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Downregulation of receptor tyrosine kinase-like orphan receptor 1 in preeclampsia placenta inhibits human trophoblast cell proliferation, migration, and invasion by PI3K/AKT/mTOR pathway accommodation

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ABSTRACT

Introduction: Invasive deficiency of the trophoblast and poor remodeling of the uterine spiral arteries were probably the primary pathogenesis causes of preeclampsia (PE). The expression of receptor tyrosine kinase-like orphan receptor 1 (ROR1) during embryogenesis had been previously confirmed and was closely related to the function of tumor cells, which was similar to the characteristics of trophoblasts. In this work, we investigated the expression profile of ROR1 in preeclampsia placentas and the functional role of ROR1 in trophoblast cells, as well as the associated molecular mechanisms.

Methods: The localization expression of ROR1 in the placenta was detected by immunohistochemistry in 20 cases of normal term pregnancy, preterm delivery, late-onset severe PE, and early-onset severe PE, respectively. The expression levels were determined by fluorescence quantitative PCR and Western blot. The influence of ROR1 on trophoblast proliferation, migration, invasion, and potential regulatory pathways was evaluated in HTR-8/SVneo cell lines by transient transfection methods.

Results: The levels of ROR1 in the placental tissues in PE were significantly lower than those in normal term pregnancy and preterm delivery. Moreover, the expression levels of ROR1 in early-onset severe PE were significantly lower than those in its late counterparts. ROR1 overexpression increased cell proliferation, migration, and invasion of HTR-8/SVneo cells, whereas its silencing had the opposite effect. Meanwhile, the phosphorylation levels of critical kinases in the PI3K/AKT/mTOR pathways were increased by ROR1 overexpression, whereas they were decreased by the silencing of ROR1.

Conclusion: ROR1 might be involved in the development of PE through regulating trophoblast viability, migration, and invasion by PI3K/AKT/mTOR signaling pathway.

1. Introduction

A complication related to pregnancy, preeclampsia (PE), is a disorder that is diagnosed by newly developed hypertension at or after 20 weeks of gestation. This syndrome is manifested by proteinuria and/or presence of maternal liver dysfunction, acute kidney injury, hematological abnormalities, neurologic symptoms, or fetal growth restriction [1]. It is categorized into two major types: early-onset PE ($<$ 34 weeks of gestation) and late-onset PE ($\geq$34 weeks of gestation) [2]. This multi-system disorder can lead to maternal convulsions, multiple organ failure, and fetal growth restriction, and even to maternal and fetal deaths. It is considered one of the main reasons for increasing maternal and infant mortality and morbidity, affecting approximately 2.5%–3.0% of women [3]. Extensive research has been conducted on this syndrome over the past few decades, but its pathological mechanism is still poorly understood, and no accurate and reliable prediction methods have been developed, although evidence supports the theories of oxidative stress, placental factors, immune maladaptation, inflammation, and endothelial injury [4]. Placenta plays a major role in PE pathogenesis, and the removal of placenta can eliminate clinical syndromes. Dysregulation in the trophoblastic behavior causes insufficient invasion into the uterus, resulting in subsequent disorder of remodeling of the maternal uterine spiral arteries [5]. This incomplete remodeling of the uterine spiral artery could further result in abnormal placenta formation [6]. Certain circulatory factors from the abnormal placenta contribute to the clinical syndromes. For this reason, the

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investigations on the trophoblastic regulatory mechanism would contribute to a better understanding of the pathophysiology and effective identification of appropriate biological markers that can be used for prediction and targeted therapy of PE.

Receptor tyrosine kinase-like Orphan Receptor 1 (ROR1) is a type-I membrane protein belonging to the evolutionarily conserved receptor tyrosine kinase (RTK) protein family. It was first identified in a human neuroblastoma cell line [7]. ROR1 gene is located on chromosomes 1 and 9, encoding a 104 kDa protein [8]. ROR1 is comprised of extracellular (cysteine-rich, frizzled-like, Ig-like, and kringle domains), transmembrane, and cytoplasmic sections (one TK domain and two serine/threonine-rich domains flanking one praline-rich domain) [9]. Extracellular cysteine-rich domain has been shown to act as a receptor for Wnt5a, which involves the activation of PI3K [10]. ROR1 is observed during the embryonic and fetal development. Additionally, ROR1 is also expressed in various human cancers and certain healthy tissues, including the pancreatic islets, esophagus, parathyroid glands, stomach, and the duodenum [11]. It is critically involved by triggering signaling cascades regulating cell invasion, migration, proliferation, differentiation, and survival. In the field of tumor study, ROR1 has attracted considerable attention because it plays an important role in PI3K/AKT/mTOR pathway. Silencing of ROR1 suppresses lung adenocarcinoma cell proliferation by PI3K/AKT/mTOR signaling pathway [12]. Moreover, ROR1 monoclonal antibodies can directly induce apoptosis of the chronic lymphocytic leukemia cells through the PI3K/AKT/mTOR pathway [13]. In addition, histological analysis revealed that ROR family proteins significantly influenced the development of nervous, cardio-respiratory, and skeletal systems during the initial stages of the embryonic development [14]. Furthermore, abnormal Wnt-ROR signaling was observed in defective placentation, which led to adverse pregnancy outcomes [15]. However, it is unknown whether ROR1 is expressed in the placental tissues, or whether its expression have biological and clinical significance.

Significant similarities between early placental development and tumorigenesis have been well documented. For example, the invasive character of trophoblast and invasive cancer cells is basically the identical [16]. However, whether ROR1 is involved in PE progression remains unclear. To date, the expression of ROR1 in placenta both under different physiological and pathological conditions is still poorly understood. This study is the first research on the expression of ROR1 in the placenta of PE women. We investigated the influence of ROR1 on the behavior of the cells in a human trophoblast cell line by gene overexpression or knockdown. Furthermore, our results identified a new pathway in HTR-8/SVneo cells, which is beneficial to the development of new treatment methods and the further elucidation of the pathogenesis of PE.

2. Materials and methods

2.1. Patients information and tissue sample collections

Written consent was obtained from all patients, and all the processes and procedures were permitted by the Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, China). The selection criteria of the patients were as follows: severe PE in women was defined as systolic blood pressures ≥160 mmHg or diastolic blood pressure ≥110 mmHg at two intervals for at least 4 h in two or more continuous occasions, and/or 24-h urinary protein quantification ≥2 g (urine protein 2 + or 3 + on urine routine) after gestation of 20 weeks in normal blood pressure previously. Furthermore, they were divided into two subgroups in accordance with gestational age at onset of PE: late-onset PE (gestational age ≥34 weeks, n = 20) and early-onset PE (gestation age < 34 weeks, n = 20).Preterm control (n = 20) was defined as normotensive women with gestation age less than 37 weeks. These women were delivered for indications that included spontaneous preterm labor, preterm premature rupture of the membranes and fetal distress. Term control (n = 20) was defined normal term pregnancy. All the women enrolled in the study were singleton pregnancies, non-smokers, non-drinkers; with no chronic hypertension, eclampsia, or gestational diabetes mellitus.

The central placenta was collected immediately after caesarean delivery under aseptic conditions. After the removal of the maternal and fetal surface, the tissues were divided into three pieces, which composed the samples, and washed separately with phosphate-buffered saline (PBS) several times to minimize contamination. One of the samples was subjected to immunohistochemistry analysis after fixation in 4% paraformaldehyde; the other two samples were immediately placed in cryopreservation tubes and stored in liquid nitrogen for subsequent real-time PCR and Western blot analyses.

2.2. Immunohistochemistry

Immunohistochemistry analysis was performed by Envision method. Four-μm-thick paraffin sections were initially prepared from the formalin-fixed and paraffin-embedded pathological tissues, which were then dewaxed with xylene and rehydrated with alcohol of gradient concentration after baking at 60 °C for 30 min. Thermal repair was performed in 250 mL of citrate acid solution at 100 °C. To inactivate endogenous enzymes, the sections were kept in 3% hydrogen peroxide methanol solution at room temperature for 30 min. Monoclonal rabbit anti-human ROR1 (1:100, Abcam) was used as a primary antibody to incubate the sections for 1 h. Further, the sections were colored with freshly configured DAB (Zhongshan, China) and re-dyed with hematoxylin. Two pathologists evaluated the histological sections and staining results using a double-blind method. Measurements were conducted by densitometry performed with image-pro plus software.

2.3. Fluorescence real-time quantitative PCR

Extraction of total RNA from the frozen placenta tissues with RNAiso Plus solution (Takara, Japan) was performed as per the user's manual. After removing contaminated genomic DNA with gDNA Eraser, PrimeScript™ RT reagent kit with Eraser (Takara, Japan) was used to reverse-transcribe 1 μg of total RNA into cDNA in 20 μL of the reaction system. A 7500 Real-time System (Applied Biosystems, USA) was used to perform real-time quantitative PCR in a 20 μL of reaction system, including 1.6 μL of primers (10 μM), 2 μL of template cDNA, 10 μL of SYBR Green Master Mix (Takara, Japan), 0.4 μL of ROX Reference Dye II (50 ×), and sterilized distilled water. The purity of real-time PCR production was monitored by agarose gel electrophoresis and melting curve analysis; β-actin was used as control. The following primers were used: ROR1, forward: 5′- CAGTCAGTGCTGAATTAGTGC-3'; reverse: 5′-TCATCGAGGGTCAGGTAAGAA-3'; β-actin, forward:5′- CCTGGCACC CAGCACAAT-3'; reverse: 5′-GGGCCGGACTCGTCATAC-3'. All procedures were repeated three times. Relative RNA expression levels were determined by the $2^{-\triangle\triangle Ct}$ method.

2.4. Western blot

The frozen placental tissues were homogenized in RIPA lysis buffer, supplemented with phenylmethylsulfonyl fluoride (Solarbio, China) on ice. The supernatant separated by centrifugation was protein extract. Before extraction with lysis buffer, HTR-8/SVneo cells were washed in PBS. Concentration of total protein was determined by the BCA method (Solarbio, China). The protein sample (40 μg) with loading buffer underwent denaturation by heating at 75 °C for 10 min. 8% separation gel and 5% concentrated gel were utilized for (SDS)-polyacrylamide gel electrophoresis. 20 μL sample solution was subjected to electrophoresis and then transferred to PVDF membranes (Millipore, USA), which were blocked for 1 h with 5% non-fat milk at room temperature before their incubation at 4 °C overnight using the following primary antibodies: ROR1 (1:100, Abcam), PI3K (1:500, Abcam), p-PI3K (1:500, Abcam),

Table 1
Clinical sample characteristics and statistical analysis.

Characteristics	Term Control (n = 20)	Preterm Control (n = 20)	Early PE (n = 20)	Late PE (n = 20)
Maternal age (years ± SD)	34.47 ± 5.03	33.45 ± 3.51	32.65 ± 4.89	32.15 ± 5.34
Pre-pregnancy BMI(kg/cm2 ± SD)	20.88 ± 3.06	22.48 ± 2.91	24.18 ± 3.52[b]	25.29 ± 3.29[b,c]
BMI(kg/cm2 ± SD)	28.02 ± 3.49	27.72 ± 0.71	30.78 ± 3.62[a,c]	32.56 ± 4.29[b,c]
Gestational age at delivery (weeks ± SD)	38.76 ± 0.89	32.22 ± 2.48	31.40 ± 1.66[b,d]	37.20 ± 1.88[b]
Systolic blood pressure (mmHg ± SD)	114.43 ± 8.96	120.04 ± 13.02	171.35 ± 21.79[b,c]	160.50 ± 15.68[b,c]
Diastolic blood pressure (mmHg ± SD)	65.66 ± 6.70	72.90 ± 8.73	107.65 ± 12.71[b,c]	97.50 ± 9.94[b,c]
24 h proteinuria (g ± SD)	0.05 ± 0.03	0.08 ± 0.04	3.11 ± 1.13[b,c,d]	2.30 ± 1.16[b,c]
Neonatal birth weight (g ± SD)	3587 ± 444.2	2308.50 ± 569.30	1555 ± 340.5[b,c,d]	2742 ± 771.5[b]
Placental weight (g ± SD)	607 ± 63.8	450 ± 76.69	362 ± 87.3[b,c,d]	497 ± 103.2[b]

[a] $p < 0.05$, versus the normal.
[b] $P < 0.01$, versus the normal.
[c] $P < 0.01$, versus preterm control.
[d] $P < 0.01$, versus late-onset PE.

AKT (1:500, Abcam), *p*-AKT (1:500, Abcam), m-TOR (1:500, Abcam), *p*-mTOR (1:1000, Abcam), p70S6K (1:1000, CST), and p-p70S6K (1:1000, CST); β-actin was used as loading control. Horseradish peroxidase-conjugated secondary antibodies were incubated the membranes for 1 h at room temperature. Protein bands were next visualized by enhanced chemiluminescence (Millipore, USA). Image J software was used to analyze the gray value of target band. Relative protein levels were normalized against β-actin.

2.5. Cell culture and transfection

HTR-8/SVneo was a first-trimester trophoblast cell line. The expression level of ROR1 in HTR-8/SVneo was moderate and was thus it used in this study. It was obtained from Zhong Qiao Xin Zhou Biotechnology (Shanghai, China). The cells were initially cultured in DMEM/F12 media (Gibco, USA) containing 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) in the incubator. The cells were subcultured at a ratio of 1:2 until a growth to approximately 90% density and then were cultured for 1 h using serum-free medium before transfection. ROR1-CMV-MCS-EGFP-SV40-Neomycin (full-length ROR1-overexpressing plasmid, GeneChem, China) and shRNA-ROR1 (Hanbio Biotechnology, Shanghai, China) were built to overexpress and knockdown endogenous ROR1 in the HTR-8/SVneo cell line. Transient transfection of ROR1-CMV-MCS-EGFP-SV40-Neomycin, CMV-MCS-EGFP-SV40-Neomycin (empty vector), and shRNA-NC (negative control) and shRNA-ROR1 into HTR-8/SVneo cells was completed with Lipofectamine 3000 reagent (Invitrogen, USA) as per the user manual. The efficiency of the overexpression and knockdown of ROR1 in the transfected cells were further validated by fluorescence real-time quantitative PCR and Western blot at 48 h post-transfection.

2.6. Cell viability assay

The Cells (3×10^3/well) were transfected in 96-well plates and tested at specific time points (24, 48, and 72 h post transfection) with Cell Counting Kit-8 (CCK-8) following the Kit instructions (Dojindo, Japan). A volume of 10 μL CCK8 reagent was put into each well before incubating the cells at 37 °C for 1 h. A Microplate Reader (ELX-800, BIOTEK, USA) was used to measure the optical density at 450 nm. Each experiment was repeated in triplicate.

2.7. Wound healing assay

The medium was replaced with serum-free medium and treated with 1 μg/μL mitomycin C for 1 h, after which a scratch was made. The cells in each of the groups were first scratched with a sterile 200 μL pipette tip, and then the cell debris washed off with serum-free medium. The cells were observed and photographed using a phase-contrast inverted microscope (IX53, Olympus, Tokyo, Japan), and the location of the cells was recorded. The cells of each group were next cultured for 48 h in serum-free medium and photographed again. The differences in the scratches on both sides between the original edge distance and the distance after 48 h were determined and defined as mobility in percentage. Each experiment was repeated in triplicate.

2.8. Transwell invasion assay

Matrigel matrix (BD, USA) was diluted at a ratio of 1:3 in serum-free media after thawed at 4 °C overnight in the super-clean table, which was performed on ice. The Transwell chamber was removed into a 24 well-plate and was coated with 40 μL of pre-diluted Matrigel matrix (BD, USA) in an incubator for 2 h at 37 °C to solidify the gel. The HTR-8/SVneo cells were transfected into the upper chamber by adding 200 μL of cell suspension (1×10^5 cells/ml) and 800 μL of culture solution containing 30% PBS was transferred into the lower chamber. The 24-well plate was placed in a cell incubator at 37 °C, 5% CO_2 for 48 h. The transwell chamber was washed with PBS twice, fixed in 4% paraformaldehyde for 20 min at room temperature, and stained with 0.5% crystal violet for 5 min. The number of the cells invading the submucosa of microporous membranes was counted in five visual fields of each hole under the inverted microscope (200 ×). The mean value was taken as the number of invasive cells in the hole. The experiments were repeated at least three times.

2.9. Statistical analysis

The experimental results were analyzed with SPSS statistical software (22.0). The data are presented as mean ± SD. Independent samples *t*-test and one-way analysis of variance (ANOVA) were employed to determine the statistical significance, set at a *P*-value of less than 0.05.

3. Results

3.1. Analysis of the clinical data

The main properties of the four groups are presented in Table 1. No distinct difference was observed in age ($P > 0.05$) among the groups. The blood pressure in the two PE groups was significantly higher than that in the two control groups ($P < 0.01$). Similarly, the 24-h urinary quantitative protein level was higher in the two PE groups than in the two control groups ($P < 0.01$), where it remained the highest in the early-onset PE women. Additionally, BMI in the two PE groups was also higher than that in two control groups ($P < 0.01$). Pre-pregnancy BMI in the two PE groups was higher than that in the control group ($P < 0.01$). However, the gestational age at delivery in the two PE groups, especially in the early-onset PE group, was lower than that in the term control group ($P < 0.01$). Similarly, the neonatal birth weight

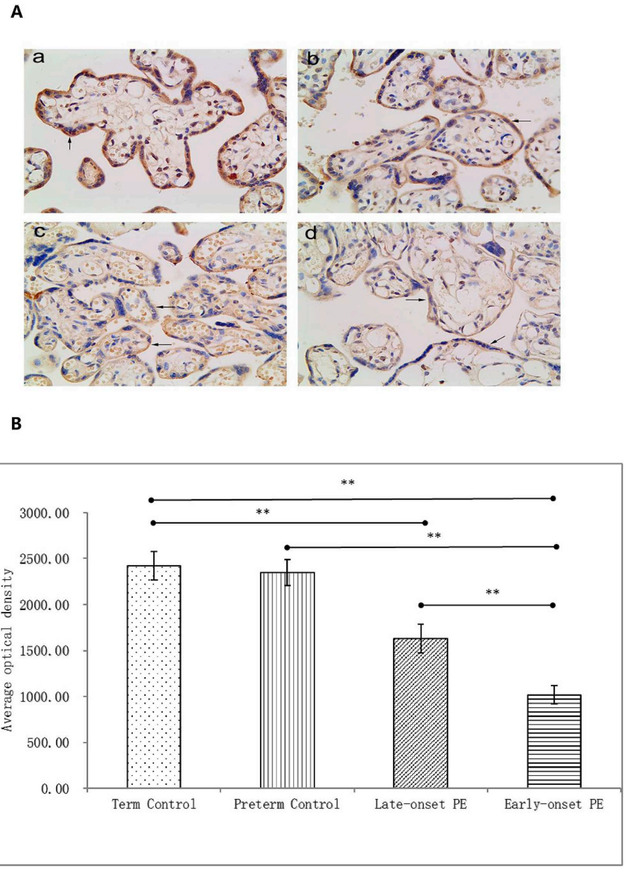

Fig. 1. Immunohistochemical results of ROR1 expression in placenta tissues. (A) The immunohistochemical pictures of ROR1 protein in the placentas of term control, preterm control, late-onset PE and early-onset PE (× 400). (a) Term control, (b) preterm control, (c) late-onset PE, and (d) early-onset PE. The arrow indicates positive staining; (B) Analysis of immunohistochemical densitometry in four groups. *$P < 0.05$, **$P < 0.01$.

and placental weight in the two PE groups were both lower than those in the term control group ($P < 0.01$). Neonatal birth weight and placental weight in the early-onset PE group were lower than the ones in the preterm control group ($P < 0.01$). Moreover, among these groups, the early-onset PE had the lowest birth weight of neonates ($P < 0.01$).

3.2. ROR1 was dysregulated in human placenta tissues of PE

First, the expression location of ROR1 protein in the placentas from the four groups was evaluated by immunohistochemistry. ROR1 was primarily located in the syncytiotrophoblast and cytotrophoblast cells (Fig. 1A). The average optical density assay revealed that in the four groups, the early-onset PE group had the lowest expression of ROR1 in the placenta, followed in a descending order by that in the late-onset PE group, and the control group (Fig. 1B).

Next, the expression level of ROR1 mRNA was analyzed in the four groups using real-time quantitative PCR, which indicated that the dysregulation of ROR1 was more significant in either of the two PE groups than that in the two control groups, with lower expression of ROR1 present in the early-onset PE group (Fig. 2A). Additionally, the protein levels of ROR1 were assessed by Western blotting, and similar results were obtained (Fig. 2B and C). These findigs were in agreement with the obtained immunohistochemistry results.

3.3. Overexpression and knockdown of ROR1 in HTR-8/SVneo cells

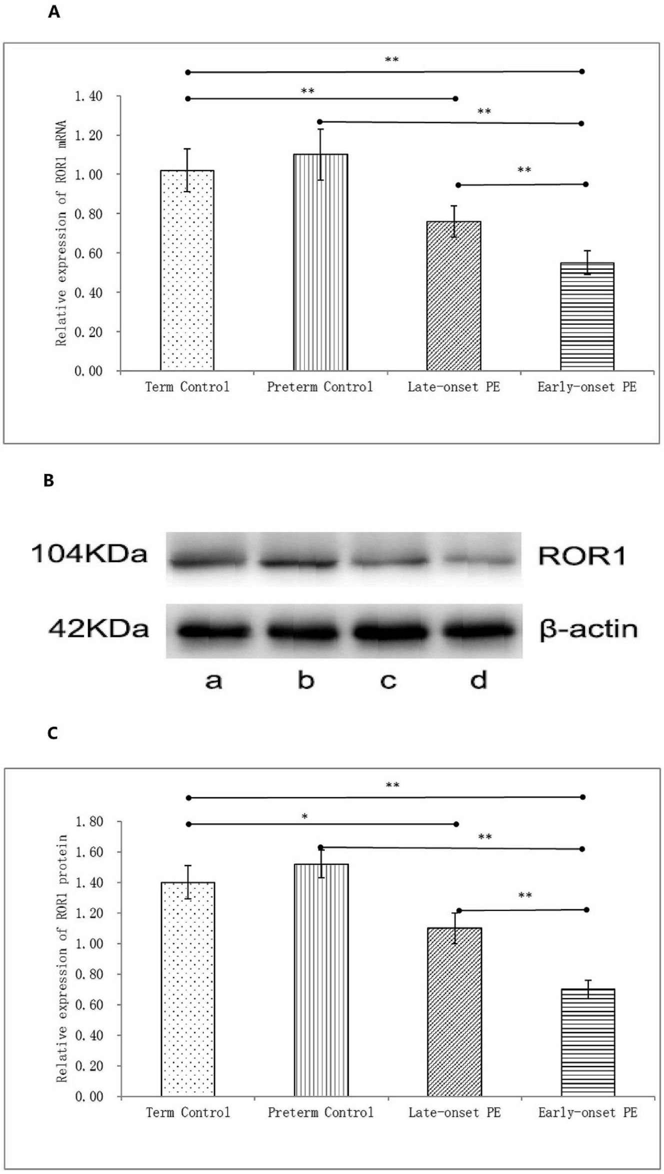

Fig. 2. Expression of ROR1 mRNA and protein in placental tissues of term control, preterm control, early-onset PE and late-onset PE. (A) ROR1 mRNA expression were quantified by fluorescence quantitative PCR. The mRNA was normalized against β-actin. **$P < 0.01$; (B) Representative bands of ROR1 protein expression from the Western blot analysis: (a) term control, (b) preterm control (c). Late-onset PE, (c) early-onset PE; (C) Densitometric values analysis of ROR1 protein from Western blot. The protein expression level was normalized against β-actin. *$P < 0.05$, **$P < 0.01$.

To further validate our results, the effects of ROR1 overexpression and knockdown were evaluated in the HTR-8/SVneo cell line. We detected the ROR1 mRNA and protein expression by real-time PCR and Western blot at 48 h post transfection, respectively. Compared to non-targeting cells, the ROR1 mRNA and protein levels were distinctly improved in the cells transfected with the ROR1-CMV-MCS-EGFP-SV40-Neomycin plasmid. In contrast, the ROR1 mRNA (Fig. 3A) and protein levels were significantly reduced in the cells transfected with shRNA-ROR1 (Fig. 3B and C).

3.4. Effects of ROR1 overexpression and knockdown on cell proliferation

The cell viability of HTR-8/SVneo cells was assessed at 24, 48, and 72 h post transfection. We found that it was significantly increased by the ROR1 overexpression but decreased by the ROR1 knockdown at 48

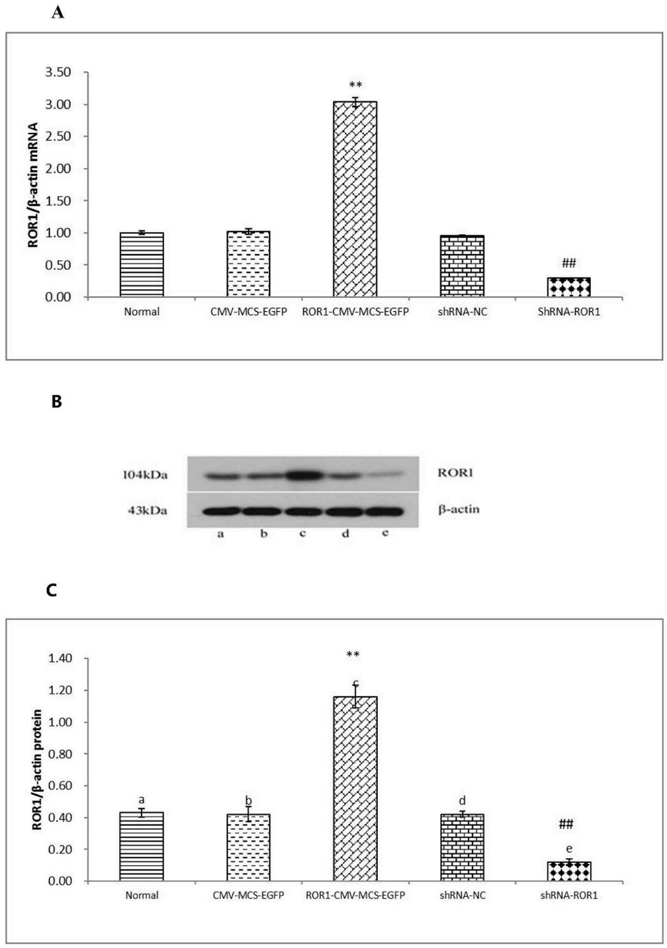

Fig. 3. ROR1 expression in HTR-8/SVneo cells. Cells were transfected with CMV-MCS-EGFP-SV40-Neomycin (b), ROR1-CMV-MCS-EGFP-SV40-Neomycin (c), shRNA-NC (d), and shRNA-ROR1 (e). The expression of ROR1 mRNA in HTR-8/SVneo cells was validated by qPCR (A) and Western blot (B and C) at 48 h post transfection. The expression of ROR1 was significantly increased in ROR1-CMV-MCS-EGFP-SV40-Neomycin group and reduced in shRNA-ROR1 group. Data were presented as means ± SD of three independent experiments. ** represents increase, $P < 0.01$, and ## represents reduction, $P < 0.01$.

and 72 h post transfection (Fig. 4A). The cell viability exhibited no distinct differences in the empty vector group and shRNA-NC group in comparison with the normal group.

3.5. Effects of ROR1 overexpression and knockdown on cell migration

The ROR1 overexpression significantly increased wound healing migration, whereas the ROR1 knockdown caused a significant decrease in this parameter (Fig. 4B and C). No marked difference was found among the normal, CMV-MCS-EGFP (empty vector), and shRNA-NC groups.

3.6. Effects of ROR1 overexpression and knockdown on cell migration

We then attempted to examine the effect of ROR1 on HTR-8/SVneo cells invasion by Matrigel-coated transwells. The ROR1 overexpression led to a significant improvement in cell invasion, whereas the ROR1 knockdown markedly inhibited it (Fig. 4D and E). However, the cell invasive ability did not change obviously in the empty vector and shRNA-NC groups as compared to the normal group.

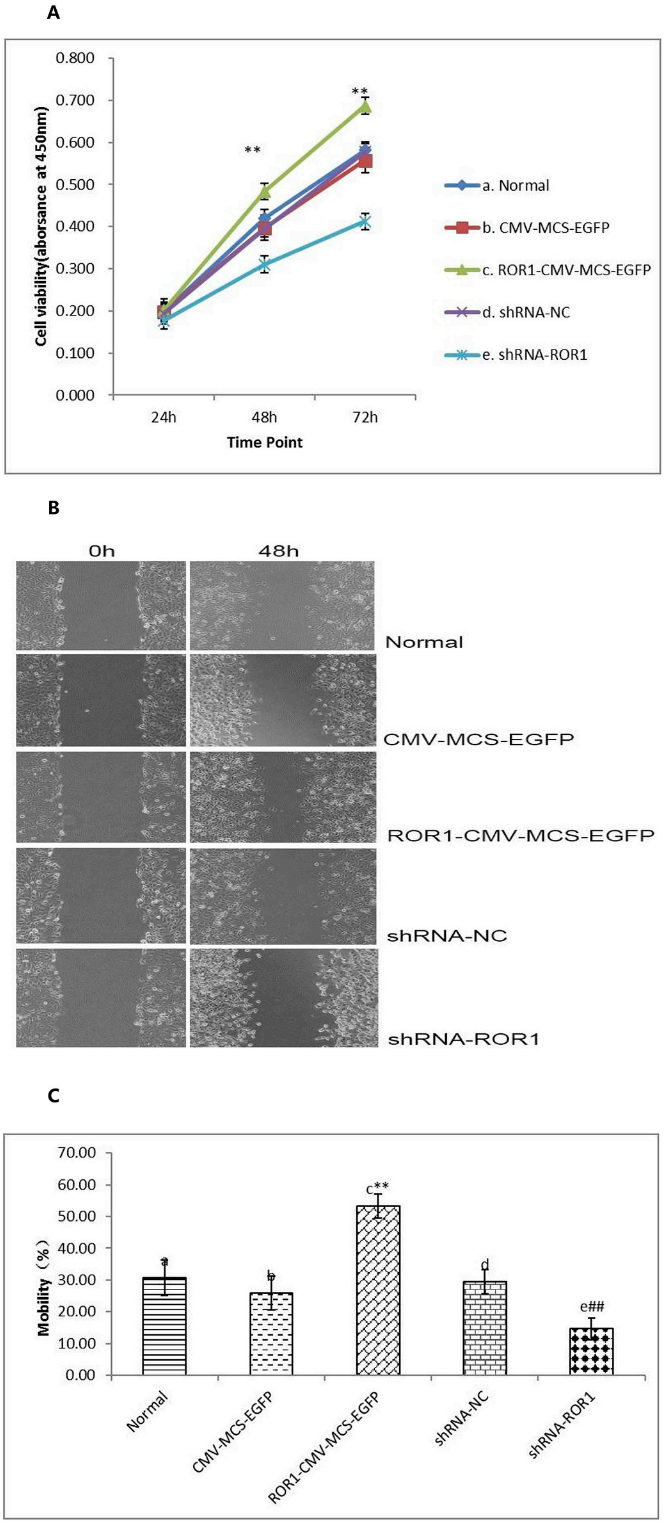

Fig. 4. Effect of ROR1 on the proliferation, migration and invasion of HTR-8/SVneo cells. (A) Cell viability was analyzed at 24, 48, and 72 h post transfection; (B) Representative photographs of wound healing assays were taken at 0 and 48 h (× 200); (C) Mobility assay of HTR-8/SVneo cells at 48 h post transfection; (D) Representative photographs of transwell invasion assay were taken at 0 and 48 h (× 200). Normal cell group (a), CMV-MCS-EGFP-SV40-Neomycin group (b), ROR1-CMV-MCS-EGFP-SV40-Neomycin group (c), shRNA-NC group (d), and shRNA-ROR1 group (e); (E) The invasive numbers of HTR-8/SVneo cells were analyzed at 48 h post transfection. The cell proliferation, migration and invasion were increased in ROR1-CMV-MCS-EGFP-SV40-Neomycin group (c) and reduced in shRNA-ROR1 group (e) at 48 h post transfection. The results were presented as mean ± SD of three independent experiments. **represents increase, $P < 0.01$, and ## represents reduction, $P < 0.01$.

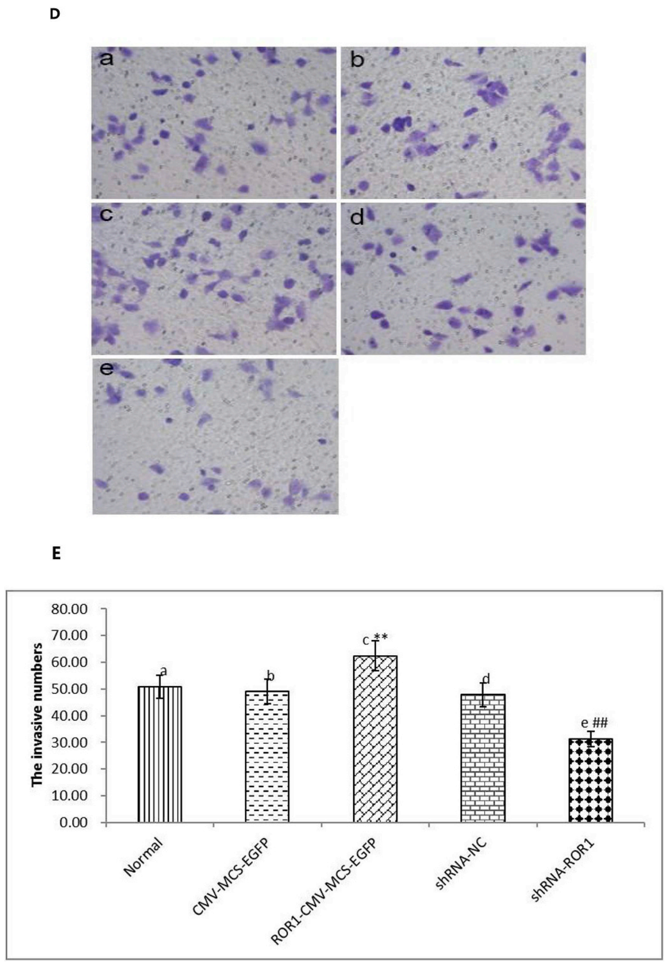

Fig. 4. (*continued*)

3.7. *Changes of crucial kinases in the PI3K/AKT/mTOR pathways induced by ROR1 overexpression and ROR1 knockdown in HTR-8/SVneo cells*

The expression of crucial kinases in PI3K/AKT/mTOR pathway was detected to demonstrate the potential mechanism underlying ROR1 related regulation. The expression levels of p-P70S6K, *p*-mTOR, *p*-AKT, and p-PI3K were significantly upregulated by ROR1 overexpression, but drastically downregulated through the ROR1 knockdown (Fig. 5). The levels of PI3K, AKT, TOR, and P70S6K displayed no significant change.

4. Discussion

This is the first study that analyzed the localization and expression levels of ROR1 in placental tissue of late-onset PE, early-onset PE, preterm delivery and normal term pregnancy. Our immunocytochemistry results showed that ROR1 was strongly expressed in the cytotrophoblast and syncytiotrophoblast of normal placenta tissues. Moreover, the expression of ROR1 mRNA and protein in the PE placenta was significantly lower than that of the normal placenta as determined though qPCR and Western blotting, which was most significantly manifested in the early-onset PE. Previous studies confirmed that ROR1 is expressed during embryogenesis and plays an important role in skeletal and neural organogenesis [17]. However, this influence on most normal post-partum tissues is negligible. In another investigation, abnormal Wnt5a-ROR signaling resulted in defects of the decidua and placenta formation in late pregnancy, ultimately leading to adverse pregnancy outcomes [15]. Therefore, the distribution of ROR1 in the placental tissue in our examination revealed that the ROR1 protein was probably potentially critically involved in the embryonic development and placenta formation.

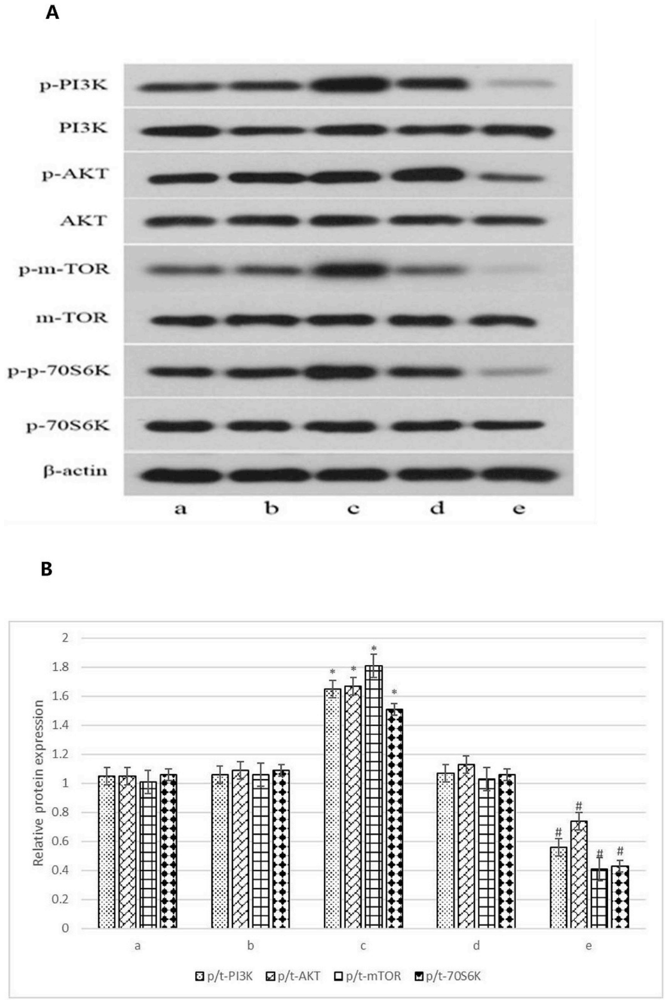

Fig. 5. Effect of ROR1 overexpression and knockdown on PI3K/AKT/m-TOR pathway. (A) Representative bands of crucial kinases protein in PI3K/AKT/mTOR pathway from Western blot: normal cell group (a), CMV-MCS-EGFP-SV40-Neomycin group (b), ROR1-CMV-MCS-EGFP-SV40-Neomycin group (c), shRNA-NC group (d), and shRNA-ROR1 group (e); (B) Relative expression assay of phosphorylated protein/total protein. The results are presented as mean ± SD of three independent experiments. The PI3K/AKT/mTOR signaling pathway was activated by ROR1 overexpression, while inhibited by ROR1 knockdown. **represents activation, $P < 0.01$. ## represents inhibition, $P < 0.01$.

The pathogenesis in early-onset PE was attributed to the placental abnormality due to reduced invasion of extravillous trophoblast [18]. Moldenhauer et al. reported that placental lesions were founded to have significantly higher rates in PE women with gestation between 28 and 32 weeks [19]. Moreover, poor spiral artery remodeling was more obvious in early-onset PE than in late-onset PE [20]. Early-onset PE is mainly associated with the expression of a high total vascular resistance (TVR)-low cardiac output (CO) response. Uterine Doppler studies were accurate in predicting the occurrence of early-onset PE than that of late-onset PE [21]. Compared with late onset, early-onset PE had a higher risk of multiple organ dysfunction and fetal complications [22]. Crispi et al. reported similar placenta-mediated complications between early-onset PE and fetal growth restriction (FGR) [23]. However, late-onset PE might be closely related to the maternal constitutional factors and was marked by a low TVR-high CO [24]. Late-onset PE arises due to the interaction between a predisposed maternal constitution and a normal placenta, reflecting a microvascular disease [18]. Moreover, the uterine resistance index was normal or slightly increased, and the perinatal outcome was more favorable [25]. Although the pathogenesis of the two subtypes of PE is perhaps different, it is still difficult to distinguish them due to their frequent significant overlapping, and most PE patients have elements of both pathologies. In agreement with previous reports, our study demonstrated shorter gestation time, lower placental weight, and neonatal birth weight in early-onset PE in comparison with late-onset PE and normal pregnant women. Conversely, higher pressure and urine protein were observed in early-onset PE.

Normal pregnancy requires increased perfusion of the placental intervillous space by recasting of maternal uterine spiral arteries. The proliferation and invasion of extravillous trophoblasts are necessary for vascular remodeling [26]. Numerous studies suggest that PE is related to the defect of trophoblast invasion. Reduced invasion and insufficient spiral artery remodeling were closely associated with pregnancy-related syndrome, *e.g.*, PE and fetal growth restriction [24]. Abnormalities in spiral artery remodeling caused low blood perfusion, leading to consequent dysfunctions of placental villous tree and fetal placental circulation [27]. The processes associated with trophoblast proliferation and invasion are governed by numerous factors, which in turn can generate signaling cascades, and regulate the effect or the participation of molecules. Our findings suggest that ROR1 can regulate the proliferation, migration, and invasion of human trophoblast cells. From here we see that downregulated expression of ROR1 in placenta may be an important factor in causing the onset of PE, and that the expression level is closely related to the occurrence time and severity of the disease.

Concerning the etiology and pathogenesis of PE, we further discovered that ROR1 may activate PI3K/AKT/mTOR signaling pathway, which is involved in the biological behavior of multiple cells in processes, such as proliferation, migration, and invasion [12,28,29]. PI3K/AKT signaling pathway is activated after binding of ROR1 with its ligand WNT5a [30]. As a downstream cascade protein of PI3K/AKT, mTOR and S kinase isoforms are involved in BeWo and JEG-3 trophoblastic cells [31]. Jin Xu et al. confirmed that PI3K/AKT/mTOR pathway is involved in regulation of trophoblast viability and invasion by LncRNA-H19 [32]. Kirs Gene lyn et al. found that the inhibition of placental mTOR activity provided a connection between placental malaria-associated intervillositis and decreased the birth weight [33]. Moreover, a defective AKT/mTOR signaling pathway in response to insulin is the key mechanism underlying insulin resistance in PE, gestational diabetes mellitus, and pregestational maternal obesity [34]. Our results revealed that the overexpression of ROR1 significantly increased the levels of p-P70S6K, phosphor-mTOR, phosphor-AKT, and phosphor-PI3K, whereas the knockdown of ROR1 produced the opposite results. Thus, it can be seen that PI3K/AKT/mTOR signaling pathway is probably the key mechanism underlying deregulated ROR1 leading to PE.

In conclusion, we demonstrated for the first time that decreased ROR1 was likely involved in the development of PE through reduced viability and invasion in trophoblast cells by activating PI3K/AKT/mTOR signaling pathways. Our research findings provide new insights into the development of diagnosis, prevention, and treatment strategy to PE. However, the experimental conditions *in vitro* are different from those *in vivo,* and further experiments are required to verify the results. The specific mechanism underlying PI3K/AKT/mTOR regulating trophoblast and ROR1 ligand are the goal of our further research.

Conflicts of interest

We declare that we have no conflict of interest in this work.

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