Trophoblastic Proliferation and Invasion Regulated by ACTN4 is Impaired in Early-onset Preeclampsia

Short Title: Preeclampsia is Associated with ACTN4 Deficiency

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Nonstandard Abbreviations:

PE (preeclampsia); EO-PE (early-onset preeclampsia); CCT (cell column trophoblast); CTB (cytotrophoblast); STB (syncytiotrophoblasts); iEVT (interstitial extravillous trophoblast); AV (anchoring villi); FV (floating villi); BP (basal plate); DS (decidual side); NS (nonsignificant); GD (gestation day); NP (nonpregnant); Lz (labyrinth zone); Jz (junctional zone); SBP (systolic blood pressure); GCT (glucose challenge test); BMI (body mass index).
Abstract

Successful pregnancy requires normal placentation, which largely depends on the tight regulation of proliferation, invasion and migration of trophoblast cells. Abnormal functioning of trophoblast cells may cause failure of uterine spiral artery remodeling, which may be related to pregnancy-related disorders, such as preeclampsia. Here, we reported that an actin-binding protein, alpha-actinin-4 (ACTN4), was dysregulated in placentas from early-onset preeclampsia. Moreover, knockdown of ACTN4 markedly inhibited trophoblast cell proliferation by reducing AKT membrane translocation. Furthermore, E-cadherin regulated ACTN4 and β-catenin co-localization on trophoblast cell podosomes, and ACTN4 downregulation suppressed the E-cadherin-induced cell invasion increase via depolymerizing actin filaments. Moreover, loss of ACTN4 recapitulated a number of the features of human preeclampsia. Therefore, our data indicate that ACTN4 plays a role in trophoblast function and is required for normal placental development.

Key words: trophoblast, AKT, podosome, placenta

Introduction

Preeclampsia (PE), a pregnancy-specific disorder characterized by new onset of hypertension and proteinuria after 20 weeks of gestation, impacts 2%~7% of all pregnancies worldwide (1). Defined by the timing of occurrence, early-onset PE (EO-PE, ≤34 week) is associated with higher risks of eclampsia and fetal growth restriction, both of which severely threaten maternal and fetal health (2, 3). Currently, the only definitive cure is delivery of the placenta and therefore the infant. Although the full understanding of PE pathogenesis remains elusive, it is widely believed to be a placenta-origin disease in which inadequate cytotrophoblast (CTB) invasion in early pregnancy may play a pivotal role and thus cause abnormal placentation, followed by systemic endothelial cell dysfunction in the late gestation (4). It is known that cellular invasion depends on the formation of invasive structures, such as invadopodia and podosomes (5); moreover, the differentiation of CTBs into extravillous trophoblasts (EVTs) is associated with changes in cell behavior, cytoskeletal organization, and adhesion structures (6, 7), suggesting that cytoskeletal integrity and appropriate remodeling may be critical for trophoblasts acquiring invasiveness. However, links between the regulation of the trophoblastic cytoskeleton and PE development remain to be elucidated.

Alpha-actinins (ACTNs) maintain cytoskeleton integrity and regulate cell movement (8). ACTNs are located at cell-cell and cell-matrix contact sites, cellular protrusions, and stress fiber-dense regions and regulate
diverse signaling pathways by linking membrane receptors with the cytoskeleton (9, 10). In humans, there are four highly homologous forms of ACTNs encoded by different genes, including actinin-2 and actinin-3, which are encoded by ACTN2 and ACTN3, respectively. These genes are muscle specific and predominantly localized in Z lines of striated muscle cells. In addition, there are two non-muscle isoforms, actinin-1 and actinin-4, which are encoded by ACTN1 and ACTN4, respectively. Although they share high similarity in protein sequences, the subcellular distribution and function are distinct (11, 12). ACTN1 is associated with adherent junctions, which play an important role in cytoskeleton regulation. In contrast, ACTN4 is largely expressed at the leading edge of moving cells, suggesting that ACTN4 may be involved in cell motility (13, 14). ACTN4 deficiency significantly attenuates metastasis of various invasive cancer cells (15-18). Moreover, as a signaling molecule, ACTN4 linked integrin with the actin cytoskeleton and increased trophoblast invasion in bovine placenta (19). A yeast two-hybrid assay screened by a human placenta cDNA library identified plasminogen activator inhibitor type-1 (PAI-1) as a modulator of biological processes involving fibrinolysis, cell migration or tissue remodeling by binding with ACTN4 (20). Additionally, ACTN4 is involved in epithelial-to-mesenchymal transition (EMT) and β-catenin stabilization in cervical cancer (21, 22), indicating it may participate in acquiring motility. On the other hand, previous studies have reported that ACTN4 directly binds to and enhances AKT membrane translocation in HeLa cells (23). Moreover, as a central regulator of cell proliferation, AKT was reduced in EO-PE placentas (24), suggesting that ACTN4 may be involved in trophoblast cell proliferation. Accumulated evidence strongly implies that ACTN4 participates in the processes of cell movement and cell proliferation (25-27). Nevertheless, the role of ACTN4 in trophoblast and EO-PE development is still largely unknown.

We therefore hypothesized that EO-PE is associated with aberrant expression and distribution patterns of ACTN4 in human term placental trophoblasts, which could influence trophoblast proliferation and invasion through AKT and β-catenin, respectively, and thus ultimately contribute to poor placentation and uteroplacental hypoperfusion.

Materials and Methods

Patient and sample collection

Placental tissues at the chorionic plate and basal plate from normal pregnancies (n=10) and EO-PE patients (n=7) who were admitted to the First Affiliated Hospital of Chongqing Medical University for cesarean
deliveries were collected as previously described (28). EO-PE was diagnosed by new onset of systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg on 2 separate readings after the 20th week of gestation but earlier than the 34th week in the presence of significant proteinuria (at least 2+) in the absence of pre-existing renal disease or chronic hypertension. Patients with other major pregnancy complications, such as gestational diabetes mellitus, spontaneous abortion and renal disease, were excluded. First trimester villous tissue (n=10) were collected from women who underwent legal termination during 6-10 weeks of gestational age not due to medical reasons, while patients with a history of spontaneous abortion or ectopic pregnancy were excluded. A portion of each biopsy was immediately snap frozen in liquid nitrogen and stored at -80 °C for further use, while the rest of the tissue was fixed in 4% paraformaldehyde and then embedded in paraffin. The patients’ clinical features are characterized in Table S1. This study was in accordance with the principles set out in the Declaration of Helsinki and approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. All samples were collected with written informed consent provided by the participants.

**Cell culture**

Immortalized human trophoblast cell line HTR8/SVneo was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The human choriocarcinoma cell lines JAR and JEG3 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The HTR-8/SVneo and JAR cells were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) medium, and the JEG3 cell lines were cultured in DMEM/F12 medium (Gibco), which were supplemented with 10% fetal bovine serum (FBS, Gemini, California, USA) at 37 °C under 5% CO2 humidified air.

**Primary cytotrophoblast cell isolation**

CTBs were isolated from human term placentas as previously described (protocol from Sadovsky’ Perinatal Biology Lab, https://mageewomens.org/wp-content/uploads/2016/06/Sadovsky_PHT_Protocol.pdf). Briefly, placental tissue was thoroughly rinsed with ice-cold saline, and then the tissue was drained before soaking in 200 ml DMEM medium (Gibco) overnight at 4 °C. Then, 1.28 mg per gram tissue of dispase was added (Worthington, Lakewood, NJ, USA) to 6.8 ml 0.25% trypsin (Gibco) and 0.75 ml 5% DNase (Sigma, Missouri, USA) into 168 ml Hank’s balanced salt solution (HBSS, Gibco), and tissue was enzymatically digested in a 37 °C water bath for 45 min to remove the outer layer of syncytium, releasing the underlying CTBs. Cells were purified by centrifuging at 1400 rpm for 30 min in Percoll gradient containing 40%
Percoll (GE Healthcare Bio-sciences, USA) and 10% 10×HBSS buffer in ddH_2O, pre-centrifuged at 15000 rpm for 50 min. The cytotrophoblast layer was just above the red blood cell layer. CTB purity reached up to 95% (Figure S1). The isolated CTBs were used immediately or cultured at 37 °C under 5% CO_2 in an incubator for 3 hrs to adhere.

**Transfection**

si-ACTN4#1 (5′-GUUCAUCGUCCAUAACCAUC-3′), si-ACTN4#2 (5′-AAAGCCUCAUUCGCAAGCA C-3′), si-ACTN4#3 (5′- ACAAGCGCUGGACUUA-3′) (29) and a scrambled control siRNA (si-NC) were synthesized by GenePharma, Inc. (Shanghai, China). Then, 50 nM siRNA was transfected into 70% confluent HTR8/SVneo cells, JEG3 or JAR cells in the presence of Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) in 6-well plates according to the manufacturer’s instructions. An ACTN4 overexpression plasmid was constructed by GenePharma (Shanghai, China) and transfected into 70% confluent HTR8/SVneo cells by the use of Lipofectamine™ 3000 in 6-well plates according to the manufacturer’s protocol. All cells were cultured for 48 hrs after transfection before other treatments.

**Lentivirus infection**

Lentiviruses carrying shRNA targeting human CDH1 lentiviral vectors (GV248) were purchased from GeneChem (Shanghai, China). A total of 1×10^5 JEG3 or JAR cells were transfected with 2×10^6 virus (MOI=20) for 48 hrs in the presence of polybrene according to the manufacturer’s instructions. Then, the cells were transferred to fresh medium containing puromycin (0.2 µg/ml) for the selection of stable clones. The shRNA sequences are listed below: CDH1#1: 5′-AAGCAGAATTGCTCACATT-3′; #2, 5′-ATTCAAAGTGGCAGCATATT-3′; #3, 5′-AACCCAAGGAATCTATCATT-3′; the scrambled control shRNA, 5′-TTCTCCGAACGTGTCACGT-3′.

**Immunohistochemistry (IHC)**

The villous and placenta tissues were washed with PBS and fixed overnight with 4% paraformaldehyde at room temperature. Then, the samples were dehydrated and embedded in paraffin before sectioning into 4 µm thick sections. For IHC, the sections were deparaffinized in xylene, rehydrated in a serial ethanol gradient, blocked with 3% H_2O_2 for 10 min, and then microwaved in 10 mM citric sodium (pH 6.0) for 15 min to retrieve antigens. After that, sections were incubated with rabbit polyclonal primary antibodies against
ACTN4 (1:200, Proteintech, Wuhan, China), mouse monoclonal antibody against CK7 (1:100, Abcam, Cambridge, UK), mouse monoclonal antibody against HLA-G (1:100, Proteintech) and mouse monoclonal antibody against Ki67 (1:200, Bioss, Beijing, China) at 4 ºC overnight. Then, secondary antibody conjugated with horseradish peroxidase was applied for 30 min at room temperature, followed by DAB solution development.

**Immunofluorescence (IF) staining**

Cells were fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and then incubated with the indicated antibodies overnight at 4 ºC, followed by incubation with FITC-conjugated or Cy3-conjugated goat anti-rabbit or mouse fluorescent antibodies (Proteintech, 1:100) for 1 hour at room temperature. For actin filament staining, permeabilized cells were incubated with 100 nM TRITC-phalloidin (Solarbio, Beijing, China) solutions directly for 30 min at room temperature. The nuclei were stained with DAPI, and images were visualized using an EVOS FL Color Imaging System (Thermo Fisher Scientific, Waltham, USA).

**Western blotting**

Protein extracts were prepared from placental tissues and cells using RIPA lysis buffer supplemented with PMSF (Beyotime Institute of Biotechnology, JiangSu, China). The lysates were then separated by SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, California, USA) and transferred onto PVDF membranes (Millipore, Darmstadt, Germany). After blocking with 5% non-fat dry milk (Bio-Rad) in Tris-buffer containing Tween-20 (TBST) for 1 hour at room temperature, the membranes were immunoblotted with primary antibodies against ACTN4 (1:3000, Proteintech), AKT (1:1000, Abcam), phosphorylated AKT at serine 473 (1:1000, Cell Signaling Technology, Danvers, MA, USA), p-GSK3β (1:1000, Cell Signaling Technology), GSK3β (1:1000, Proteintech), Cyclin D1 and p21 (1:1000, Proteintech), β-catenin (1:1000, Cell Signaling Technology), E-cadherin (1:1000, Cell Signaling Technology), Na, K-ATPase (1:1000, Abcam) or β-actin (1:1000, Proteintech) overnight at 4 ºC. After rinsing with TBST, the membranes were then incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Zhong San Golden Bridge Crop, Beijing, China) at room temperature for 1 hr. Immunoreactive bands were developed using an enhanced chemiluminescent substrate (Millipore), and the images were captured and analyzed by a ChemiDoc XRS+ system (Bio-Rad).
Co-immunoprecipitation (Co-IP)

Anti-ACTN4 antibody (Proteintech) or a matched isotype antibody IgG (Beyotime) were incubated with Surebeads Protein G Magnetic Beads (Bio-Rad) for 1.5 hrs at room temperature. Cells were lysed with IP lysis buffer (Beyotime) supplemented with protease inhibitor mixture (Beyotime) and incubated with an antibody-beads complex for another 1 hour. Then, the protein extracts were precipitated by the antibody-beads complex using a magnetic rack. After being washed three times with IP lysis buffer, Co-IP products were boiled at 95 °C for 15 min with diluted 4× laemmli protein sample buffer (Bio-Rad) in lysis buffer, and then the supernatant was analyzed by immunoblotting.

Extraction of plasma membrane and cytoplasm

Cellular plasma membrane and cytoplasm were separated by using a commercial kit (Invent Biotechnologies, Plymouth, MN, USA) according to the manufacturer’s instructions. Briefly, $1 \times 10^6$ cells were first sensitized by 200 µl buffer A (Invent Biotechnologies) and then passed through to a zigzag path filter by centrifuging at 14000 rpm for 30 sec. The pellet was resuspended and centrifuged at 3000 rpm for 1 min; then, the supernatant was transferred and centrifuged at 14000 rpm for 30 min at 4 °C. The supernatant was the cytosol fraction. Next, the pellet was resuspended in 200 µl buffer B (Invent Biotechnologies) before centrifuging at 10000 rpm for 5 min at 4 °C. The supernatant was transferred and 1.6 ml cold PBS was added, followed by centrifugation at 14000 rpm for 30 min. The pellet was the isolated plasma membrane protein. Finally, the plasma membrane proteins were denatured in detergent-containing buffer (Invent Biotechnologies) for Western blotting.

Cell proliferation assays

A total of $5 \times 10^3$ cells suspended in 100 µl medium was added to one well of 96-well plates for Cell Counting Kit-8 (CCK-8) assays (Boster, Wuhan, China). Cell proliferation was determined at the time points of 0, 24, 48 and 72 hrs after siRNA treatment. The 10 µl CCK-8 solution was added to HTR8/SVneo cells at 0, 24, 48 and 72 hrs after siRNA treatment and then incubated at 37 °C for 4 hrs. Absorbance was measured at 450 nm using a Multiskan Go microplate reader (Thermo Fisher Scientific). For JEG3 cell proliferation assay, $3 \times 10^4$ E-cadherin deficient cells were seeded onto matrigel pre-coated 48-well plate with presence of si-NC or si-ACTN4 (50 nM) for 24 hrs. Then, the cells were stained by crystal violet, Images were captured by using an EVOS FL Color Imaging System (Thermo Fisher Scientific).
Flow cytometry

The cell cycle distribution was analyzed by flow cytometry using a BD FACS Vantage SE Cell Sorter (Becton-Dickinson, CA, USA). Briefly, HTR8/SVneo cells were pre-treated with siRNA for 48 hrs. Then, each sample was collected and fixed in 70% ethanol overnight at 4 °C. Next, cells were mixed with a fluorochrome solution composed of propidium iodide (PI), 0.25 mg/ml RNase and 0.1% Triton X-100 before analyzing. The cell population of interest was gated on the basis of the forward and side-scatter properties. Cut-off values (i.e., vertical and horizontal lines on the resulting scatter plots) were designated based on the autofluorescence of control cells. The results are expressed in terms of the percentage relative to controls. For cell apoptosis, JEG3 cells were harvested and exposed to the Annexin V-APC and DAPI binding buffer for 20 min. The stained cells were then analyzed using a CytoFLEX flow cytometer (BD Biosciences).

DNA synthesis assay

For EdU assays, cells were planted in 96-well plates. A total of 100 µl culture medium containing 50 µM EdU was added to each well for 2 hrs. Then, cells were fixed with 4% formaldehyde for 30 min. After washing, cells were incubated with a Click-iTR EdU Kit (RiboBio, Guangzhou, China) for 30 min, then stained with 100 µl DAPI (RiboBio) at room temperature for 30 min, and then imagined with a fluorescent microscope (EVOS FL Color Imaging System). EdU-positive cells were counted in five random view fields of each treatment in three independent experiments.

Matrigel invasion and transwell migration assay

The invasion assay was performed in a transwell chamber consisting of a 24-well plate with membrane inserts (Corning, New York, USA) containing polycarbonate filters with 8 µm pore sizes pre-coated with 60 µl of 1 mg/ml Matrigel matrix solution (BD Biosciences, California, USA). A suspension of 5×10^4 cells in 300 µl serum-free culture medium was added to the inserts, and each insert was placed in the lower chamber containing 600 µl 10% FBS culture medium. After 24 hrs, the cells that penetrated the membrane were fixed by 4% paraformaldehyde and stained by crystal violet. The cell migration assay was similar to the invasion assay, but the insert was not coated with matrigel. Images were captured by using an EVOS FL Color Imaging System (Thermo Fisher Scientific).
Establishment of ACTN4-deficient mice during pregnancy

The 8-12-week-old CD1 female mice weighing 25-35 g from the Experimental Animal Center of Chongqing Medical University were mated with age-matched male mice. Pregnant mice were randomly assigned into four groups (GD14.5, Ad-Ctrl, n=4; Ad-ACTN4, n=5; GD18.5, Ad-Ctrl, n=6; Ad-ACTN4, n=6). Adenoviral carrying shRNA targeting mouse ACTN4 was purchased from Cyagen Biosciences (Guangzhou, China). Age-matched nonpregnant mice were divided into two groups (Ad-Ctrl, n=5; Ad-ACTN4, n=5). The presence of a vaginal plug was considered as GD0.5. On GD8.5, the pregnant mice were injected with Ad-EGFP-Ctrl or Ad-EGFP-ACTN4 (2 × 10⁹ PFU, 100 µl) through the tail vein. The SBP was monitored by tail-cuff photoplethysmography BP-2000 SERIES II (Visitech System, Apex North Carolina, USA) daily from GD6.5 to GD18.5. The mice were euthanized on GD14.5 and GD18.5 to collect placentas or on GD18.5 to examine the weights of fetuses and placentas.

Statistics

Statistical data were analyzed by Student’s t test (2 groups) and ANOVA test (above 2 groups). \( P < 0.05 \) was considered to significant differences. The statistical analyses were performed by using GraphPad Prism 7 software (La Jolla, California, USA).

Results

Trophoblastic ACTN4 is downregulated in EO-PE complicated placentas

We first evaluated the protein expression levels of ACTN4 in human placentas at early stage of pregnancy by immunohistochemistry (IHC), which showed that ACTN4 was highly expressed in various subtypes of trophoblast cells of the first trimester human villus, including villous cytotrophoblasts (CTB), cell column trophoblasts (CCT) and interstitial extravillous trophoblast cells (iEVT) in decidua (Figure 1A). PE, especially EO-PE, is a pregnancy complication that has long been attributed to inadequate trophoblast invasion. Therefore, we detected the expression levels of ACTN4 in placentas by Western blotting. The results demonstrated that ACTN4 protein was significantly lower in EO-PE placentas than in normal placentas (Figure 1B). Accordantly, IHC staining further revealed that unlike the strong staining of ACTN4 in CTBs and iEVTs in floating villi (FV) and basal plates (BP) in normal placentas, ACTN4 expression was compromised in those locations in EO-PE placentas (Figure 1C). To be more specific, primary CTBs were isolated (Figure S1), and Western blotting showed a 50% reduction of ACTN4 protein level in CTBs from
EO-PE placentas compare to that from normal pregnancies (Figure 1D). Collectively, the results indicated that downregulation of trophoblastic ACTN4 is associated with EO-PE complicated placentas.

**ACTN4 deficiency inhibits proliferation in trophoblasts**

To ascertain the causality between trophoblastic ACTN4 deficiency and EO-PE development, the role of ACTN4 in trophoblast proliferation was explored. First, ACTN4 expression levels in HTR-8/SVneo, JAR and JEG3 cells were determined. The results suggested that ACTN4 expression was significantly higher in HTR-8/SVneo cells than in the others (Figure 2A). Next, ACTN4 expression in HTR-8/SVneo cells was downregulated by transfection of three siRNAs that specifically targeted ACTN4 mRNA, and Western blotting results confirmed that ACTN4 expression in HTR-8/SVneo cells was silenced by si-ACTN4#1 or si-ACTN4#2 (Figure 2B). Cell counting kit-8 (CCK8) assays demonstrated that ACTN4 deficiency strikingly decreases trophoblast proliferation ability compared with control cells 48 hrs later (Figure 2C). In line with these data, flow cytometry results revealed that cell cycle arrest in G1 phase was induced after administration of ACTN4 siRNA to HTR8/SVneo cells for 48 hrs (Figure 2D). Consistently, EdU staining showed that knockdown of ACTN4 significantly reduces DNA synthesis in HTR8 cells at 48 hrs (Figure 2E).

**ACTN4 silencing inhibits AKT phosphorylation, signaling and cell cycle**

Numerous reports have demonstrated that ACTN4 is a functional partner of AKT, and it may play significant roles in AKT translocation, phosphorylation and signaling transduction in cancer cells (23, 30). Therefore, to investigate the involvement of AKT in the regulation of trophoblast proliferation by ACTN4, we examined the changes in the AKT pathway in HTR8/SVneo cells in response to manipulations of ACTN4 expression. As expected, Western blotting showed that ACTN4 downregulation resulted in decreased AKT phosphorylation at Ser473 in HTR8 cells; accordingly, the phosphorylation level of its downstream effector, GSK-3β, was also significantly compromised (Figure 3A). Moreover, ACTN4 silencing decreased the expression level of cyclin D1, the checkpoint for cell cycle progression from G1- to S-phase, but augmented the cyclin-dependent kinase inhibitor p21, which is known to be suppressed by AKT. Conversely, ACTN4 overexpression significantly elevated the levels of p-AKT\textsuperscript{Ser473}, p-GSK3β, and cyclin D1, but ameliorated p21 expression. However, in the presence of a specific PI3K inhibitor, LY294002, the changes in p-AKT\textsuperscript{Ser473}, p-GSK3β, cyclin D1, and p21 due to ACTN4 were largely blocked.
without interfering with ACTN4 expression. These results indicated that ACTN4 might regulate trophoblast proliferation through the PI3K/AKT/GSK3β signaling pathway.

**ACTN4 is required for AKT trafficking and activation in trophoblasts**

Previous reports have shown that ACTN4 interacts with AKT in various cancer cells, perhaps by facilitating AKT translocation onto the plasma membrane, thus playing a pivotal role in AKT activation (31-33). However, such interaction between ACTN4 and AKT has yet to be identified in trophoblasts. In this study, IF staining results demonstrated the co-localization of ACTN4 and AKT throughout the cytoplasm and plasma membrane in HTR8/SVneo cells and primary CTBs (Figure 4A). Furthermore, the protein-protein interaction between ACTN4 and AKT was confirmed by co-IP in HTR8/SVneo cells and primary CTBs (Figure 4B). To gain insight into whether ACTN4 mediated AKT translocation to the plasma membrane, and consequently phosphorylation, we investigated the effects of ACTN4 on the subcellular distribution of AKT in trophoblasts. The results showed that inhibition of ACTN4 expression reduced membranous AKT but increased cytoplasmic AKT. In contrast, the AKT level in the membrane fraction of HTR8/SVneo cells was augmented by ACTN4 overexpression, while cytoplasmic AKT was impaired (Figure 4C). Taken together, these data clearly suggested that ACTN4 is required for AKT trafficking from the cytosol to the cell membrane in trophoblasts.

**ACTN4-β-catenin complex is essential for E-cadherin-induced podosome formation**

ACTN4 is an actin-binding protein involved in cell motility (34, 35). It is reported that the association of ACTN4 and β-catenin evokes cell movement and mediates cancer metastasis (29), while the E-cadherin/β-catenin system is important for epithelial-mesenchymal transition. Therefore, it is important to understand whether ACTN4 interacts with E-cadherin/β-catenin in trophoblasts for acquiring motility. For this purpose, the interaction between ACTN4 and β-catenin was examined in JEG3, an epithelial-like trophoblast cell line. IF staining showed that ACTN4 and β-catenin co-localized in cell-cell adhesion (Figure 5A), and then co-IP confirmed the physical interaction between ACTN4 and β-catenin in JEG3 cells (Figure 5B). To determine the involvement of E-cadherin in the formation of the ACTN4-β-catenin complex, three specific shRNAs targeting CDH1, the gene encoding E-cadherin, were applied to JEG3 cells, and shCDH1#2 demonstrated the highest efficiency in interfering with E-cadherin expression (Figure S2). Intriguingly, downregulation of E-cadherin resulted in increased ACTN4 and β-catenin co-localization
mainly at trophoblastic podosomes, including filopodia and lamellipodia (Figure 5C). Consistently, the interaction between ACTN4 and β-catenin in JEG3 cells was also enhanced by knockdown of E-cadherin (Figure 5D). These data strongly indicated that E-cadherin had an inhibitory effect on the interaction between ACTN4 and β-catenin, which may be critical for filopodia and lamellipodia formation in trophoblasts. Dynamic formation and disassembly of actin filament-based structures, such as filopodia and lamellipodia, are essential for cell invasion and migration. We therefore assessed the roles of the ACTN4-β-catenin complex in the regulation of trophoblast invasiveness and migration. In line with previous findings, TRITC-phalloidin staining of actin filaments was highly organized and concentrated at the cell periphery in E-cadherin deficient JEG3 cells, which was associated with improved invasion and migration. However, this elevation in invasiveness and migration were totally inhibited in the presence of si-ACTN4#1, which also result in marked disruption of actin filaments (Figure 5E, F), while cell proliferation and apoptosis weren’t differ among those groups (Figure S3). In addition, similar results were also observed in JAR cells (Figure S4). In summary, these observations indicated that the ACTN4-β-catenin complex is essential for podosome formation and acquiring invasiveness in trophoblasts, which is negatively regulated by E-cadherin.

**ACTN4-deficient mice demonstrated compromised placentation and PE-like phenotype**

To further validate our *in vitro* findings, ACTN4-deficient mice were generated by intravenous injection of EGFP-tagged ACTN4 interfering adenovirus on GD8.5. ACTN4 expression and the levels of p-AKT<sup>Ser473</sup> and p-GSK3β were significantly downregulated in the Ad-ACTN4 group on GD14.5 and GD18.5 (Figure 6A), respectively. The transfection efficiency was similar between placentas from Ad-Ctrl and Ad-ACTN4 injected mice, as comparable levels of EGFP were detected in the two groups on both GD14.5 and GD18.5 (Figure S5). Histological analyses of the uteroplacental unit on GD14.5 revealed significant reduction of the junctional zone area in ACTN4-deficient mice, while the labyrinth area was not affected (Figure 6B). As the result, the labyrinth area to junctional zone area ratio was increased in placentas from ACTN4-deficient mice compared to control mice (Figure 6B). Further assessment of proliferation showed that the number of Ki67 stained cells in the junctional zone was decreased in the ACTN4-deficient group (Figure 6B). Meanwhile, the systolic blood pressure (SBP) sharply increased in ACTN4-deficient mice from GD12.5 until GD18.5, but the hypertensive phenotype was not observed in either Ad-Ctrl dams or Ad-ACTN4 dams of age-matched nonpregnant (NP) female mice (Figure 6C). Moreover, placental and fetal weights on
GD18.5 were both significantly lower in ACTN4-deficient mice compared to Ad-Ctrl mice (Figure 6D, E). Furthermore, pathological examination revealed that pregnant ACTN4 deficient mice are associated with histological and morphological alterations in kidney, such as swollen glomeruli with narrowed capillary and Bowman’s spaces (Figure S6). These facts indicated that ACTN4 deficiency impairs placentation and induces the PE-like phenotype in vivo.

**Discussion**

The current study found that ACTN4 is mainly expressed in placental CTBs but is compromised in EO-PE complicated pregnancies. Downregulation of ACTN4 suppresses trophoblast proliferation in vitro, likely through the inhibition of AKT phosphorylation. In addition, we provided evidence that the ACTN4-β-catenin complex is essential for mediating the inhibitory effects of E-cadherin on podosome formation and invasion in trophoblasts. Moreover, in vivo experiments further confirmed that ACTN4 deficiency impairs placental development and mimics the PE-like phenotype in mouse. Appropriate development of the placenta or its component lineages at early stages is critical for successful pregnancy (36). Dysregulation of CTB differentiation into EVT disrupts the invasion of trophoblasts into the uterus, which consequently leads to insufficient spiral artery remodeling and placental hypoperfusion (37, 38). Poor early placentation is especially associated with early-onset PE, which was based on the onset time earlier than 34 weeks. In the present study, although the normal pregnancy and EO-PE participants were not gestationally age-matched, ACTN4 expression remained stable throughout pregnancy when comparing the first trimester villi to full-term placentas (Figure S7), suggesting that the loss of ACTN4 expression in EO-PE placentas was not due to early gestational age but was a pathological feature.

It is reported that trophoblastic proliferation is essential for placental development during early pregnant (39). Here, we found that suppression of ACTN4 expression inhibits trophoblast proliferation. Numerous studies have shown that AKT plays a critical role in the regulation of cell proliferation (24, 40, 41). Moreover, AKT membrane translocation is essential for its phosphorylation and subsequent activation (31-33). Interestingly, ACTN4 has a functional link between the transporter and actin filaments by contributing to molecular or vesicle translocation toward the cell surface (42-46). However, the role of ACTN4 in regulating AKT has yet to be discovered. To the best of our knowledge, this study is the first to report that ACTN4 facilitates the translocation of AKT to the cell membrane by direct interaction. Furthermore, downregulation of ACTN4 resulted in decreased AKT phosphorylation at Ser473 in
trophoblast cells. This finding strongly suggests that ACTN4 modulates AKT activation by regulating its subcellular distribution. Although inhibition of PI3K promotes ACTN4 nuclear translocation in certain types of cancer cells (47, 48), which might be required for binding to transcriptional factors (25), we did not observe ACTN4 translocation into nuclei upon inhibition of PI3K in trophoblast cells (data not shown). This result confirmed that ACTN4 is an upstream regulator of the AKT pathway in trophoblasts.

In addition to the involvement in trophoblastic proliferation, this study also revealed the participation of ACTN4 in cytoskeletal remodeling, critical for acquisition of cell motility (5, 6). E-cadherin/β-catenin is associated with the actin cytoskeleton and regulates cell motility by binding with actin filaments (49), and our data revealed that E-cadherin negatively regulates the ACTN4-β-catenin complex, which is involved in the regulation of cell invasion. Therefore, ACTN4 plays a crucial role in the cytoskeletal organization of the actin network by cross-linking with actin filament, which in turn promotes podosome formation and enhances invasiveness. Accordantly, TRITC-phalloidin staining of primary CTBs isolated from normal and EO-PE placentas showed that the organization of actin filaments was disrupted in EO-PE placentas (Figure S8). Collectively, impaired ACTN4 expression and consequent actin filament disarrangement may be involved in the aberrant trophoblastic invasion in EO-PE.

Adenovirus-based transfection via tail vein injection on GD8.5 successfully induced sFlt-1 overexpression and PE-like symptoms in mice, as reported by other groups (50, 51). Thus, ACTN4-deficient dams were established by the same approach in this study, and placental ACTN4 expression was significantly downregulated from GD14.5 to the end of pregnancy (Figure 6A). Notably, ACTN4-deficient mice were associated with abnormal placentation that mainly manifested as a decrease of junctional zone size; as a result, smaller placentas and fetuses were obtained. Most importantly, the blood pressure of ACTN4-deficient mice escalated in mid-gestation, which replicates the timing at which hypertension appears in human EO-PE. Nonetheless, adenovirus delivered intravenously could lead to a global knockdown that influenced ACTN4 expression in various cell types and organs; therefore, the aforementioned phenotypes may not solely derive from ACTN4 deficiency in trophoblasts. To further solidify the findings from this study, trophoblast-targeted virus delivery can be achieved by a lentiviral vector or a newly reported lab-engineered nanoparticle that contains a placenta-homing peptide (52-55). Alternatively, placenta-specific ACTN4-deficient mice can be generated from transgenic mice harboring a Cre gene regulated by a trophoblast-specific promoter (56, 57). These tools could be extremely helpful for extending our understanding of ACTN4 in trophoblasts.
In conclusion, ACTN4 participates in trophoblast proliferation and motility through its involvement in AKT translocation and its interactions with β-catenin, respectively. Loss of ACTN4 results in inhibition of proliferation, aberrant cytoskeletal remodeling and compromised invasiveness in trophoblasts, which may contribute to the etiology of early-onset PE.

Acknowledgments
We thank Xue Zhang (School of Public Health and Management, Chongqing Medical University) and Junwei Gu (School of Public Health and Management, Chongqing Medical University) for technical assistance of animal study and primary CTBs isolation. This work was supported by the National Key Research and Development Program of Reproductive Health & Major Birth Defects Control and Prevention (2016YFC1000407, 2018YFC1002901), the National Natural Science Foundation of China (81520108013, 81771613, 81671488, 81871189, 81801482, 81601305, 81601304), Chongqing Municipal Education Commission (CXTDX201601014), Science and Technology Commission of Chongqing (cstc2017jcyjBX0045), and Chongqing Entrepreneurship and Innovation Supporting Program for Returned Overseas Students (cx2017104). We would also like to appreciate the support from “111 program” of Ministry of Education P.R.C and State Administration of Foreign Experts Affairs P.R.C.

Declaration of Interest
The authors declare no conflicts of interest.

Author contributions

References


actinin alpha 4 (ACTN4), is a nuclear receptor coactivator that promotes proliferation of MCF-7 breast cancer cells. *J Biol Chem* **286**, 1850-1859


38. Plaks, V., Rinkenberjer, J., Dai, J., Flannery, M., Sund, M., Kanasaki, K., Ni, W., Kalluri, R., and


circular ruffling and macropinocytosis in mouse macrophages: analysis by fluorescence ratio imaging. *J Cell Sci* **113 ( Pt 18)**, 3329-3340


Figure legends

**Figure 1. ACTN4 expression pattern in placenta.** A. IHC staining of ACTN4 in first trimester human placenta villi (a-b, d-e) and decidua (c, f). iEVTs and CTBs were stained by HLA-G and CK7, respectively; AV (anchoring villi), FV (floating villi), CCT (cell column trophoblast), STB (syncytiotrophoblasts). Scale bars, 100 µm. B. Western blotting of ACTN4 in normal and EO-PE placentas, Data are presented as the median + range, *P* < 0.05, Mann-Whitney U tests. C. IHC staining of ACTN4 in FV and basal plate (BP) of normal and EO-PE placentas on serial sections. CCTs, iEVTs and CTBs were stained by HLA-G and CK7, respectively, DS, decidual side. Scale bars, 100 µm in a-b, e-f; 200 µm in c-d, g-h. D. Western blotting of ACTN4 in primary cytotrophoblast cells isolated from normal and EO-PE placentas. Data are presented as the means ± SEM, **P** < 0.01, ***P** < 0.001.
Figure 2. ACTN4 regulates trophoblast proliferation. A. Western blotting of ACTN4 in HTR-8/SVneo, JAR and JEG3 cells. B. Interference efficiency of siRNAs targeting ACTN4 in HTR-8/SVneo cells was assessed by Western blotting. C. CCK8 assay on HTR-8/SVneo cells after 0, 24, 48, and 72 hrs of si-ACTN4#1 and si-ACTN4#2 transfection. D. Cell cycle analysis of HTR-8/SVneo cells by flow cytometry after 48 hrs of si-ACTN4#1 and si-ACTN4#2 transfection. E. EdU (pink) staining on HTR-8/SVneo cells after 48 hrs of si-ACTN4#1 and si-ACTN4#2 transfection. Nuclei were counterstained with DAPI (blue). Scale bars, 200 µm. All data are means ± SEM of three independent experiments performed in triplicate, *P < 0.05, **P < 0.01, ***P < 0.001, NS, nonsignificant.
Figure 3. ACTN4 regulates PI3K/AKT/ GSK3β pathway in trophoblasts. A. Western blotting of ACTN4, p-AKT<sup>ser473</sup>, AKT, p-GSK3β, GSK3β, Cyclin D1 and p21 in HTR-8/SVneo cells transfected with si-ACTN4#1 or ACTN4-OE plasmid for 48 hrs in the presence and absence of LY294002 (20 µM) for 24 hrs after transfection. B. Densitometry quantification of Western blots. Data are means ± SEM of three independent experiments performed in triplicate, *P < 0.05, **P < 0.01, ***P < 0.001, NS, nonsignificant.
Figure 4. ACTN4 binds to and facilitates AKT translocation onto the plasma membrane in trophoblasts. A. IF staining of AKT (red) and ACTN4 (green) in HTR-8/SVneo and primary human CTB cells. Nuclei were counterstained by DAPI (blue). Scale bars, 200 µm. B. IP of ACTN4 in HTR-8/SVneo cells and primary human CTBs by the use of anti-ACTN4 rabbit polyclonal antibody or control rabbit IgG. The IP products and input control were then blotted with antibodies against ACTN4 and AKT. C. Western blotting of membranous and cytoplasmic AKT in HTR-8/SVneo cells that were transfected with si-ACTN4#1 or ACTN4 overexpression plasmid (ACTN4-OE). Na, K-ATPase and β-actin were applied for loading controls.
Figure 5. The ACTN4-β-catenin complex is essential for the regulation of podosome formation by E-cadherin. A. IF staining of ACTN4 (green) and β-catenin (red) in JEG3 cells. Scale bars, 200 µm. B. Co-IP of ACTN4 and β-catenin in JEG3 cells. C. IF staining of ACTN4 (green) and β-catenin (red) in E-cadherin deficient JEG3 cells; white arrows: podosomes. Scale bars, 100 µm. D. Co-IP of ACTN4 and β-catenin in E-cadherin deficient JEG3 cells. E. TRITC-phalloidin staining (red) of actin filament in E-cadherin deficient JEG3 cells in the presence of si-ACTN4#1 or control (si-NC) for 24 hrs. Nuclei were counterstained by DAPI (blue). Scale bars, 200 µm. Insets: higher magnification views of the boxed areas. F. Matrigel invasion assays (upper panel) and transwell migration assays (lower panel) of E-cadherin deficient JEG3 cells in the presence of si-ACTN4#1 or si-NC. All data are means ± SEM of three independent experiments performed in triplicate, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS, nonsignificant.
Figure 6. ACTN4-deficient mice have abnormal placentation and PE-like phenotypes.

A. Western blotting of ACTN4, p-AKT<sup>Ser473</sup>, AKT, p-GSK3β and GSK3β in placentas collected from Ad-Ctrl (n=4) or Ad-ACTN4 (n=5) injected mice on GD14.5 and Ad-Ctrl (n=5) or Ad-ACTN4 (n=5) injected mice on GD18.5. B. H&E staining (upper panel) and IHC staining of Ki67 (lower panel) on GD14.5 placentas from Ad-Ctrl (n=4) and Ad-ACTN4 (n=5) injected mice. Lz, labyrinth zone, Jz, junctional zone. The area of Lz and Jz, and the Lz to Jz ratio were analyzed. C. SBP was monitored daily in Ad-Ctrl (n=6) and Ad-ACTN4 (n=6) groups during GD6.5 and GD18.5, as well as in nonpregnant female mice treated with Ad-Ctrl (n=5) and Ad-ACTN4 (n=5). P, pregnant; NP, nonpregnant. D. Placental weight on GD18.5. E. Fetal weight on GD18.5 (Ad-Ctrl, n=73 pups from 5 dams; Ad-ACTN4, n=69 pups from 5 dams). Data are expressed as the means ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, NS, nonsignificant.
Figure 1
Figure 2

A

HTR8  JAR  JEG3

ACTN4

β-actin

105 kDa

42 kDa

B

HTR8

si-NC  si-ACTN4#1  si-ACTN4#2

ACTN4

β-actin

105 kDa

42 kDa

C

0 24 48 72

Time (hours)

OD (460 nm)

0.0 0.5 1.0 1.5

D

Cell number (%)

G1/G0  S  G2/M

si-NC  si-ACTN4#1  si-ACTN4#2

E

DAPI  EdU  MERGE

si-NC

si-ACTN4#1

si-ACTN4#2

EdU positive cells (%)

si-NC  si-ACTN4#1  si-ACTN4#2
Figure 5

A. DAPI, ACTN4, β-catenin, MERGE

B. IP produces

Input IgG ACTN4
β-catenin
ACTN4
IB
92 kDa
105 kDa

C. shCtrl, shCDH1#2

D. IP produces

Input Anti-ACTN4
β-catenin
shCtrl shCDH1#2
ACTN4
IB
92 kDa
105 kDa

E. Con, shCtrl, shCDH1#2, shCDH1#2 + si-NC, shCDH1#2 + si-ACTN4#1

F. Relative cell invasion

Con shCtrl shCDH1#2 shCDH1#2 + si-NC shCDH1#2 + si-ACTN4#1

Relative cell migration

Con shCtrl shCDH1#2 shCDH1#2 + si-NC shCDH1#2 + si-ACTN4#1

*** NS ****

** NS **
Figure 6

A

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<tr>
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<th>GD14.5</th>
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<td>Ad-Ctrl</td>
<td>Ad-ACTN4</td>
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<td>ACTN4</td>
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| p-AKT
(WT)       |        |             |        |             |
| p-GSK3β       |        |             |        |             |
| GSK3β         |        |             |        |             |
| β-actin       |        |             |        |             |

B

H&E

Ki67

C

Area of junctional zone (arbitrary unit)

D

Placenta weight (g)

E

Fetal weight (g)