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SRC-3 Plays a Critical Role in Human Umbilical Vein Endothelial Cells by Regulating the PI3K/Akt/mTOR Pathway in Preeclampsia

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Abstract

Preeclampsia (PE) is currently thought to be characterized by oxidative stress which may lead to endothelial dysfunction. The normal function of vascular endothelium is essential to vascular homeostasis. Previous studies have shown that steroid receptor coactivator 3 (SRC-3) interacts with estrogen receptors (ERs) which are involved in the vasoprotective effects of estrogen and is also associated with cell migration, invasion, and inflammation; however, its role in PE remains unclear. The main purpose of this study is to identify the role of SRC-3 in the function of human umbilical vein endothelial cells (HUVECs) during the development of PE. Our study demonstrated that the expression of SRC-3 was significantly decreased in PE placentas compared to normal placentas. Additionally, lentivirus short hairpin RNA against SRC-3 and hypoxia/reoxygenation treatments attenuated migration and tube formation abilities and enhanced HUVEC apoptosis. Furthermore, we detected possible downstream in the PI3K/Akt/mammalian target of rapamycin (mTOR) signal pathway activity, which is involved in SRC-3-mediated HUVEC function. Our data suggest that oxidative stress plays a crucial role in controlling SRC-3 expression, which influences the migration and tube formation abilities of endothelial cells through the PI3K/Akt/mTOR signaling pathways. This action may then result in PE pathogenesis.

Keywords

SRC-3, human umbilical vein endothelial cells, preeclampsia, PI3K/Akt/mTOR signaling pathway

Introduction

Preeclampsia (PE), which is a pregnancy-specific disorder primarily characterized by hypertension and significant proteinuria after 20 weeks of pregnancy, is one of the major causes of maternal morbidity and mortality.[1] The incidence of PE worldwide is 3% to 8%.[2] Although the exact etiology of PE remains unclear, several varying hypotheses exist, including oxidative stress.[3] In PE pathology, oxidative stress is caused by reactive oxygen species (ROS) that overwhelms the antioxidant defences.[4]

Steroid receptor coactivator 3 (SRC-3), also known as amplified in breast cancer 1 (AIB1), nuclear receptor coactivator 3 (NCOA3), receptor-associated coactivator 3 (RAC3), p300/CBP/co-integrator protein (pCIP), activator of thyroid hormone and retinoid receptor (ACTR), and thyroid hormone receptor activator molecule 1 (TRAM-1), is located on chromosome 20q12 and belongs to the p160 SRC family .[5,6] As an oncogene, SRC-3 has been studied in various cancers, such as ovarian cancer, esophageal cancer, colorectal cancer, lung cancer, and breast cancer. Accumulating evidence suggests that SRC-3 mediates the development of cancer by promoting cancer cell proliferation and invasion.[7-11] The SRC-3 is also shown to be important in various growth factor signaling pathways, such as epidermal growth factor and human epidermal growth factor receptor 2 signaling in cancer epithelial cells and insulin-like growth factor 1 in breast cancer epithelium.[12-14] In addition, previous studies have shown that SRC-3 is associated with

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angiogenesis and interacts with estrogen receptors (ERs) which mediate the vasoprotection of estrogen.[15,16] However, the role of SRC-3 in the pathogenesis of PE is largely unknown. Therefore, the aim of this study is to elucidate the effect of SRC-3 during the development of PE.

Many vital vascular endothelium functions are essential to vascular homeostasis, such as the release of vasoconstrictors and vasodilatory substances to mediate smooth muscle tone, and the release of soluble factors to regulate anticoagulation, antiplatelet formation, and fibrinolysis.[17,18] However, intermittent placental perfusion will induce an ischemia–reperfusion (I/R) injury to the placenta, which may further cause intense oxidative stress to the placental endothelium.[19] This defect in endothelial homeostasis is fundamental to the initiation and development of PE.[20] A number of studies have demonstrated that the PI3K pathway downregulation is associated with PE.[21-23] As a member of the lipid kinase family, PI3K is divided into 3 classes which include class I (IA and IB), class II, and class III. The class IA PI3K enzymes catalyze the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) from phosphatidylinositol-4,5-bisphosphate (PIP2), which phosphorylates the downstream protein kinase B (Akt) and then activates further downstream signaling events, such as the mammalian target of rapamycin (mTOR). Studies have shown that SRC-3 regulates cellular function, such as cell growth and cell proliferation, through the PI3K/Akt/mTOR signaling pathway.[24,25] However, the potential function of SRC-3 and the relationship between SRC-3 and the PI3K/Akt/mTOR signaling pathway in the process of placental oxidative stress need further validation. Therefore, our study primarily focuses on oxidative stress induced aberrant expression of SRC-3, which may regulate the PI3K/Akt/mTOR signaling pathway, further affecting the migration and tube formation abilities of endothelial cells, which are associated with PE.

In this study, the hypoxia–reoxygenation (H/R) condition was used to mimic the oxidative stress status of PE, and lentivirus short hairpin RNA transfection was used to detect the effects of SRC-3 on endothelial cell migration and tube formation in vitro. Moreover, the PI3K inhibitor LY294002 was applied to explore whether the PI3K/Akt/mTOR signaling pathway participated in the underlying molecular mechanisms of SRC-3 in human umbilical vein endothelial cells (HUVECs). The aim of our study was to investigate the hypothesis that SRC-3 has an effect on HUVEC migration and tube formation and that the dysregulation of SRC-3 expression is associated with PE.

Materials and Methods

Patients and Tissue Collection

Term placental tissues were collected from patients with PE ($n = 26$) and normotensive control pregnant women ($n = 31$) by cesarean delivery in the third trimester. PE was defined according to the American College of Obstetrics and Gynecology. Patients with chronic renal diseases, diabetes mellitus, chronic hypertension, or other metabolic diseases were excluded.

Ethical approval was granted by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. All participants signed the informed consent form. Placenta tissues were dissected from 5 cotyledons on the maternal side without visible infarction, calcification, hematoma, or tears. Tissues were selected for approximately 1 cm × 1 cm × 1 cm dimension from midway between the umbilical cord insertion site and the peripheral edge of the placenta. Placenta tissues were washed in ice-cold 0.9% saline immediately after collection. For immunofluorescence, 1 piece of the tissue was fixed in 4% paraformaldehyde for 1 hour, then cryoprotected in 25% sucrose overnight. Then tissues were placed in optimal cutting temperature compound and frozen in liquid nitrogen and cryosectioned at 5 μm. The samples were stored at −80°C until use. For RNA and protein extraction, tissues were stored at −80°C until use.

Cell Culture and H/R Treatment

The HUVECs was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The HUVECs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (#11875093; Gibco, Carlsbad, California, USA) containing 10% fetal bovine serum (#10099141; Gibco, Carlsbad, California, USA). The cells were routinely cultured at 37°C in a humidified atmosphere with 5% CO_2. The H/R intervention was performed as follows: 2 hours under 2% oxygen followed by transfer to the standard culture condition with 20% oxygen for 6 hours.[26] Before treatment, HUVECs were switched to serum-free RPMI 1640 medium for 24 hours.

RNA Interference and PI3K Inhibition

To obtain stably transfected cells, HUVECs were transfected with lentivirus short hairpin RNA (shRNA) against SRC-3 (shSRC-3: 5′-AGACTCCTTAGGACCGCTT-3′) and control vector (shNC; GenePharma, Shanghai, China), according to the manufacturer's protocol. Stably transfected cells were established by treatment with 5 ug/mL puromycin. The shRNA transfection efficiency as measured by flow cytometry was 99.34%.

The HUVECs were preincubated with highly selective PI3K inhibitor LY294002 (#S1105; Selleck Chemicals, Houston, USA) at 20 μM for 24 hours. LY294002 was diluted with dimethyl sulfoxide (DMSO). Before treatment, HUVECs were switched to serum-free RPMI 1640 medium for 24 hours.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from placenta using a TRIzol regent (#15596026; Invitrogen Life Technologies, Carlsbad,

California, USA), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using Transcriptor First Strand cDNA Synthesis kit (#04897030001; Roche, Basel, Switzerland). The quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, California, USA) by using FastStart Essential DNA Green Master (#06924204001; Roche, Basel, Switzerland). The qRT-PCR primer sequences were as follows: SRC-3 sense: 5′-CCCCCAAGCAACTTAGAATG-3′, antisense: 5′-CCACC AGCAGTAGGGTTTTC-3′; β-actin sense: 5′-GGCACCCAG CACAATGAA-3′, antisense: 5′-CTAAGTCATAGTCCGCC TAGAAGCA-3′. The PCR conditions were as follows: 95°C for 10 minutes, 40 cycles at 95°C for 10 seconds, and 63.3°C for 30 seconds, and extension at 72°C for 10 seconds. The relative quantification of the PCR products was normalized to the level of β-actin.

Immunofluorescence

The placenta tissue and 4 HUVEC cell groups were permeabilized with 0.2% Triton X-100 and blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 1 hour. An anti-SRC-3 antibody (1:100; #5765; Cell Signaling Technology, Massachusetts, USA) was used as the primary antibody in 4°C overnight. Then, the placentas were incubated with the appropriate secondary antibody for immunofluorescence for 1 hour. The nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI; #ZLI-9557; ZSGB-BIO, Beijing, China) for 5 minutes. A negative control was created by replacing the primary antibody with normal immunoglobulin G. Images were acquired with EVOS FL Auto Imaging System (Life Technologies, Carlsbad, California, USA).

Western Blotting

Protein extracts were prepared from placentas and HUVECs using radio immunoprecipitation assay lysis (RIPA) buffer (#P0013B; Beyotime Biotechnology, Shanghai, China). Protein concentration was measured using the bicinchoninic acid protein assay (#P0010; Beyotime Biotechnology, Shanghai, China). Samples containing equal amounts of proteins (40 μg) were separated by 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride membranes (#ISEQ00010; Millipore, Massachusetts, USA). After blocking with 5% nonfat dry milk (#1706404; Bio-Rad, California, USA) in a tris-buffer containing 0.05% Tween-20 (TBST) for 1 hour, the membranes were incubated with the primary antibodies overnight at 4°C, including rabbit antibodies against SRC-3 (1:1000; #5765; Cell Signaling Technology, Massachusetts, USA), p-Akt(Ser^{473}) (1:1000; #9271; Cell Signaling Technology, Massachusetts, USA), Akt (1:1000; #9272; Cell Signaling Technology, Massachusetts, USA), P-mTOR (Ser^{2481}) (1:1000; ab137133; Abcam, Cambridge, UK), mTOR (1:1000; #2983; Cell Signaling Technology, Massachusetts, USA), vascular endothelial growth factor receptor 2 (VEGFR2; 1:500; #A5609; ABclonal, Houston, USA), vascular cell adhesion molecule 1 (VCAM-1; 1:500; #BA0406; Boster Biological Technology, Wuhan, Hubei, China), and mouse antibodies against β-actin antibody (1:1000; #BM0627; Boster Biological Technology, Wuhan, Hubei, China). Then, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; ZSGB-BIO, Beijing, China) at room temperature for 1 hour. Positive bands were detected using chemiluminescence reagents (#WBKLS0500; Millipore, Massachusetts, USA) and analyzed using ChemiDoc image analyzer (Bio-Rad, California, USA).

In Vitro Cell Migration Assay

A migration assay was performed to assess the effect of HUVEC migration. The outer chamber was a 24-well plate, and the inner chamber was a polycarbonate filter (8 μm pores; #353097; Corning, Steuben County, New York, USA). The HUVECs (1×10^4 per chamber) were added to each inner chamber with 200 μL serum-free RPMI-1640 medium, and the outer chamber was immediately loaded with 600 μL RPMI-1640 medium containing 10% fetal bovine serum. After 8 hours of incubation at 37°C, nonmigrating cells in the upper surface of the filters were removed using a sterile cotton swab, then the migrating 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 detect the HUVEC tube formation ability, a tube formation assay was performed. BD matrigel (10 mg/mL; #356234; BD Biosciences, San Diego, California, USA) was distributed in a 96-well plate (50 μL/well) on ice and allowed to solidify at 37°C for at least 30 minutes. After the matrigel mixture solidified, HUVECs (3.0×10^4 per well) were gently added to each of the triplicate wells. Tube formation was quantitatively measured by calculating the total tube 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 tube length (millimeter) per millimeter square area.

Cell Apoptosis Flow Cytometry Analysis

The HUVEC apoptotic rates were measured using an annexin V-phycoerythrin/ 7-amino-actinomycin D (Annexin V-PE/ 7-AAD) Apoptosis Detection kit (#KGA1017; KeyGen

Table 1. Clinical Characteristics of Normal Patient Group and Preeclampsia Patient Group.

Category	Normal	Preeclampsia
Number	31	26
Patient age (years)	30.39 ± 3.35	29.88 ± 5.05
Gestational age (weeks)	39.21 ± 0.82	36.60 ± 2.25[c]
Body mass index (BMI; kg/m^2)	27.63 ± 2.84	30.56 ± 3.52[b]
Gravidity	2.42 ± 1.41	2.35 ± 1.09
Parity	0.42 ± 0.49	0.15 ± 0.37[a]
Proteinuria (g/24 hours)	0.05 ± 0.01	2.722 ± 1.26[c]
Systolic blood pressure (mm Hg)	119.9 ± 8.38	162.0 ± 17.79[c]
Diastolic blood pressure (mm Hg)	66.45 ± 6.60	101.3 ± 12.16[c]
Neonatal birth weight (g)	3402 ± 292.04	2669 ± 475.32[c]
Neonatal birth length (cm)	49.79 ± 1.18	47.00 ± 2.00[c]
Placental weight (g)	568.1 ± 46.89	467.6 ± 42.93[c]

[a]$P < .05$.
[b]$P < .01$.
[c]$P < .001$.

Biotech, Nanjing, Jiangsu, China). After treatment, the HUVECs were washed with ice-cold PBS and stained in annexin V-PE and 7-AAD binding buffer for 20 minutes. Apoptosis rates were quantified with a fluorescence activated cell sorter (FACS) Vantage SE flow cytometer (BD Biosciences, San Diego, California, USA). Annexin PE+/7-AAD− (upper right quadrant, Q2) was predicted to be early apoptotic cells, and Annexin PE+/7-AAD+ (lower right quadrant, Q3) was predicted to be late apoptotic cells. Therefore, the total percentage of apoptotic cells was represented by the sum of Q2 and Q3.

Statistical Analysis

The data were collected from several independent experiments, with 3 replicates per experiment. All data were expressed as the mean ± standard deviation. All statistical analyses were performed using GraphPad Prism. Comparisons of continuous variables between the 2 groups were conducted using independent *t* tests. Statistical differences among multiple groups were evaluated using 1-way analysis of variance (ANOVA), followed by the least significant difference multiple comparison, as appropriate. $P < .05$ was considered statistically significant.

Results

Clinical Characteristics

Clinical data from all patients are shown in Table 1. No significant differences in patient age and gravidity were observed between the normal patient group and patient group with PE. However, the gestational age ($P < .001$), parity ($P < .05$), neonatal birth weight ($P < .001$), neonatal birth length ($P < .001$), and placental weight ($P < .001$) were lower in patients with PE. Furthermore, PE was associated with significantly higher body mass index ($P < .01$), systolic blood pressure ($P < .001$), diastolic blood pressure ($P < .001$), and 24-hour proteinuria ($P < .001$) compared to the normal patient group.

SRC-3 was Expressed in Human Placenta Endothelial Cells and the Expression of SRC-3 was Decreased in PE Placentas

First, SRC-3 protein expression in human placentas was detected using immunofluorescence. As shown in Figure 1, intense SRC-3 expression was observed in the endothelial cells of third-trimester placental tissues. The identification of endothelial cells and cell nuclei was confirmed by platelet endothelial cell adhesion molecule-1 (CD31) and 4',6-diamidino-2-phenylindole (DAPI) staining, respectively (Figure 1B and F). In addition, the expression of SRC-3 in normal placentas was higher than in PE placentas (Figure 1A and D). To quantify SRC-3 expression in placentas, the tissues were assessed using qRT-PCR and western blotting analyses. As a result, the SRC-3 messenger RNA (mRNA) level ($P < .05$) and protein level ($P < .05$) in PE placentas were significantly lower than in normal placentas (Figure 2A-C).

The H/R Condition Suppressed HUVEC Migration and Tube Formation

To mimic the oxidative stress environment during PE development, HUVECs were cultured under H/R condition.[14,26,27] Immunofluorescence images showed that SRC-3 protein expression in H/R-treated HUVECs was reduced compared to normal HUVECs (Figure 3A and B). Western blotting also consistently showed that the SRC-3 protein level decreased after H/R exposure (Figure 4A and B; $P < .001$)

In addition, our results revealed that the migration capacity of H/R-treated HUVEC diminished compared to normal HUVEC (Figure 5A, B, and G; $P < .001$). Moreover, H/R exposure also reduced HUVEC tube formation which potentially compared to the normal cell group (Figure 6A, B, and G; $P < .01$).

Furthermore, the effect of H/R on apoptosis in HUVEC was measured by flow cytometry. A representative flow cytometric image in Figure 7 shows that H/R treatment promoted cell apoptosis (Figure 7A, B, and G; $P < .001$).

Short Hairpin RNA-Mediated SRC-3 Knockdown Inhibited HUVEC Migration and Tube Formation

To further explore the role of SRC-3 in endothelial cells, we infected HUVEC with lentivirus shRNA against SRC-3. As a result, shRNA depleted SRC-3 protein expression compared to the normal cell group and control vector group (shNC) as validated by immunofluorescence (Figure 3A-C and G-I) and western blotting (Figure 4A and B; $P < .001$), indicating high-efficiency SRC-3 gene silencing.

Vascular endothelial growth factors (VEGFs) are the main regulators of vascular development and vessel

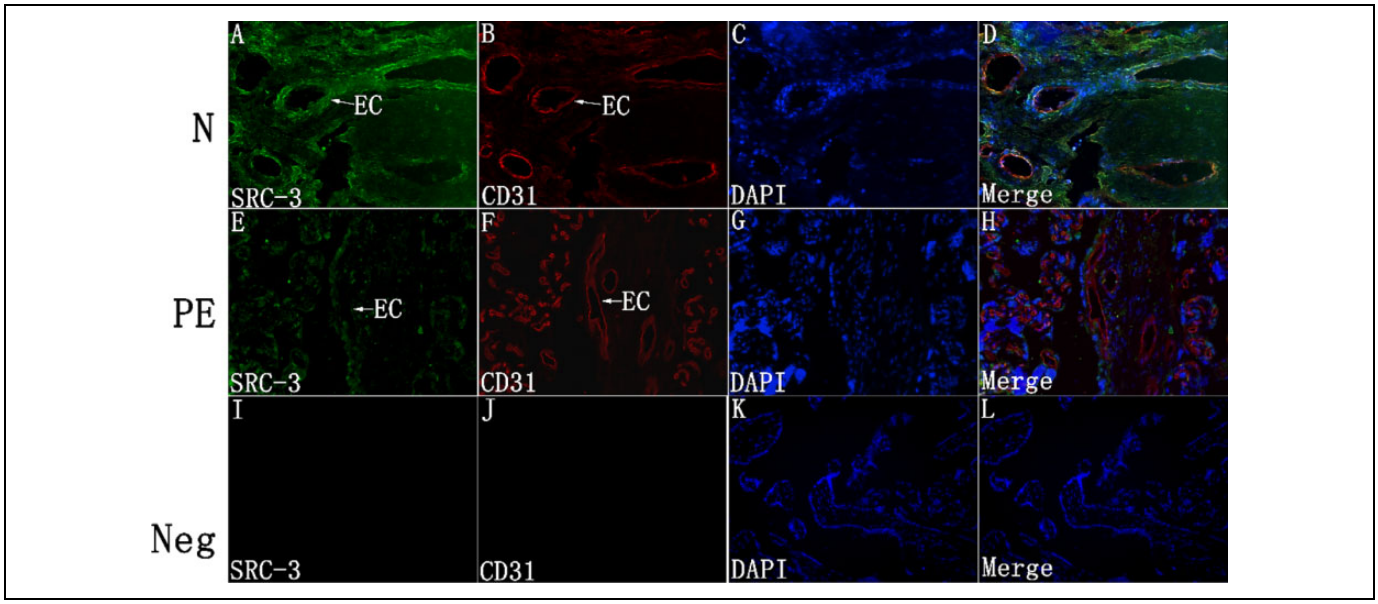

Figure 1. Immunofluorescent detection of SRC-3 proteins in placenta tissues (200×). A, SRC-3 protein (green) was localized within the endothelial cells in normal placentas. E, SRC-3 protein was rarely expressed in preeclampsia placentas. B, F, Immunofluorescence for CD31 (red), which serves as a marker for endothelial cells. C, G, K, The nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI; blue). D, H, L, Merge of the SRC-3, CD31, and DAPI images. A-D, Normal term placenta. E-H, Preeclampsia placenta. I-L, Negative controls (Neg) on sections in which normal immunoglobulin G (IgG) was used in place of the primary antibody. EC indicates vascular endothelial cells; SRC-3, steroid receptor coactivator 3.

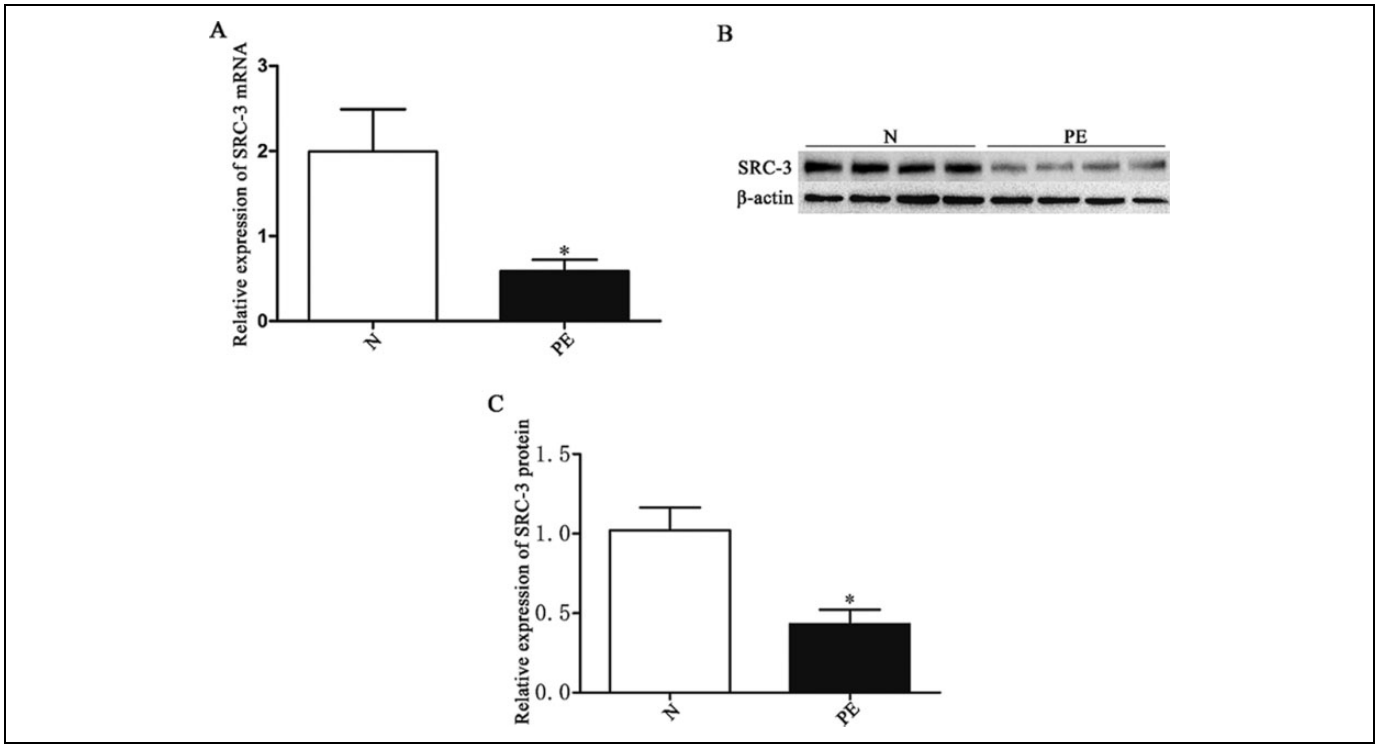

Figure 2. Quantitative expression of SRC-3 in placental tissues by qRT-PCR and western blotting. A, Statistical bar graphs of SRC-3 mRNA expression as determined by qRT-PCR in the normal control group (n = 31) and preeclampsia group (n = 29). B, Western blotting image of SRC-3 expression in the normal control group (N = 31) and preeclampsia group (n = 29). C, Statistical bar graphs of the western blotting results in (B). *$P < .05$ compared with the normal control group. N indicates normal cell group; PE, preeclampsia group; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; SRC-3, steroid receptor coactivator 3.

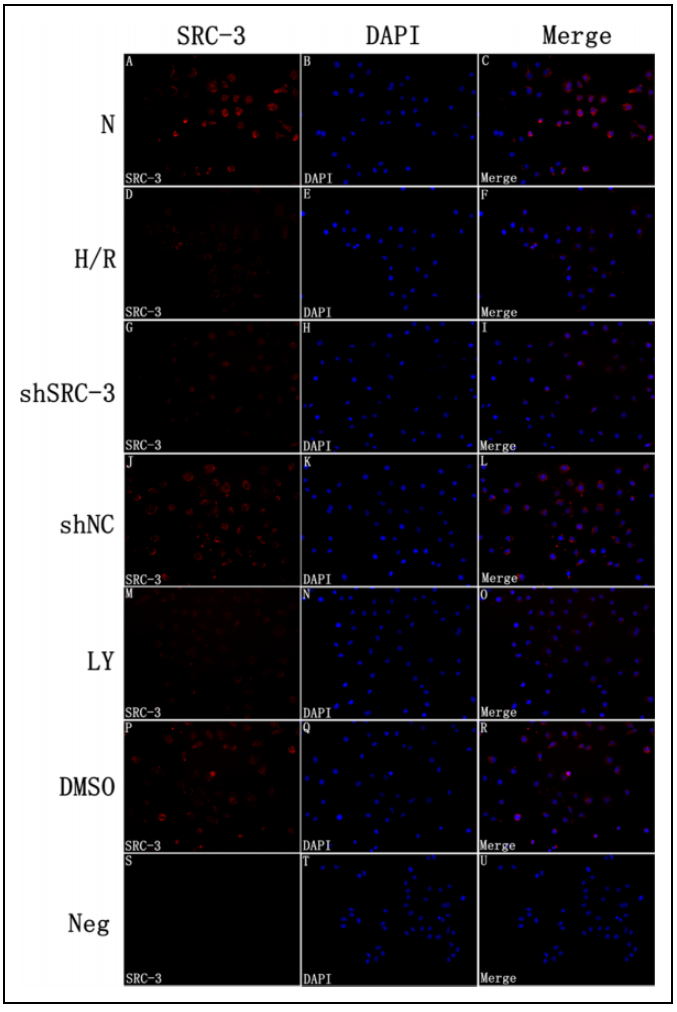

Figure 3. Immunofluorescence of SRC-3 expression in HUVECs (200×). Fluorescence signals specific to the SRC-3 antibody were visualized as red, and the nuclei were stained with DAPI (blue). A-C, SRC-3 protein expression in normal control cells. D-F, SRC-3 expression decreased after H/R treatment. G-I, SRC-3 protein expression decreased after lentivirus shRNA transfection. J-L, shNC transfection had no effect on SRC-3 protein expression in HUVECs. M-O, SRC-3 protein expression was decreased after LY treatment. P-R, DMSO treatment did not influence SRC-3 protein expression in HUVECs. S-U, Negative control. HUVECs indicates human umbilical vein endothelial cell line; N, normal cell group; H/R, hypoxia/reoxygenation cell group; shSRC-3, lentivirus short hairpin RNA against SRC-3 in HUVECs; shNC, control vector in HUVECs; LY, PI3K inhibitor LY294002 treatment in HUVECs; DMSO, dimethyl sulfoxide treatment in HUVECs; Neg, negative control; SRC-3, steroid receptor coactivator 3.

function. As the main VEGFR on endothelial cells, VEGFR2 is highly expressed during embryonic vasculogenesis and angiogenesis and is associated with neovascularization in tumors.[28-30] The VEGFR2 is believed to have effects on endothelial cell differentiation, proliferation, and migration and formation of the vascular tube.[31] On the other hand, VCAM-1, which is expressed in endothelial cells, regulates leukocyte adhesion and initiates the inflammatory response after vascular injury. A number of studies have demonstrated that expression of VCAM-1 is upregulated in endothelial cells during inflammatory diseases by various mediators, such as ROS.[32,33] Therefore, we tried to investigate the relationships between SRC-3 and VEGFR2/VCAM-1. The decreased expression of VEGFR2 and the increased expression of VCAM-1 was observed in HUVEC after SRC-3 downregulation (Figure 4A and E; $P < .001$; Figure 4A and F; $P < .01$).

Since SRC-3 was decreased in PE placentas and under H/R condition, we further investigated whether SRC-3 had an effect on endothelial cell functions. First, the migration assay was performed to detect the migration ability of normal HUVECs and shRNA against SRC-3 transfected HUVECs. The results showed that HUVEC migration was reduced by shRNA treatment (Figure 5A, C, and G; $P < .001$). Moreover, we also examined whether SRC-3 influenced HUVEC tube formation, and the results demonstrated that shRNA transfected HUVEC possessed reduced tube formation capability (Figure 6A, C, and G; $P < .01$).

In addition, flow cytometry analysis was used to measure the effect of shRNA on HUVEC apoptosis. The representative flow cytometry image in Figure 7 reveals that treatment with shRNA against SRC-3 dramatically enhanced the HUVEC apoptotic rate (Figure 7A, C, and G; $P < .05$). Moreover, compared with normal cells, shNC treatment had no effect on the migration ability, tube formation capacity, and apoptosis rate in HUVECs (Figure 5A, D, and G; Figure 6A, D, and G; and Figure 7A, D, and G).

Interactions Between the PI3K Signaling Pathway and SRC-3 Expression in HUVECs

Since the PI3K/Akt/mTOR signal pathway is associated with many cellular events including cell proliferation, apoptosis, differentiation, and angiogenesis, we used the highly selective PI3K inhibitor LY294002 to treat HUVECs and investigated the expressions of the PI3K downstream proteins p-Akt and p-mTOR in HUVECs. Compared to the normal group, LY294002 observably reduced p-Akt(Ser^{473})/Akt (Figure 4A and C; $P < .001$) and p-mTOR(Ser^{2481})/mTOR (Figure 4A and D; $P < .001$) expression in HUVEC, indicating that LY294002 successfully inhibited PI3K protein expression.

Moreover, H/R treatment decreased p-Akt(Ser^{473})/Akt (Figure 4A and C; $P < .01$) and p-mTOR(Ser^{2481})/mTOR (Figure 4A and D; $P < .001$) expression compared to normal HUVEC. Similarly, p-Akt(Ser^{473})/Akt (Figure 4A and C; $P < .001$) and p-mTOR(Ser^{2481})/mTOR (Figure 4A and D; $P < .001$) expression was reduced by shRNA-treated HUVECs compared to the normal group.

Intriguingly, immunofluorescence (Figure 3A-C and M-O) demonstrated that SRC-3 protein expression was diminished by LY294002 treatment in HUVECs compared to the normal cell group. This finding was confirmed by western blotting (Figure 4A and B; $P < .001$).

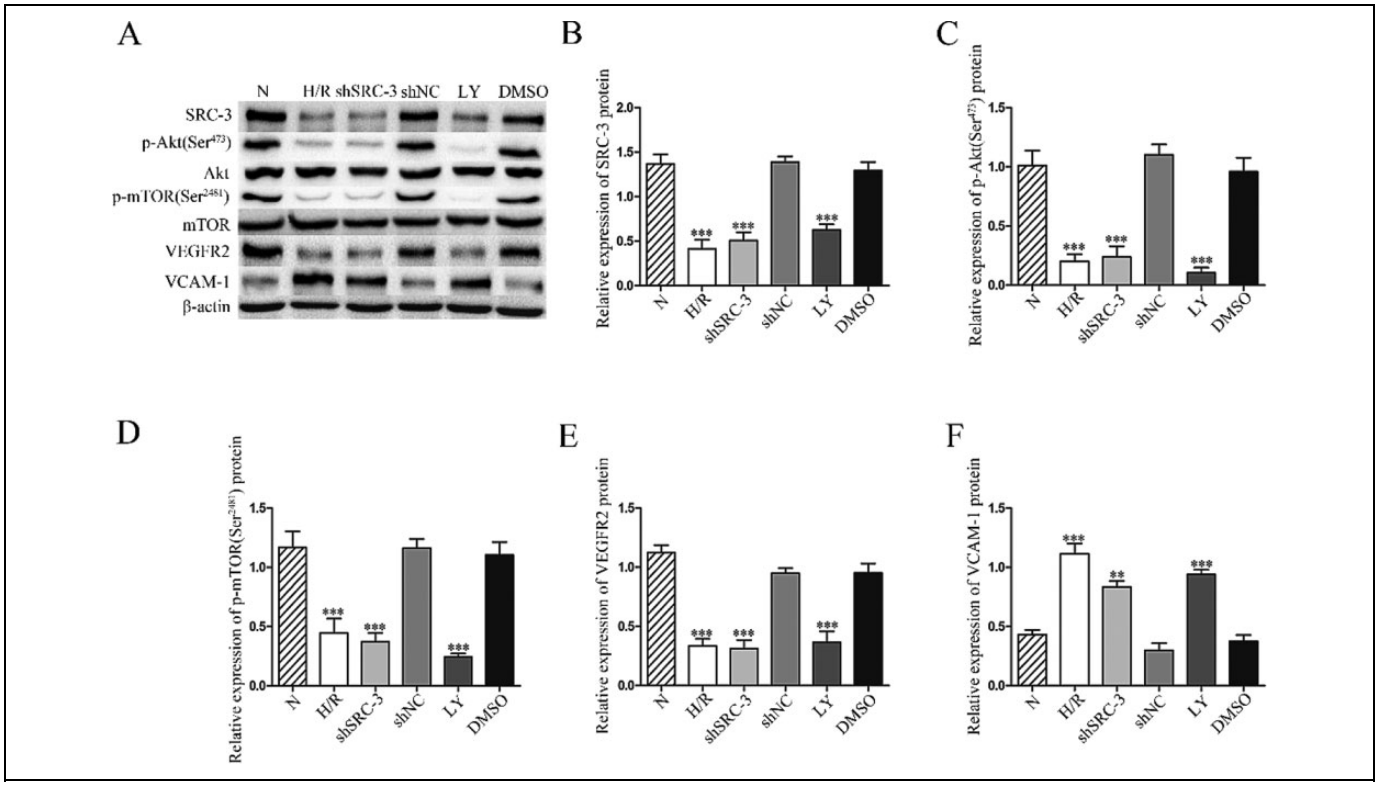

Figure 4. Effects of H/R, shRNA, and LY294002 on expressions of SRC-3, PI3K signaling pathways, VEGFR2, and VCAM-1 protein in HUVECs. A, Representative western blotting images of SRC-3, p-Akt(Ser^{473}), p-mTOR(Ser^{2481}), VEGFR2, and VCAM-1 in HUVECs with the indicated treatments. B-F, Statistical analysis of the western blotting results in (A). Data were analyzed by 1-way ANOVA to assess significant differences ($n = 3$ in triplicate). $^{**}P < .01$ compared to the normal control group, $^{***}P < .001$ compared to the normal control group. HUVECs indicates human umbilical vein endothelial cell line; N, normal cell group; H/R, hypoxia/reoxygenation cell group; shSRC-3, lentivirus short hairpin RNA against SRC-3 in HUVECs; shNC, control vector in HUVECs; LY, PI3K inhibitor LY294002 treatment in HUVECs; DMSO, dimethyl sulfoxide treatment in HUVECs; Akt, protein kinase B; p-Akt, phosphorylated Akt; m-TOR, mammalian target of rapamycin; p-mTOR, phosphorylated mTOR; VEGFR2, vascular endothelial growth factor receptor 2; VCAM-1, vascular cell adhesion molecule 1; ANOVA, analysis of variance.

In addition, as shown in Figure 5, a significantly decreased migration potential was observed in LY294002-treated HUVECs compared to normal cells (Figure 5A, E, and G; $P < .001$). The HUVEC tube formation ability was also diminished by LY294002 treatment (Figure 6A, E, and G; $P < .01$). However, compared to normal HUVEC, LY294002 treatment had no effect on the cell apoptosis rate as detected by flow cytometry (Figure 7A, E, and G). Additionally, treatment with DMSO failed to affect the migration ability, tube formation capacity, and apoptosis rate of HUVECs (Figure 5A, F, and G; Figure 6A, F, and G; and Figure 7A, F, and G) compared to the normal cell group.

Discussion

Oxidative stress occurs when the balance between antioxidant host defenses and ROS is broken. This results in endothelial dysfunction and further affects angiogenesis.[4,34] Angiogenesis impairment is a crucial event in PE pathophysiology.[35]

The SRC-3 concentration dramatically increases following conception and reaches its peak at term.[36] The SRC-3 depletion may reduce cell proliferation and migration and attenuate vascular formation activity. Thus, we proposed a hypothesis that SRC-3 dysregulation may play a role in PE development.

In our study, we demonstrated that SRC-3 enhanced endothelial cell functions, including cell migration and angiogenesis. Meanwhile, the mechanism of SRC-3 action on endothelial cells may be through the PI3K/Akt/mTOR signaling pathway.

First, we demonstrated that SRC-3 is expressed in placental endothelial cells. The lower SRC-3 expression in PE placentas compared to normal third-trimester placentas was confirmed by qRT-PCR and western blotting. To further verify whether SRC-3 was altered as a result of oxidative stress, we used H/R treatment to mimic I/R insult on the placenta, which resulted in intense oxidative stress in the placental endothelium. We revealed that HUVEC migration and tube formation potential were dramatically weakened after H/R treatment, accompanied by reduced expressions of SRC-3. The findings provided evidence that SRC-3 might be involved in PE HUVEC dysfunction.

To further investigate the effect of SRC-3 on HUVEC, lentivirus shRNA was used to knockdown SRC-3 expression. The

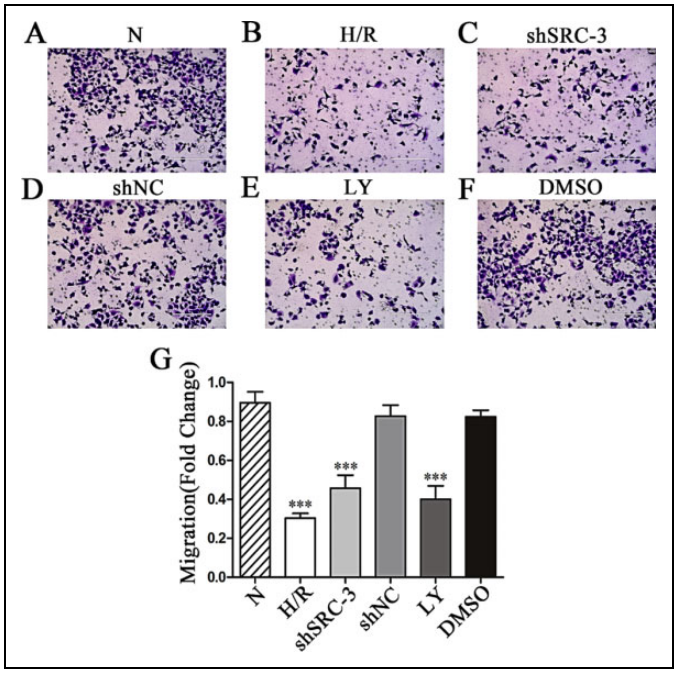

Figure 5. Migratory ability of HUVECs detected by migration assay (200×). A-F, Representative images of filters containing cells from the migration assay. A, Normal cell group (N). B, Hypoxia/reoxygenation cell group (H/R). C, Lentivirus short hairpin RNA against SRC-3 in HUVECs (shSRC-3). D, Control vector in HUVECs (shNC). E, PI3K inhibitor LY294002 treatment in HUVECs (LY). F, DMSO treatment in HUVECs (DMSO). G, Statistical bar graphs show a summary of the migration assay. Data were analyzed by 1-way ANOVA to assess significant differences (n = 3 in triplicate). ***$P < .001$ compared to the normal cell group. HUVECs indicates human umbilical vein endothelial cell line; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.

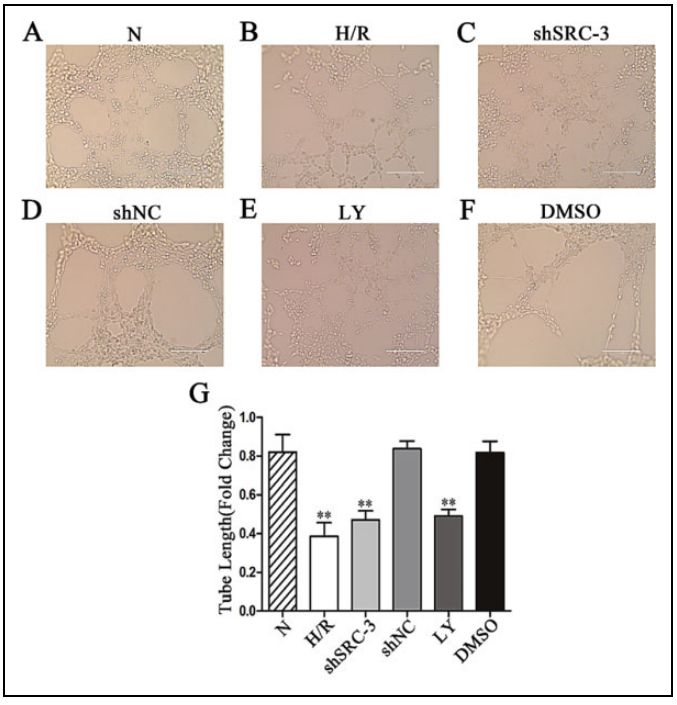

Figure 6. The HUVEC tube formation ability detected by migration assay (200×). A-D, Representative images revealed tube formation in HUVECs. A, Normal cell group (N). B, Hypoxia/reoxygenation cell group (H/R). C, Lentivirus short hairpin RNA against SRC-3 in HUVECs (shSRC-3). D, Control vector in HUVECs (shNC). E, PI3K inhibitor LY294002 treatment in HUVECs (LY). F, DMSO treatment in HUVECs (DMSO). G, Statistical bar graphs show quantification of tube formation index in the tube formation assay. Scale bar: 200 μm. The tube formation index was expressed as tube length (mm) per area (mm^2). Data were analyzed by 1-way ANOVA to assess significant differences (n = 3 in triplicate). **$P < .01$ compared to the normal cell group. HUVECs indicates human umbilical vein endothelial cell line; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.

VEGFR2 is essential for endothelial cell biological function during development.[37] Additionally, expression of VCAM-1 increased on endothelial cells damaged by ROS.[32] Thus, we investigated the expressions of VEGFR2 and VCAM-1. Our results showed that VEGFR2 expression was reduced by shRNA transfection, which was consistent with the decrease in SRC-3. In contrast, the expression of VCAM-1 was increased. Moreover, HUVECs treated with shRNA were subjected to migration and tube formation assays. Our study demonstrated that HUVEC formation and migration ability were significantly decreased by shRNA treatment. These data suggested that the downregulation of SRC-3 may impair the migration and angiogenesis functions of endothelial cells.

The PI3K signaling pathway combines both intracellular and extracellular signals to regulate various cell biological responses, including cell metabolism, growth, proliferation, and survival.[38] Previous studies revealed that SRC-3 was an upstream molecule in the PI3K signaling pathway.[24,25,39] In addition, the PI3K signaling pathway desensitization was detected in PE.[21,22,40] The serine/threonine kinase Akt is a direct downstream effector of PI3K, and the activation of Akt can further stimulate the activation of mTOR. Thus, we further investigated the relationship between SRC-3 and the PI3K/Akt/mTOR signaling pathway in PE. We found that oxidative stress led to p-Akt and p-mTOR reduction, accompanied by the downregulation of SRC-3 expression. Moreover, p-Akt and p-mTOR expressions were decreased after SRC-3 shRNA transfection. These data prove that downregulation of SRC-3 induced by oxidative stress can attenuate the PI3K/Akt/mTOR pathway activation in HUVEC. Additionally, inhibition of PI3K attenuated VEGFR2 expression and increased VCAM-1 expression in HUVEC. The HUVEC migration and tube formation potential were also reduced after PI3K inhibition. Taken together, the above findings indicate that SRC-3 may serve as a positive mediator of the PI3K/Akt/mTOR signaling pathway during oxidative stress. Interestingly, our data show that inhibition of the PI3K pathway abated the expressions of SRC-3, which means PI3K may have a cross talk with SRC-3. This finding requires further authentication.

It is well-known that estrogen possesses a vasoprotective function. Estrogen may increase vasodilatation and inhibit the response to vascular injury.[40] Estrogen regulation of expression in target tissues is mediated by ERs, including ERα and

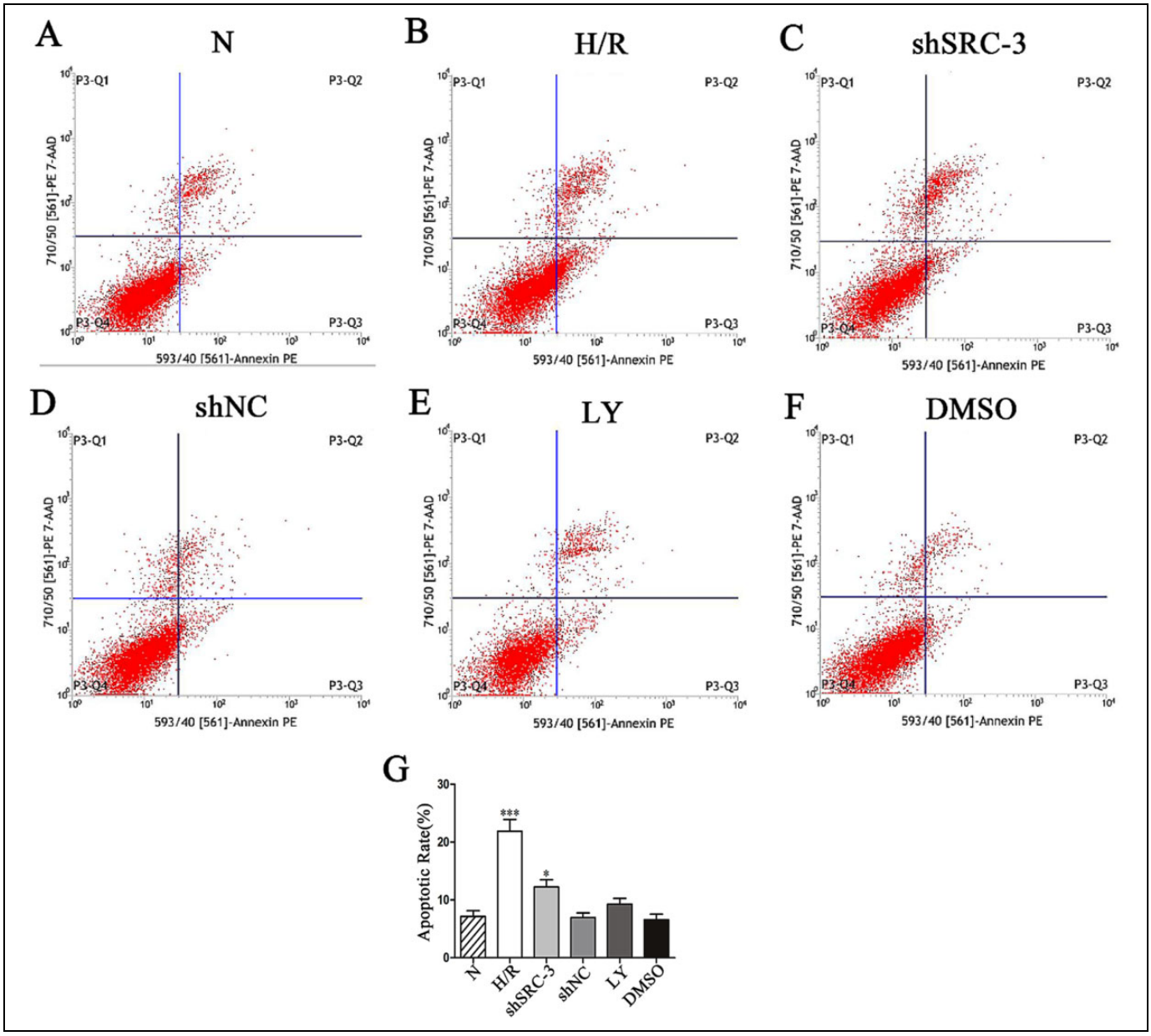

Figure 7. Apoptosis rates of HUVECs assessed by flow cytometry. A-D, Representative pictures of flow cytometry for the apoptosis rate in HUVECs. A, Normal cell group (N). B, Hypoxia/reoxygenation cell group (H/R). C, Lentivirus short hairpin RNA against SRC-3 in HUVECs (shSRC-3). D, Control vector in HUVECs (shNC). E, PI3K inhibitor LY294002 treatment in HUVECs (LY). F, DMSO treatment in HUVECs (DMSO). G, Statistical bar graphs show the quantification of the apoptotic rates in HUVECs. Annexin PE+/7-AAD− (upper right quadrant, Q2) was predicted to be early apoptotic cells; Annexin PE+/7-AAD+ (lower right quadrant, Q3) was predicted to be late apoptotic cells; the total percentage of apoptotic cells = Q2 + Q3. Data were analyzed by 1-way ANOVA to assess significant differences (n = 3 in triplicate).*$P < .05$ compared with the normal cell group, ***$P < .001$ compared with the normal cell group. HUVECs indicates human umbilical vein endothelial cell line; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.

ERβ.[41,42] Numerous studies have reported that the dysregulation of estrogen and ERs is involved in the development of PE.[43,44] Moreover, SRC-3, an ER coactivator, interacts with ERs, which in turn mediates the vasoprotection of estrogen.[16] Thus, further studies should focus on the underlying association between SRC-3 and ERs in PE.

In addition, future studies should clarify the relationship between SRC-3 and trophoblast function in the setting of PE. To conduct more intensive research into the role of SRC-3 in PE, more elaborate models, including placental explants and PE animal models, are needed.

In summary, our present study revealed that decreased expression of SRC-3 induced by oxidative stress influences the function of endothelial cells through the PI3K/Akt/mTOR signaling pathway, which participates in the pathogenesis of PE.

Authors' Note

Yu Yuan and Nan Shan contributed equally to this work and should be considered first coauthors.

Declaration of Conflicting Interests

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