulating mesodermal cell migration via Cxcr4a-signaling [53]. It has been suggested the expression of ELA in BeWo and JEG3 cell lines decreases significantly within hypoxic conditions, associated with a decrease in the invasiveness of trophoblasts [54]. Consistent with previous studies, we found that knockdown of ELA prevented trophoblast migration and invasion in HTR8/SVneo cells (Fig. 3), while over-expression of ELA promoted trophoblast migration and invasion (Fig. 4). MMP2 and MMP9 are currently the most widely studied factors that promote trophoblast invasion [55–57]. Many factors, including PI3K, affect the invasion of trophoblasts through MMP2/MMP9 [58]. The present study established that knockdown of ELA decreased the expression of MMP2 and MMP9, while over-expression of ELA had the opposite effect in HTR8/SVneo cells (Figs. 3 and 4). These results indicate that ELA affects the invasion of trophoblasts.

Several signaling pathways play key roles in regulating cellular invasion, including the PI3K/Akt/mTOR signaling pathway which is considered to be a key regulator of invasive activity [59]. Activated APJ signaling can activate PI3K [16], and so ELA could be linked to the

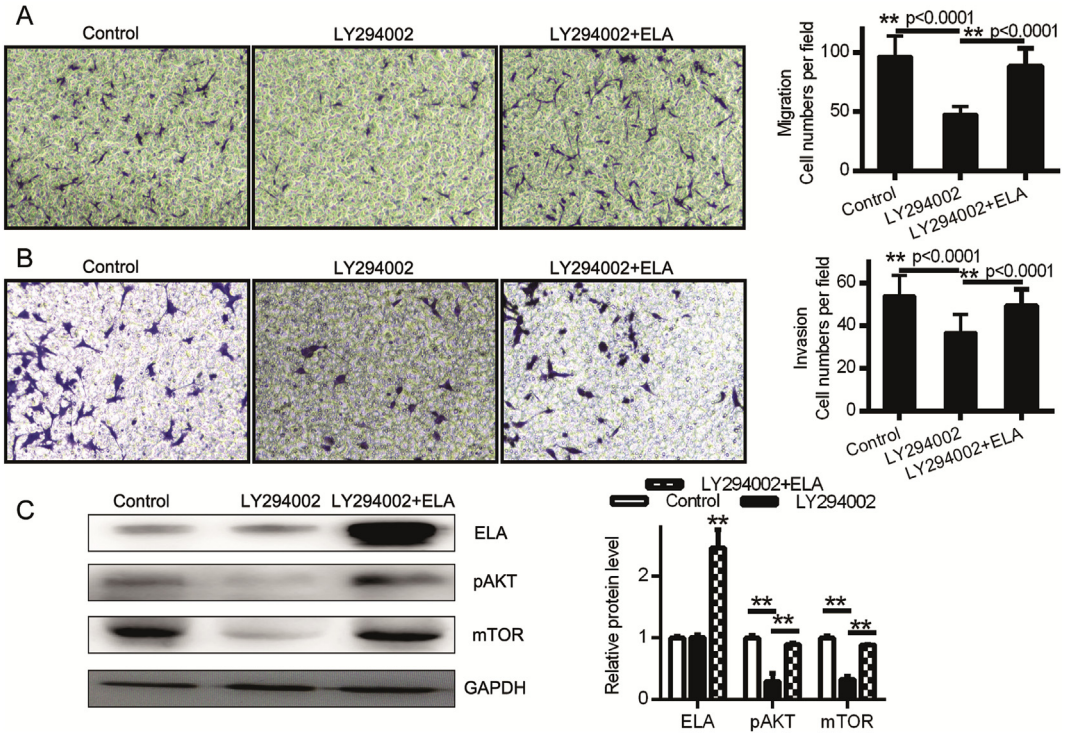

Fig. 6. We used PI3K inhibitor LY294002 to treat HTR8/SVneo cells. A, Cells migratory abilities were markedly reduced in LY294002 group compared with control and LY294002 + ELA group. B, Cells invasion were inhibited in LY 294002 group using the Matrigel invasion assay compared with control and LY294002 + ELA group. C, We used western blot to determine the Expression of pAKT and mTOR in HTR8/SVneo cells. (N = 3) (*P < 0.05, **P < 0.01).

pathogenesis of preeclampsia through the PI3K/Akt/mTOR pathway. Consistent with this hypothesis, we found that levels of pPI3K, pAKT and mTOR were markedly decreased and migration and invasion also decreased significantly in HTR8/SVneo cells following ELA knockdown (Fig. 5). LY294002 is currently the most widely used PI3K blocker [28,60]. After blockade of PI3K the ability of cells to undergo invasion and migration was significantly reduced and the expression of pAKT and mTOR significantly decreased in HTR8/SVneo cells (Fig. 6). Figs. 5 and 6 suggest that Elabela was involved in the trophoblastic migration and invasion through the PI3K/Akt/mTOR pathway. After blocking PI3K and adding Elabela, as shown in Fig. 6, the migration and invasion capability of cells increased, as did expression of AKT and mTOR, suggesting that overexpression of Elabela would result in a reduction in inhibition of PI3K, with Akt/mTOR possibly activated through other pathways, promoting cell migration and invasion, possibilities that will be studied in more detail in the future. Partially consistent with this study, Zhou et al. has reported that administration of ELA can lead to an increase in pAKT and that treatment with ELA can promote trophoblast invasion and MMP9 mRNA expression [51]. Taken together, our findings indicate that ELA may influence trophoblast migration and invasion by affecting the PI3K/AKT/mTOR pathway.

In summary, ELA mRNA and protein levels were decreased in the placentas of women suffering early onset preeclampsia. In addition, ELA affects the invasion and migration of trophoblasts in HTR8/SVneo cells through the PI3K/AKT/mTOR pathway. This study suggests a possible mechanism by which ELA can induce early-onset preeclampsia, rendering ELA a potential target for the prevention and treatment of this condition.

Conflict of interest

We have no conflicts of interest to declare.

Funding

This work was supported by the grants from Science and Technology Benefit People Special of Qingdao Science and Technology Bureau in China [Grant number: 19-6-1-56-nsh].

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