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ORIGINAL ARTICLE

Dysregulated expression of ACTN4 contributes to endothelial cell injury via the activation of the p38-MAPK/p53 apoptosis pathway in preeclampsia

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Abstract

Preeclampsia (PE) is a hypertensive disease associated with increased endothelial cell dysfunction caused by systemic oxidative stress. Alpha-actinin-4 (ACTN4) is a member of the α-actinin family of actin crosslinking proteins that are upregulated in several types of cancer. However, its role in PE remains unclear. In this study, we found that ACTN4 was localized in placenta vascular endothelial cells (ECs), and its expression was downregulated in primary human umbilical vein endothelial cells (HUVECs) from severe preeclamptic patients compared to that in HUVECs from normotensive pregnant women. ACTN4 expression was also decreased in normotensive HUVECs treated with H_2O_2. Downregulation of ACTN4 by siRNA or H_2O_2 treatment promoted normotensive HUVEC apoptosis and increased p38-MAPK phosphorylation along with elevated levels of p53 phosphorylation, caspase cascade proteins, and bax and repressed expression of bcl-2. Conversely, upregulation of ACTN4 in PE HUVECs significantly inhibited apoptosis and decreased p38-MAPK phosphorylation compared to that of the PE HUVEC controls. In addition, overexpression of ACTN4 in normotensive HUVECs attenuated H_2O_2 treatment-induced apoptosis with decreased p53 phosphorylation, caspase cascade, and bax expression levels and increased expression of bcl-2 compared to that of only H_2O_2 treatment. Moreover, the suppression of ACTN4 induced apoptosis, which could be blocked by the p38-MAPK inhibitor SB202190. Collectively, these results demonstrate that dysregulated ACTN4 expression may be associated with PE due to its effects on endothelial cell apoptosis via the p38-MAPK/p53 apoptosis pathway.

Keywords Preeclampsia · Endothelial cell dysfunction · Oxidative stress · Apoptosis · P38-MAPK/p53 pathway

Introduction

The vascular endothelium is a type of epithelium that lines the blood vessels, forming an interface between circulating blood and the vessel wall. The vascular endothelium regulates vascular wall functions, such as fluid filtration, homeostasis, hormone trafficking, and vascular tone regulation [5]. Changes in the structure and function of the vascular endothelium

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(endothelial dysfunction) are aetiological factors in many cardiovascular diseases, such as coronary arterial disease, atherosclerosis, and preeclampsia (PE) [1, 4, 20].

Preeclampsia is a pregnancy-related disorder characterized by new-onset hypertension, proteinuria, and systematic maternal endothelial dysfunction. PE affects 3–8% of pregnant women each year and is a significant cause of maternal and foetal morbidity and mortality worldwide [10]. It has been speculated that poor trophoblast invasion of the spiral arteries during placentation may be the primary cause of the disease, followed by systemic endothelial cell dysfunction in late gestation, which may lead to final clinical manifestations [24]. Moreover, in this pathological development, excessive endothelial cell (EC) apoptosis caused by oxidative stress has also been observed in some studies [3, 22, 25], and many apoptosis-related pathways, such as the p38 mitogen-activated protein kinase (p38-MAPK) pathway and NF-κB pathway, are involved in this pathology [12, 18].

α-Actinins (ACTNs) are cytoskeletal proteins that maintain cytoskeleton integrity and control cell movement [23, 27]. There are four isoforms of ACTN in human tissue [13]. ACTN2 and ACTN3 (muscle isoforms) are highly expressed in the muscle, whereas ACTN1 and ACTN4 (non-muscle isoforms) are ubiquitously expressed in numerous cell types [9]. As one of two non-muscle isoforms, ACTN4 has mostly been described as a metastasis-related gene in several types of malignant tumors that promotes invasion and metastasis [8]. Moreover, ACTN4 is also involved in signal transduction, nuclear translocation, and gene expression regulation [9]. Additionally, as a widespread isoform, ACTN4 is also present in ECs and is involved in their functional regulation [7, 32]. Endothelial NOS activity in bovine aortic endothelial cells can be tonically and dynamically regulated by competitive interaction with ACTN4 and calmodulin [7]; additionally, another study showed that a monoclonal C-7 antibody can inhibit endothelium-dependent vasorelaxation by inhibiting ACTN4 activity. These results indicate that ACTN4 may participate in EC regulation. Moreover, recent studies have shown that ACTN4 is involved in cell apoptosis [16, 17]. Since EC apoptosis plays an important role in the progression of PE, we speculate that ACTN4 may also play an important role in this process; however, no studies have focused on the role of ACT4 in EC apoptosis.

We therefore hypothesized that PE is associated with aberrant expression of ACTN4 in human umbilical vein endothelial cells (HUVECs), which could influence EC function through apoptosis, and preliminarily elucidated the underlying mechanisms.

Materials and methods

Placental tissue collection, cell isolation and identification, cell culture

Placental tissues and umbilical cords from nulliparous women with severe PE ($n = 20$) and nulliparous women with normotensive pregnancies ($n = 20$) matched by comparable gestational age were obtained after delivery by caesarean section. PE was diagnosed based on clinical and laboratory findings according to the AJOG criteria [10]. Pregnant women with multiple pregnancies and/or with chronic medical disorders, such as diabetes mellitus, cardiovascular disease, chronic renal disease, collagen disorders, chronic hypertension, or metabolic diseases, were excluded. Small pieces (1 cm^3) were cut from the maternal side with the non-calcified area under aseptic conditions and washed briefly in ice-cold sterile normal saline. For immunofluorescence, pretreatment was carried out as described previously [33].

For cell isolation, HUVECs from normal placentas and placentas from PE patients were isolated as previously described [11]. Isolated endothelial cells were then cultured in DMEM/F-12 (HyClone, SH30023.02) containing 20% foetal bovine serum at 37 °C with 5% CO_2 and 95% air in a humidified incubator. HUVECs at passages 2–6 were used for the experiments. Human factor VIII (FVIII) was used to identify isolated cells by immunofluorescence [28], and the results can be found in the attachment to the manuscript.

Informed consent was obtained from all participants. Approval was also granted by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. All participants signed the informed consent form. The clinical characteristics of all participants are summarized in Table 1.

Immunofluorescence

In regard to the frozen placental tissues, detection of ACTN4 activity in normotensive and preeclamptic placenta vascular endothelium was performed as described previously [33]. Briefly, the primary antibodies were diluted in the appropriate blocking solution at the following concentrations: mouse anti-ACTN4 (1:100; Proteintech, 66628-1-Ig) and rabbit anti-CD31 (1:100; Abcam, ab32457). The secondary antibody was a FITC-labelled goat anti-rabbit IgG (1:200; Beyotime, A0562) and a Cy3-labelled goat anti-mouse IgG (1:200; Beyotime, A0521). The nuclei were stained with DAPI (1 μg/mL, Beyotime, C1005). Images were acquired with an

Table 1 Clinical characteristics of the study subjects

Characteristics	Normal pregnancy $n = 20$	Preeclampsia $n = 20$
Maternal age	27.30 ± 3.9	27.60 ± 2.9
BMI (kg/m^2)	28.57 ± 1.69	28.77 ± 1.81
Smoking history	None	None
Gestational age (weeks)	36.26 ± 1.13	36.48 ± 1.07
Neonatal birth weight (g)	3204.35 ± 475.97	2573.50 ± 435.79*
Systolic blood pressure (mmHg)	119.15 ± 14.68	162.10 ± 7.86*
Diastolic blood pressure (mmHg)	74.70 ± 4.31	103.20 ± 5.65*
Proteinuria level (g/24 h)	++ to +++	–

Values are shown as means ± SEM. All statistical analyses were performed using non-parametric Mann–Whitney test on SPSS 19.0

*$P < 0.05$

EVOS FL Auto Imaging System (Life Technologies, Carlsbad, California, USA).

Cell transfection, p38 inhibition, and H_2O_2 treatment

The sequences of the ACTN4 siRNA (5′-GUUCAUCGUCCAUACCAUC-3′) and a negative control siRNA were obtained from Hayashida's article [6] and synthesized by GenePharma Biotechnology (Shanghai, China). To knockdown ACTN4, 50 nM siRNA was transfected into 70% confluent ECs in the presence of Lipofectamine® 2000 (Thermo Fisher Scientific) in 6-well plates according to a protocol outlined by the manufacturer. A plasmid overexpressing ACTN4 and the empty vector were purchased from Hanbio Biotechnology (Shanghai, China). In regard to the overexpression experiments, ECs were grown to approximately 60–70% confluence in 35-mm dishes and transfected with 2 μg of the ACTN4 plasmid or the empty vector (Hanbio), using Lipofectamine® 2000 Transfection Reagent as recommended by the manufacturer. The transfection efficiency was examined using Western blot.

For the pathway inhibitor experiment, HUVECs were pre-incubated with 20 μM highly selective p38-MAPK inhibitor SB202190 (Selleck Chemicals, S1077) or 0.1% dimethyl sulfoxide (DMSO) as a control for 2 h, followed by siRNA or control transfection for another 48 h. The post-treatment cell lysates were then subjected to Western blot analysis with the indicated antibodies. SB202190 was diluted with DMSO.

In regard to the H_2O_2 experiments, HUVECs with or without transfection were exposed to 100 μM H_2O_2 in complete medium for 24 h and were subjected to the following experiments.

Migration assay

A migration assay was performed to assess the effect of HUVEC migration [19]. The outer chamber was a 24-well plate, and the inner chamber was a polycarbonate filter (8 μm pores; Corning, 3422). HUVECs (1.0×10^4 per chamber) with different treatments were added to each inner chamber with 200 μL of serum-free DMEM, and the outer chamber was immediately loaded with 600 μL of DMEM containing 20% foetal bovine serum. After 8 h of incubation at 37 °C, non-migrating cells on the upper surface of the filters were removed using a sterile cotton swab, and the migrated cells on the lower surface of the filters were fixed in ice-cold methanol and dyed with 0.1% crystal violet. The number of migratory cells was recorded using an EVOS FL Auto Imaging System.

Tube formation assay

To investigate the tube formation ability of HUVECs, a tube formation assay was performed. The Matrigel matrix (Corning, 356234) was distributed in a 48-well plate (150 μL/well) on ice and allowed to solidify at 37 °C for at least 30 min. After the Matrigel had solidified, post-treatment HUVECs (2.0×10^4 per well) were gently added to each of the triplicate wells. Tube formation was quantitatively measured after 8 h of incubation by calculating the total length of tube-like structures using the EVOS FL Auto Imaging System interfaced with Image-Pro Plus image analysis. Tracks of endothelial cells that were organized into networks of cellular cords (tubes) were counted in 5 random fields. The tube formation index was calculated as the total tube length (millimetre) per millimetre squared area.

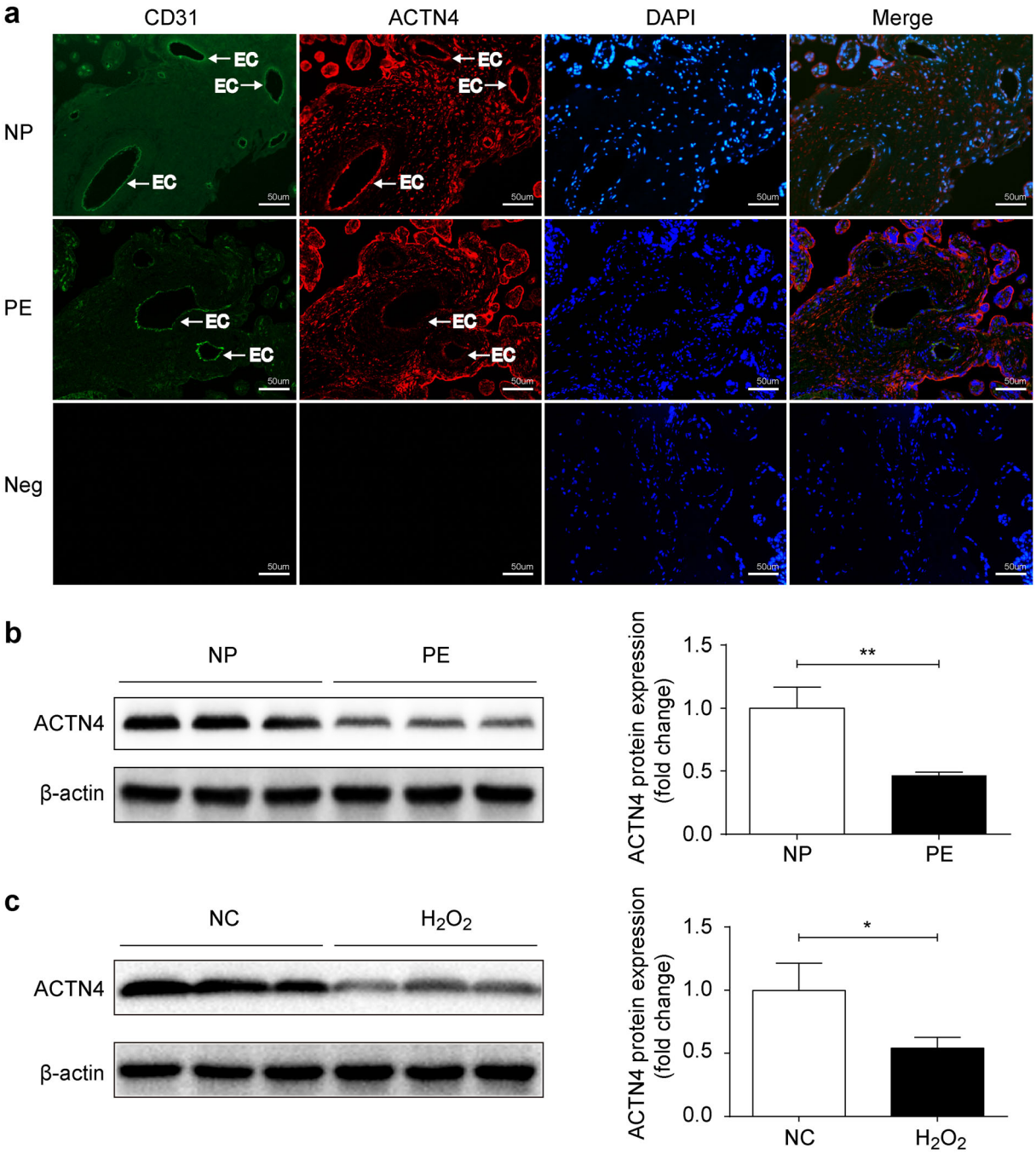


Fig. 1 Downregulated expression of ACTN4 in preeclamptic endothelial cells and in HUVEC treated with H_2O_2. **a** Double labelling the immunofluorescence analysis of ACTN4 protein expression in the endothelial cells in placenta from normotensive patients (NP) and PE patients (PE). CD31 serves as a marker for endothelial cell (EC). Bar = 50 μm. **b** Western blot analysis of ACTN4 expression in HUVEC isolated from normotensive pregnancies ($n = 20$) and PE patients ($n = 20$). **c** ACTN4 in HUVEC isolated from normotensive pregnancies were treated with H_2O_2 (100 μM) for 24 h, and, then, the lysates were subjected to Western blot analysis. Statistical analysis of protein densitometry quantification in Western blot by Student's t test. Data are means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$

Western blot analysis

HUVECs were lysed in RIPA lysis buffer (Beyotime, P0013B) in accordance with the manufacturer's instructions. The extracted proteins were quantified with a BCA protein assay (Beyotime, P0010S) and subjected to Western blot analysis. Primary antibodies against the following proteins were used: ACTN4 (1:2000; Proteintech, 66628-1-Ig), β-actin (1:1000;

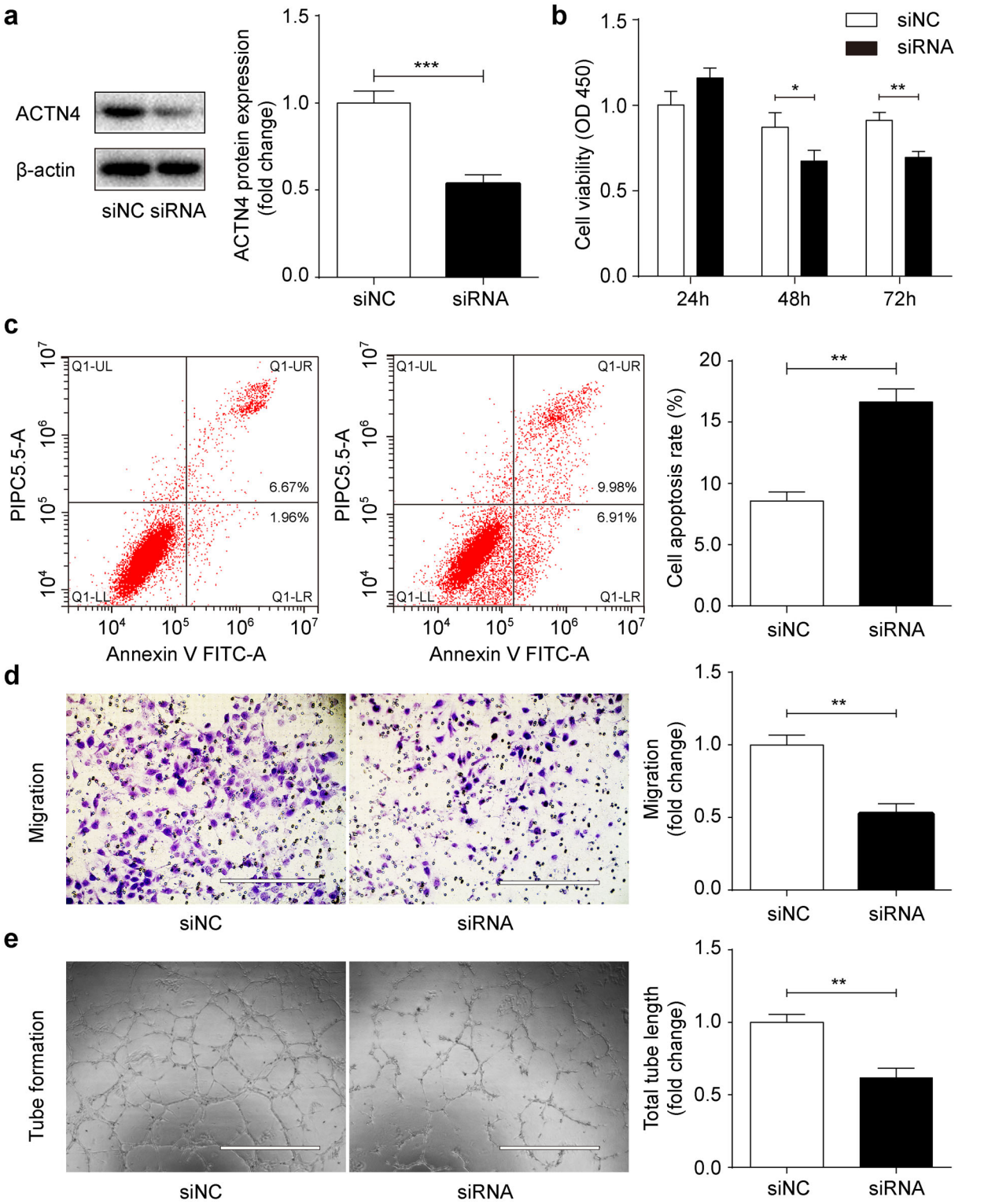
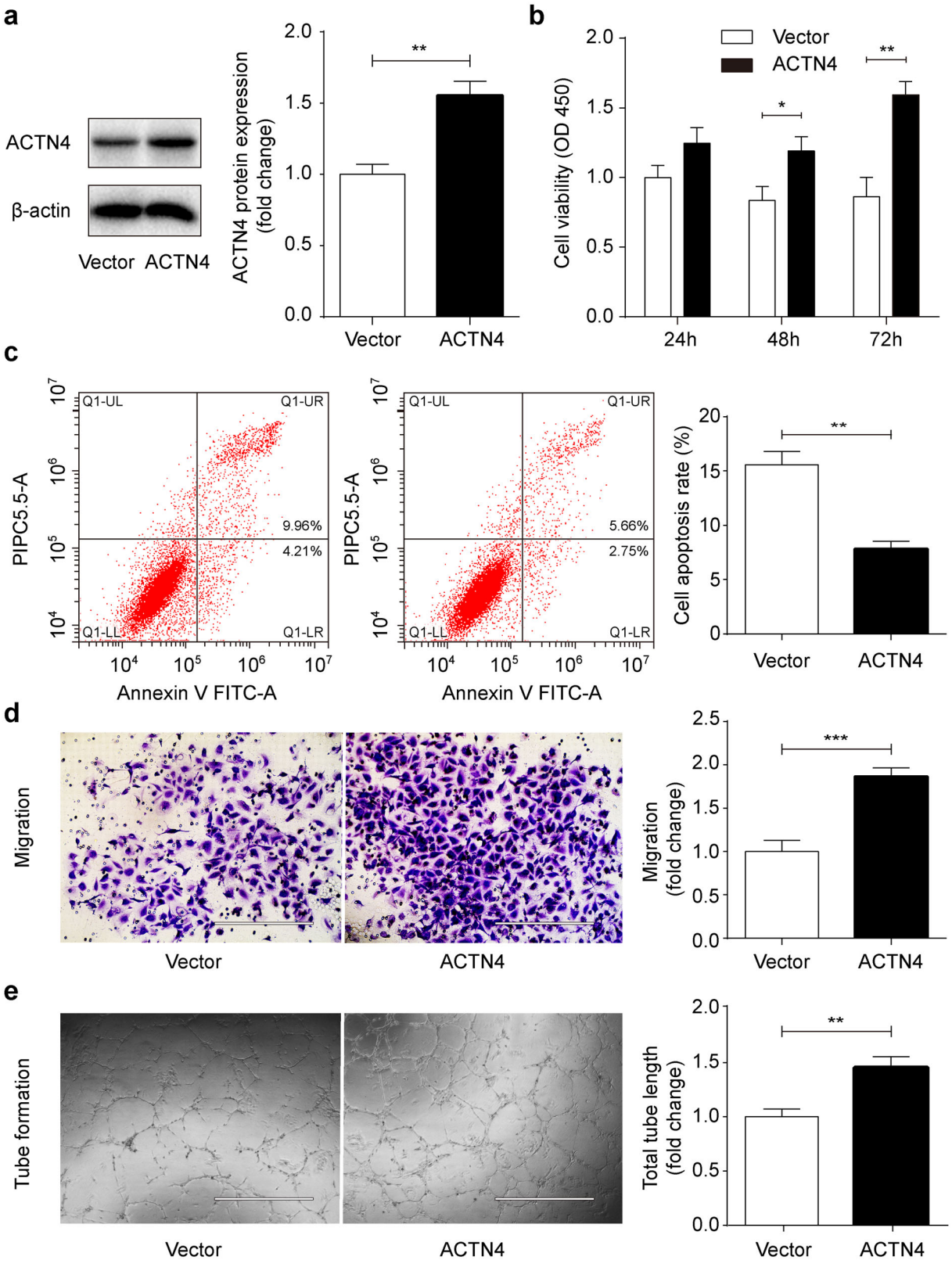


Fig. 2 Downregulation of ACTN4 promoted HUVECs from normotensive pregnancy apoptosis. **a** HUVEC isolated from normotensive pregnancies were transfected with ACTN4 siRNA or negative control siRNA (siNC) for 48 h. Confirmation of ACTN4 knockdown by RNA interference is shown by Western blot. **b** Viability of the cells after knockdown was detected by CCK-8 (Cell Counting Kit-8) assay at different time points. **c** Ratio of apoptotic cells was confirmed by flow cytometric analysis. **d** Migration of HUVECs was determined by migration assays. Representative images are shown. Bar = 200 μm. The relative fold changes in cell migration were counted. **e** Tube formation ability of HUVECs was detected by Matrigel migration assays. Representative images are shown. Bar = 1000 μm. The relative fold changes in total tube length were counted. All the statistical data were analyzed by Student's *t* test. All data are means ± SEM of 3 independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$. OD450, optical density at 450 nm


a
ACTN4
β-actin
Vector ACTN4
ACTN4 protein expression (fold change)
2.0
1.5
1.0
0.5
0.0
**
Vector
ACTN4
b
Vector
ACTN4
Cell viability (OD 450)
2.0
1.5
1.0
0.5
0.0
*
**
24h
48h
72h
c
PIPC5.5-A
Q1-UL
Q1-UR
9.96%
4.21%
Q1-LL
Q1-LR
Annexin V FITC-A
5.66%
2.75%
Cell apoptosis rate (%)
20
15
10
5
0
**
Vector
ACTN4
d
Migration
Vector
ACTN4
Migration (fold change)
2.5
2.0
1.5
1.0
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Vector
ACTN4
e
Tube formation
Vector
ACTN4
Total tube length (fold change)
2.0
1.5
1.0
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Vector
ACTN4

◀ **Fig. 3** Upregulation of ACTN4 suppressed HUVEC apoptosis and rescued cell functions. HUVECs isolated from PE patients were transfected with ACTN4 plasmids or empty vector as control for 48 h. **a** The lysates were subjected to Western blot analysis with the indicated antibodies. **b** Cell viability was detected by CCK-8 assay. **c** Ratio of apoptotic cells in a population of HUVECs transfected with the indicated plasmids, as confirmed by flow cytometric analysis. **d** Migration of HUVECs was determined by migration assays. Representative images are shown. Bar = 200 μm. The relative fold changes in cell migration were counted. **e** Tube formation of HUVECs was detected by Matrigel migration assays. Representative images are shown. Bar = 1000 μm. The relative fold changes in total tube length were counted. All the statistical data were analyzed by Student's *t* test. All data are means ± SEM of 3 independent experiments performed in triplicate. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. OD450, optical density at 450 nm

Proteintech, 60008-1-Ig), P38 (1:1000; Cell Signaling Technology, 8690), p-P38 (1:1000; Cell Signaling Technology, 4511), JNK (1:1000; Cell Signaling Technology, 9252), p-JNK (1:1000; Cell Signaling Technology, 9251), ERK (1:1000; Cell Signaling Technology, 9102), p-ERK (1:1000; Cell Signaling Technology, 9101), Bax (1:1000; Proteintech, 50599-2-Ig), Bcl-2 (1:1000; Proteintech, 50599-2-Ig), caspase3 (1:500; Abcam, ab13847), cleaved-caspase3 (1:500; Abcam, ab2302), caspase9 (1:1000; Abcam, ab32539) and cleaved-caspase9 (1:1000; Abcam, ab2324). Polyvinylidene fluoride membranes were incubated with the primary antibody at 4 °C overnight and then incubated with the appropriate HRP-conjugated secondary antibody (1:5000; Proteintech, SA00001-1 and SA00001-2). Immunoreactive signals were detected by enhanced chemiluminescence reagents and were analyzed by a Chemi-doc image analyzer (Bio-Rad).

Cell viability assessment

Cell viability was measured by the Cell Counting Kit-8 (CCK-8) assay (Solarbio, CA1210) and performed according to the manufacturer's instructions. HUVECs (0.8 × 104) transfected with siRNA or plasmids were diluted in 100 μL of serum-free medium and then seeded in a chamber of a 96-well plate. The cell viability was determined after 24, 48, and 72 h by using a microplate reader (Bio-Rad) at a 450 nm wavelength. The experiment was performed in triplicate.

Flow cytometry analysis

The apoptosis rate of HUVECs pre-treated with siRNA or plasmids was determined using flow cytometry. Annexin V-FITC-A and PI Apoptosis Detection Kits (Key-Gen Biotech,

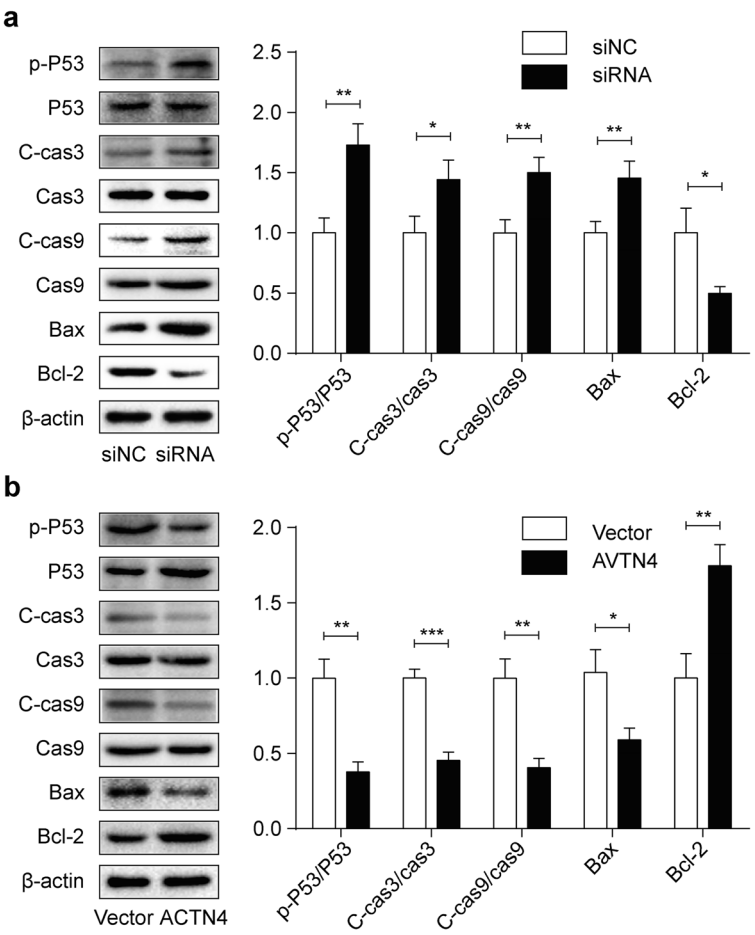

Fig. 4 Apoptosis-related proteins were regulated by ACTN4. **a** HUVECs isolated from normotensive pregnancies after knockdown for 48 h were subjected to Western blot analysis with the indicated antibodies. **b** HUVECs isolated from PE patients after transfection with the empty vector or ACTN4 expression plasmids for 48 h were subjected to Western blot analysis with the indicated antibodies. Densitometry quantification of these protein levels is shown. All the statistical data were analyzed by Student's *t* test. All data are means ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

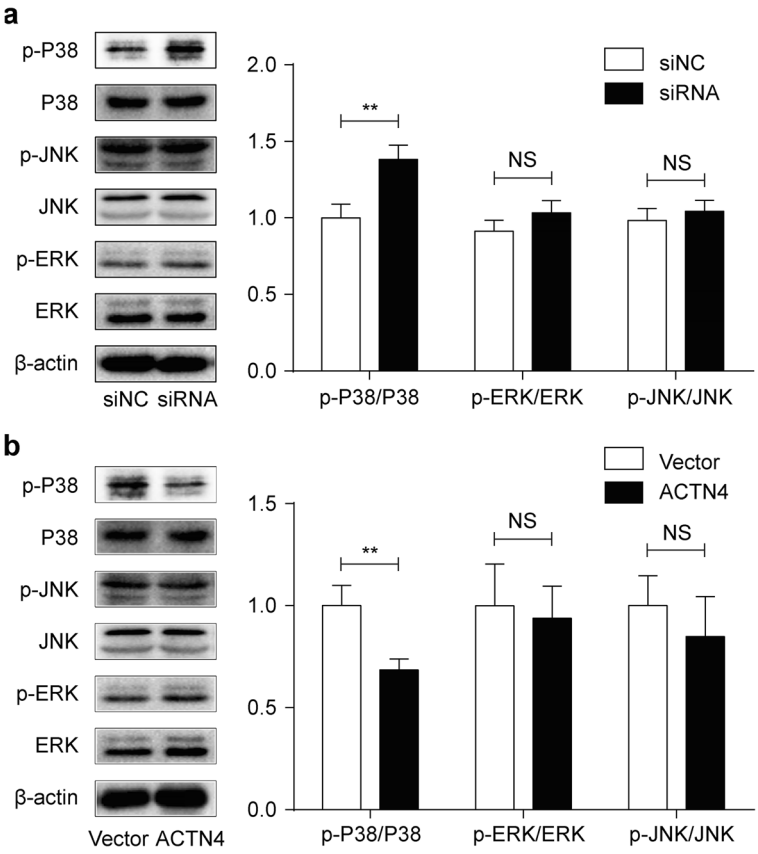

Fig. 5 ACTN4 operated p38-MAPK through changing its phosphorylation in HUVECs. **a** HUVECs isolated from normotensive pregnancies after knockdown for 48 h were subjected to Western blot analysis with the indicated antibodies. **b** HUVECs isolated from PE patients after transfection with the empty vector or ACTN4 expression plasmids for 48 h were subjected to Western blot analysis with the indicated antibodies. Densitometry quantification of these protein levels is shown. All the statistical data were analyzed by Student's *t* test. All data are means ± SEM. $^{**}P < 0.01$. NS, non-significance

KGA1017) were used as previously described [21]. Briefly, the cells were grown in a six-well plate and incubated at 37 °C for 48 h after transfection. After two washes with PBS, the cells were incubated in binding buffer for 30 min in the dark. The apoptosis rate was quantified with a FACS Vantage SE flow cytometer (BD Biosciences).

Statistical analysis

Values are shown as the mean ± SEM. All statistical analyses were performed using SPSS 19.0 software (SPSS Statistics, Inc.). The differences between the 2 groups were analyzed by Student's *t* test. Statistical significance was achieved at $P < 0.05$.

Results

ACTN4 was aberrantly expressed and localized in placenta vascular endothelial cell, and H_2O_2 treatment downregulated ACTN4 expression

We analyzed ACTN4 protein expression levels in the placenta vascular endothelium of women with normotensive pregnancy and severe PE using immunofluorescence (Fig. 1a). The results demonstrated that ACTN4 protein was intensely expressed in the vascular endothelium, which was defined by the positive expression of CD31 (platelet endothelial cell adhesion molecule—1). In addition, the ACTN4 expression was altered in placental endothelium between normotensive pregnancies and PE pregnancies. To quantify ACTN4 expression levels in the vascular endothelium, HUVECs isolated from women with normotensive pregnancy (normal HUVECs, $n = 20$) and severe PE (PE HUVECs, $n = 20$) were assessed using Western blotting. As shown in Fig. 1b, the expression of ACTN4 in the PE group was significantly lower than that in the normotensive pregnancy group (NP).

As oxidative stress is a major aetiological factor of endothelial cell dysfunction in PE development, we further investigated the effect of oxidative stress on ACTN4 expression in HUVECs by H_2O_2 treatment. As shown in Fig. 1c, the expression of ACTN4 in normal HUVECs after H_2O_2 treatment was significantly lower than that in the negative control (NC) HUVECs.

The decreased expression of ACTN4 in PE vascular endothelium and HUVECs under oxidative stress injury conditions suggested that the downregulation ACTN4 might be involved in endothelial injury and the pathogenesis of PE.

ACTN4 knockdown induced normal HUVEC apoptosis

To investigate the role of ACTN4 in endothelial cells, we used an ACTN4-specific siRNA to reduce ACTN4 expression in

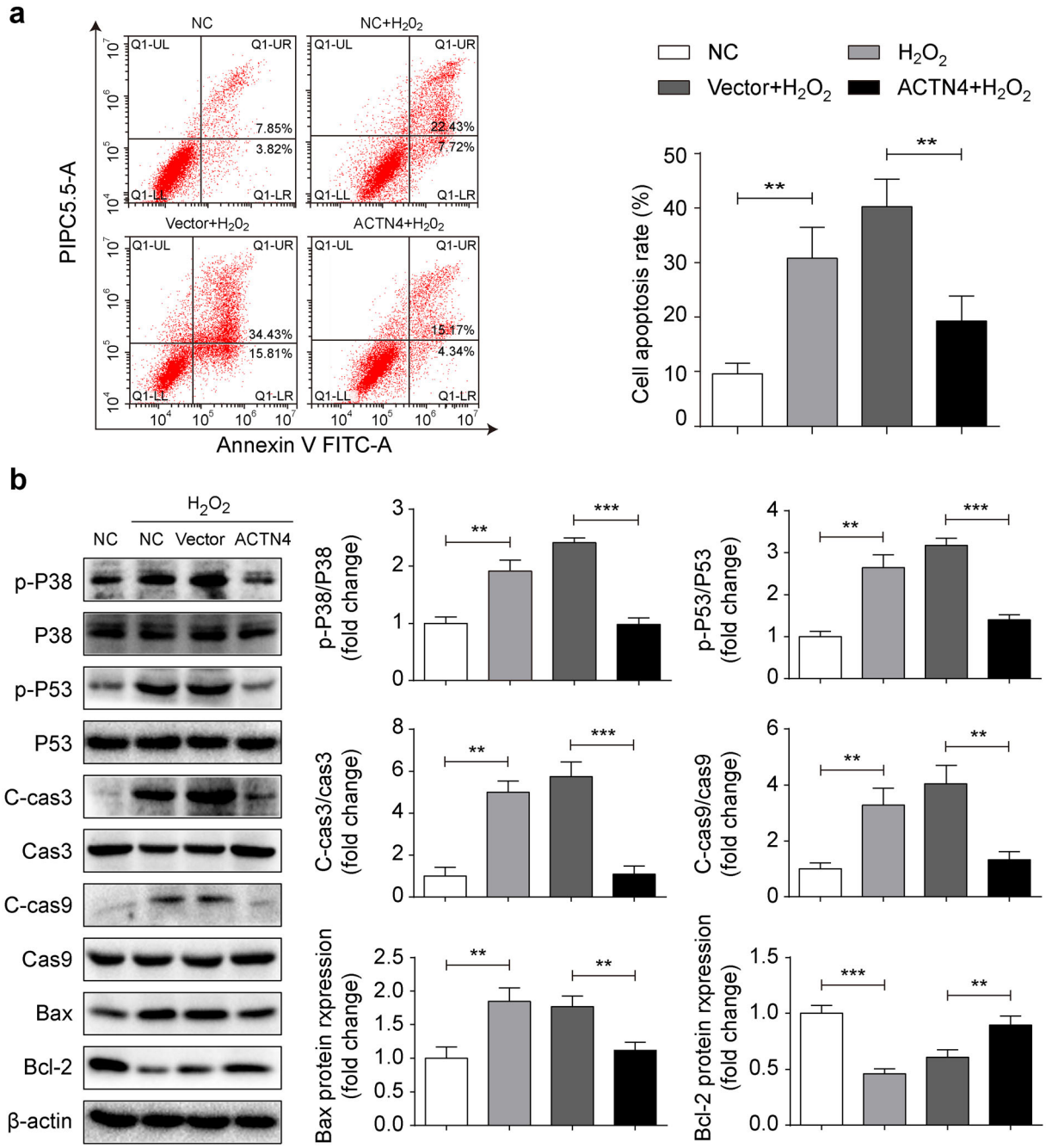

Fig. 6 H_2O_2 treatment could induce cell apoptosis and overexpression ACTN4 could against this effects. **a** HUVECs from normotensive pregnancies with/without plasmid transfection for 24 h were treated with H_2O_2 (100 μM) for another 24 h, and, then, the apoptosis rate was confirmed by flow cytometric analysis. **b** HUVECs after treatment were subjected to Western blot analysis with the indicated antibodies. Statistical analyses of relative protein levels were shown. All the statistical data were analyzed by Student's *t* test (2 groups). All data are means ± SEM of 3 independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$

normal HUVECs. The effect of ACTN4 knockdown on protein expression was verified by Western blot. RNA interference targeting ACTN4 significantly decreased the protein expression level in the knockdown cells when compared with that in the control cells (Fig. 2a).

We next investigated the effect of ACTN4 knockdown on cell viability and apoptosis in HUVECs. A significant decrease in cell viability was observed in the knockdown group at 48 h post-transfection (Fig. 2b). After the CCK-8 assays, we further investigated the effect of downregulation of ACTN4 on normal HUVEC apoptosis using flow cytometric analysis. The knockdown of ACTN4 significantly induced apoptosis compared with that of the negative control (Fig. 2c). We also detected apoptosis-related proteins, and the results showed a significant

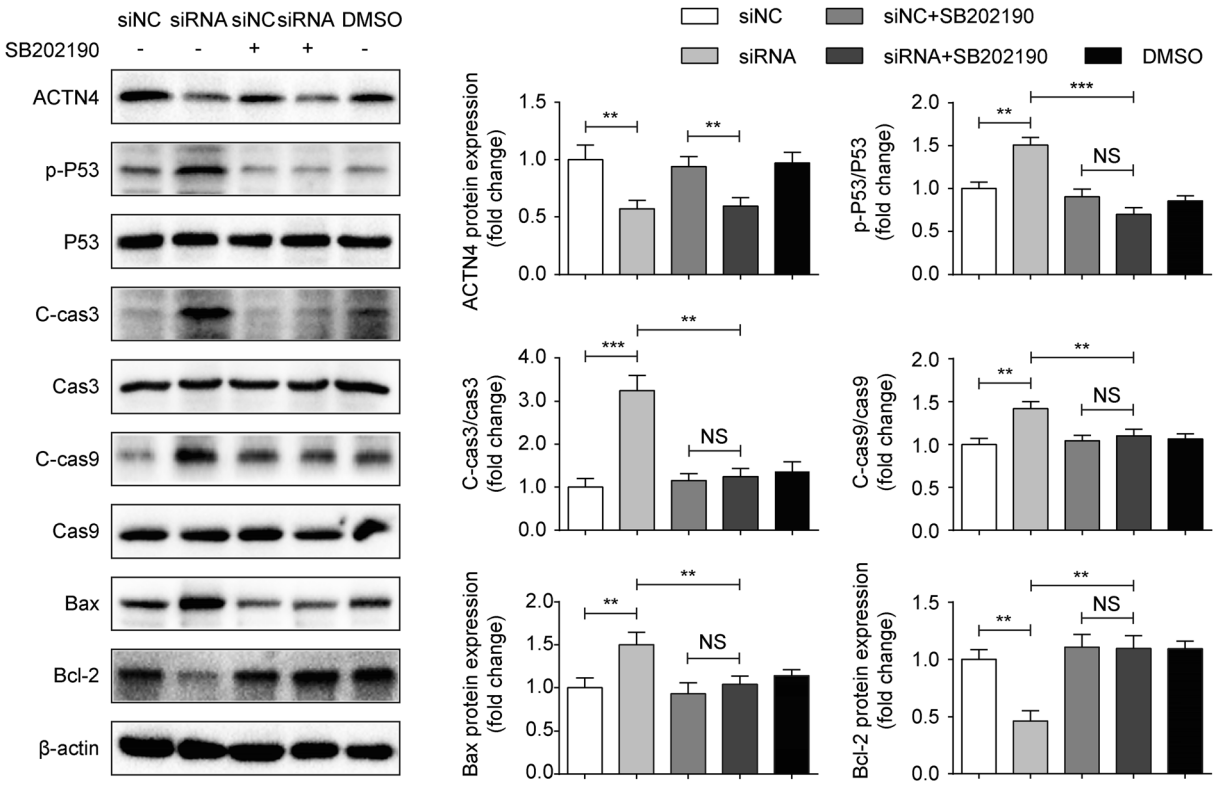

Fig. 7 ACTN4 knockdown induced HUVEC apoptosis by activating p38-MAPK/p53 apoptosis pathway. HUVECs from normotensive pregnancies were preincubated with p38 MAPK inhibitors (SB202190) for 2 h and then were transfected with ACTN4 siRNA or control siRNA for another 48 h. The lysates of HUVECs after treatment were subjected to Western blot analysis with the indicated antibodies. Statistical analyses of relative protein levels were shown. All the statistical data were analyzed by Student's *t* test (2 groups). All data are means ± SEM of 3 independent experiments performed in triplicate. $^{**}P < 0.01$; $^{***}P < 0.001$. NS, non-significance

increase in the protein ratio of p-P53/P53, cleaved-caspase3/caspase3 (c-cas3/cas3), cleaved-caspase9/caspase9 (c-cas9/cas9), and increased bax expression level with a concurrent decreased expression level of bcl-2 (Fig. 4a).

In addition, the migration and tube formation abilities of HUVECs were assessed by migration assay and tube formation assay. ACTN4 knockdown significantly inhibited these two abilities (Fig. 2d,e).

These results reveal that knockdown of ACTN4 impairs endothelial cell function by promoting apoptosis.

ACTN4 upregulation suppressed PE HUVEC apoptosis

To further confirm the vital role of ACTN4 in endothelial injury, we upregulated ACTN4 in HUVECs isolated from PE patients, in which ACTN4 expression was decreased in a previous experiment (Fig. 3a). As shown in Fig. 3b and c, the ACTN4 upregulation group had significantly increased viability and suppressed apoptosis compared to that of the vector group. The protein ratio of p-P53/P53, c-cas3/cas3, and c-cas9/cas9 and the expression level of bax were significantly decreased with increased expression of bcl-2 (Fig. 4b). In addition, overexpression of ACTN4 also significantly rescued the migration and tube formation abilities of HUVECs isolated from PE patients (Fig. 3d,e). These observations are consistent with the findings with respect to ACTN4 knockdown in HUVECs from women with normotensive pregnancy and further support the significance of ACTN4 in regulating the functions of ECs.

P38-MAPK was regulated by ACTN4 through phosphorylation alteration in HUVECs

Previous studies demonstrated that mitogen-activated protein kinases, including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38-MAPK, are responsive to stresses, including oxidative stress. Previous papers have identified a relationship between ACTN4 and the MAPK pathway [26]. To elucidate the mechanisms by which ACTN4 affects endothelial injury, we detected the protein levels of the key members of MAPK signalling pathways.

Interestingly, we found that the ratio of p-p38/p38 was significantly increased in HUVECs from normotensive pregnancies after ACTN4 knockdown (Fig. 5a) and was decreased in PE HUVECs after ACTN4 upregulation (Fig. 5b). The Western blot results revealed no significant differences in the ratios of p-JNK/JNK and p-ERK/ERK. These data suggest

that the phosphorylation of p38-MAPK is controlled by ACTN4 in HUVECs.

Overexpression of ACTN4 rescued H_2O_2-induced HUVEC apoptosis via the activation of the p38-MAPK/p53 apoptosis pathway

Previous experiments showed that ACTN4 was reduced in normal HUVECs under oxidative stress conditions (H_2O_2) and ACTN4 knockdown induced apoptosis. We next chose to determine whether ACTN4 overexpression has a protective effect on H_2O_2-induced oxidative stress and investigate its potential mechanisms. The results showed that H_2O_2 could significantly induce normal HUVEC apoptosis (Fig. 6a). Western blot analyses indicated that after H_2O_2 treatment, the expression of ACTN4 was significantly decreased and the p38-MAPK/p53 apoptosis pathway was activated (Fig. 6b). Moreover, overexpression of ACTN4 before H_2O_2 treatment attenuated apoptosis by suppressing the p38-MAPK/p53 apoptosis pathway (Fig. 6b).

Decreased phosphorylation of P38 reversed the promotion effects of ACTN4 knockdown on HUVEC apoptosis

To further strengthen the hypothesis that ACTN4 regulates apoptosis via the p38-MAPK/p53 apoptosis pathway, we performed rescue experiments using the p38-MAPK inhibitor SB202190 in combination with ACTN4 silencing, to investigate the effect of differential ACTN4 expression on the expression of pathway-related proteins in HUVECs.

Western blot analyses indicated that downregulation of ACTN4 resulted in p53 activation by phosphorylation, with increased apoptosis-related protein expression levels (bax, c-cas3, and c-cas9) and decreased expression of bcl-2 (Fig. 7). However, SB202190 treatment nearly blocked the effect of ACTN4 knockdown on p38-MAPK/p53 apoptosis pathway activation and had no effect on ACTN4 expression. Taken together, these results reveal that ACTN4, as an upstream factor, may regulate endothelial cell apoptosis through the p38-MAPK/p53 apoptosis pathway.

Discussion

Inadequate trophoblast invasion and impaired spiral artery remodelling induce repeated ischemia–reperfusion (H/R) conditions. The resulting H/R environment within the placenta stimulates oxidative stress and releases a variety of mediators in the maternal circulation, including ROS, which causes an increased rate of EC apoptosis and is an important pathological process involved in the aggravation of PE disorders [29]. It is thought that dysregulated expression of genes regulating EC apoptosis is a possible reason for the development of PE disorders, and our results indicate that ACTN4 may be involved in this process. In the present study, we first identified that ACTN4 was expressed in placental ECs by immunofluorescence analysis and was expressed at lower levels in severe PE placentas than in normal placentas. Consistent with the immunofluorescence result, a lower expression level of ACTN4 in PE HUVECs was also observed. These results indicate the potential role of ACTN4 in the pathogenesis of PE.

Intracellular ROS play an important role in oxidative stress-mediated endothelial cell dysfunction. H_2O_2 is a well-known ROS that is significantly increased in the plasma of PE patients [31]. We therefore used H_2O_2 to mimic oxidative stress conditions in PE patient circulation, and the results showed that ACTN4 expression in normal HUVECs was significantly reduced after H_2O_2 treatment. These findings provide evidence that oxidative stress induces reduced expression of ACTN4 and may participate in the pathogenesis of endothelial dysfunction associated with PE. Further experiments showed that silencing of ACTN4 expression in normal HUVECs increased apoptosis, while upregulation of ACTN4 in PE HUVECs effectively rescued cell functions by inhibiting apoptosis. Western blot analysis also showed that levels of apoptosis-related proteins changed in response to different ACTN4 expression levels. In addition, ACTN4 overexpression attenuated H_2O_2-induced apoptosis. Thus, it appears that ACTN4 is a protective factor that helps maintain homeostasis of the vascular endothelium. Consistent with its reduced expression in severe PE patients, we provide the first evidence that ACTN4 expression is reduced in HUVEC after oxidative stress injury in vitro and that reduced ACTN4 expression leads to endothelial injury via the promotion of HUVEC apoptosis. These results suggest a potential role for ACTN4 in mediating oxidative stress injury in HUVECs, and our further study revealed that ACTN4 regulates HUVEC apoptosis via the p38-MAPK/p53 apoptosis pathway.

To further explore the potential ACTN4-mediated regulatory mechanisms, diverse signalling pathways related to apoptosis and cell stress were investigated, and the results showed that the p38-MAPK/p53 apoptosis pathway is involved in this regulation. As a stress response protein, p38-MAPK is activated under various conditions, such as H/R, $PM_{2.5}$, and ROS [30]. p38-MAPK also plays key roles in regulating EC behaviour in response to oxidative stress in the development of PE. Li et al. found that PE umbilical cord tissues exhibited extensive p38 phosphorylation, and HUVECs treated with H/R showed similar levels of p38 phosphorylation [14]. Because p53 is an important downstream effector of p38, high levels of p53 phosphorylation can also be detected in PE placenta and HUVECs. Gao et al. observed increased apoptosis in cultured HUVECs from PE pregnancies compared to normotensive controls with extensive p53 phosphorylation and

subsequently the regulation of downstream proteins, including bax, bcl-2, and the caspase cascade [3]. We therefore hypothesize that the p38-MAPK/p53 apoptosis pathway may be an important pathway involved in oxidative stress-induced endothelial apoptosis. In fact, studies have found that this signalling pathway is activated in cardiovascular diseases [2, 15]. We revealed that ACTN4 knockdown promotes p38 phosphorylation and activation of the relevant downstream proteins, while overexpression of ACTN4 inhibits these effects. In addition, SB202190, a selective inhibitor of p38-MAPK, suppressed the effect of ACTN4 knockdown on p38-MAPK/p53 apoptosis pathway activation, indicating the pathway is an important intermediary between ACTN4 and HUVEC apoptosis. Recently, studies have shown that ACTN4 plays a role not only in cancer invasion but also in the regulation of apoptosis. Lomert et al. reported that overexpression of ACTN4 in H1299 cells enhanced cell proliferation by lowering apoptosis levels and that co-expression of RelA/p65 and ACTN4 induced apoptosis in non-small lung carcinoma cells [17]. Consistent with Lomert's work, our results also show that upregulation of ACTN4 alone in PE HUVECs can rescue cell viability and decrease the apoptosis rate.

As mentioned in the "Introduction" section, ACTN4 participates in the regulation of gene expression following the activation of certain transcription factors, including p38-MAPK [26]. Although we did not perform quantitative experiments, such as PCR, to detect relevant gene expression levels, and Western blotting was the main method used in our research, we believe that our results justify this conclusion because all the evidence leads to the same conclusion that ACTN4 regulates HUVEC function via the p38-MAPK pathway. However, the mechanisms involved in this process may require more research. Whether there are other signalling pathways involved in the regulatory function of ACTN4 or whether ACTN4 directly or indirectly relates to the p38 MAPK pathway requires further investigation.

In summary, this study is the first to report decreased expression of ACTN4 in severe PE endotheliocytes. Oxidative stress can downregulate ACTN4 expression, which contributes to endothelial injury by promoting apoptosis via the activation of the p38-MAPK/p53 apoptosis pathway. However, more studies are necessary to confirm whether there are other pathways related to ACTN4. To determine the effects of ACTN4 on other relevant cell types, such as trophoblast cells and animal models, further verification is required.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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