

Effects of forkhead box protein M1 on trophoblast invasion and its role in preeclampsia development

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Abstract. The present study aimed to investigate the expression of the forkhead box protein M1 (FOXM1) in the placenta of patients with preeclampsia, and its effect on trophoblasts. A total of 28 patients with preeclampsia and 30 patients without preeclampsia (controls) who underwent cesarean section and were admitted to the Affiliated Hospital of Qingdao University between June 2013 and September 2016 were enrolled in the present study. The expression of FOXM1 in placental tissues was examined by reverse transcription-quantitative polymerase chain reaction, western blotting and immunohistochemistry. HTR8/SVneo cells were used to measure the *in vitro* expression of the vascular endothelial growth factor (VEGF). The results demonstrated that FOXM1 expression was downregulated in the placental tissues of patient with preeclampsia ($P < 0.05$). Following the silencing of FOXM1 expression, the proliferation of HTR8/SVneo cells was suppressed. The results of flow cytometry demonstrated that proportion of HTR8/SVneo cells in the G0/G1 phase and the proportion of apoptotic cells increased. The expression of the apoptosis regulator BCL-2, as well as the expression of VEGF mRNA and protein expression were also downregulated following FOXM1 silencing. FOXM1 may therefore promote the development of preeclampsia via the VEGF signaling pathway.

Introduction

Preeclampsia is one of the main causes of perinatal maternal and infant mortality, with an incidence of 1-5% (1,2). Pathophysiological changes begin in early pregnancy; however symptoms usually become evident after 20 weeks gestation (3). The main symptoms of preeclampsia include hypertension, proteinuria, edema, full body spasms and in severe cases,

multiple-organ dysfunction (3). Preeclampsia is a condition associated with the placenta; clinical practice has demonstrated that disease-associated symptoms are rapidly relieved following placental delivery (4). Decreased trophoblast invasion, required to establish pregnancy, contributes to the development of preeclampsia (5) and inhibits vascular reconstruction in the uterine spiral arteries (6). Preeclampsia is a complex disease and its course is difficult to predict, therefore the termination of pregnancy may be recommended as a method of controlling disease progression (7). The etiology and pathogenesis of preeclampsia remains unclear.

Forkhead box protein M1 (FOXM1) is a protein expressed in a number of mammalian cells and serves important roles in physiological functions and pathological conditions, including mitosis, cell proliferation, differentiation, senescence, organogenesis, DNA repair and cancer invasion (8-10). FOXM1 in villous and decidual tissue is expressed in the cytotrophoblast during early pregnancy, and may affect embryo implantation and early placental formation (11-13). It has been demonstrated that the vascular endothelial growth factor (VEGF) signaling pathway is involved in the function of extravillous trophoblasts and trophoblast invasion (14,15). Furthermore, VEGF promotes endothelial cell division, vascular endothelial cell proliferation and migration, and tubular structure formation during neovascularization. It also enhances the permeability of vascular endothelial cells (16). Therefore, the current study investigated whether FOXM1 involvement during preeclampsia occurs via the VEGF signaling pathway.

In the present study, the mRNA and protein expression of FOXM1 in the placenta of patients with or without preeclampsia were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. *In vitro* function experiments were performed in HTR8/SVneo trophoblast cells. The association between FOXM1 and VEGF was investigated to explore the role of FOXM1 in preeclampsia.

Patients and methods

Patients. A total of 28 pregnant patients with preeclampsia and 30 patients without preeclampsia who underwent cesarean sections at the Affiliated Hospital of Qingdao University between June 2013 and September 2016 were enrolled in the present study. The patients without preeclampsia were used

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as controls. Participants were all female, with a median age of 33 years (range, 21-41 years) in the preeclampsia group and 35 years (range, 20-42 years) in the control group. The diagnosis of preeclampsia was made by professors from the Department of Pathology of the Affiliated Hospital of Qingdao University (Qingdao, China). This diagnosis was confirmed by serological tests and other clinical examinations, including blood pressure assessment, routine laboratory evaluation (the measurement of urine volume, creatinine, alanine aminotransferase or aspartate aminotransferase, and platelets), fetal status assessment and proteinuria detection. The mean weights of infants from the control and preeclampsia groups were 3.15 ± 0.97 and 2.79 ± 1.03 kg, respectively. The blood pressure of infants was not measured. Placental tissue samples (500 mg) were collected from each participant during cesarean section and stored in liquid nitrogen at -196°C . The protocol of the present study was approved by the Ethical Review Board of the Affiliated Hospital of Qingdao University and written informed consent was obtained from all patients.

Reagents and equipment. SuperReal PreMix (SYBR-Green; cat. no. FP204) and the TIANScript II RT kit (cat. no. KR107) were purchased from Tiangen Biotech Co., Ltd. (Beijing, China). iQTM and Image LabTM software (version 3.0) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA) for RT-qPCR. The BCA protein assay kit (cat. no. RTP7102) was purchased from Real-Times (Beijing) Biotechnology Co., Ltd. (Beijing, China). Cell preservation solution was purchased from Ruikangdi Co., Ltd. (Beijing, China). Enhanced chemiluminescence (ECL) solution (cat. no. ab65623) was purchased from Abcam (Cambridge, MA, USA). All plasmids were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Cell proliferation and cytotoxicity test kits for the MTT assay were purchased from JRDUN Biotechnology Co., Ltd. (Shanghai, China). Cell cycle and apoptosis detection kits were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The Annexin V-FITC apoptosis detection kit was purchased from Vazyme Biotech, Co., Ltd. (Nanjing, China).

RT-qPCR. Placental tissues were pulverized with liquid nitrogen. A total of 1 ml TRIzol (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added to each 100 mg tissue for adequate lysis and RNA was extracted using the phenol chloroform method as previously described (17). The integrity of the RNA bands was detected using agarose gel electrophoresis as previously described (18). RNA purity was assessed by measuring the 260/280 ratio using a spectrophotometer. RT was performed using 1 μg total RNA using a TIANScript II RT kit. Template cDNA was stored at -20°C . qPCR was performed using the SuperReal PreMix (SYBR-Green) and specific primers. The primers for FOXM1 were as follows: Forward, 5'-GGCTCCCAGC ATCAAGCA-3' and reverse, 5'-TGTTCCGGCGGAGCTCT GGA-3'. The primers for β -actin, which was used as the internal reference gene, were as follows: Forward, 5'-ATCTGGCAC CACACCTTCACAATGAGCTGCG-3' and reverse, 5'-CGT CATACTCCTGCTTGCTGATCCACATCTGC-3'. The primers for VEGF were as follows: Forward, 5'-TTGCCTTGC TGCTCTACCTC-3' and reverse, 5'-AAATGCTTTCTCCGC TCTGA-3'. The qPCR reaction conditions were as follows:

Initial denaturation for 2 min at 94°C , followed by 35 cycles of denaturation for 30 sec at 94°C , annealing for 30 sec at 55°C and extension for 1 min at 71°C , and final extension for 2 min at 71°C . Relative gene expression of FOXM1 and VEGF was calculated using the $2^{-\Delta\Delta\text{C}_q}$ method (19). All experiments were performed in triplicate.

Western blotting. The expression of FOXM1 and VEGF proteins was determined by western blotting. Total proteins were extracted with radioimmunoprecipitation lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) following the supplier protocol. Protein concentration was determined using a BCA kit. Proteins (50 μg per lane) were separated using 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% skimmed milk in Tween-20 in Tris buffer solution at room temperature for 1 h. Proteins were then incubated with rabbit anti-human primary antibodies against FOXM1 (cat. no. ab175798; 1:2,000), VEGF (cat. no. ab46154; 1:1,000), BCL-2 (cat. no. ab32124; 1:1,000) and the internal reference protein β -actin (cat. no. ab129348; 1:5,000; all Abcam) overnight at 4°C . Proteins were subsequently incubated with horseradish-conjugated secondary antibodies (goat anti-rabbit; ab6721; 1:3,000; Abcam) at room temperature for 1 h. The membrane was exposed to the ECL solution and then exposed to the Gel Doc XR gel imaging and analysis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The image signal was analyzed and quantified by Image Lab software version 3.0 (Bio-Rad Laboratories, Inc.). The relative content of the target protein was calculated by the ratio of the gray value of the target protein band to that of the internal reference band. All experiments were performed in triplicate.

Immunohistochemistry. Placental tissues were fixed in 10% formaldehyde at 4°C for 24 h, embedded in paraffin and cut into 5- μm -thick sections. Then, sections were dewaxed using xylene and rehydrated in a descending alcohol series. Antigen retrieval was achieved by incubation with sodium citrate solution (Beyotime Institute of Biotechnology) and the sample was heated in the microwave at 97°C for 12 min. Following washing of the sections, they were incubated with 3% hydrogen peroxide at room temperature for 10 min. Following blocking with 10% goat serum (Beyotime Institute of Biotechnology) at room temperature for 1 h, sections were incubated with rabbit anti-human FOXM1 primary antibodies (cat. no. ab83097; 1:50; Abcam) overnight at 4°C . Samples were then washed with PBS, incubated with the goat anti-rabbit horseradish peroxidase conjugated secondary antibodies (cat. no. ab6721; 1:1,000; Abcam) for 1 h at room temperature and washed with PBS. Finally, sections were developed with chromogenic reagent 3'3'-diaminobenzidine for ~ 10 sec at room temperature, counterstained with hematoxylin for 20 sec at room temperature, mounted with neutral gum and observed using a Nikon TS100-F optical microscope (Nikon Corporation, Tokyo, Japan; magnification, $\times 500$).

Human HTR8/SVneo transfection. HTR8/SVneo cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured at 37°C in 5% CO_2 with

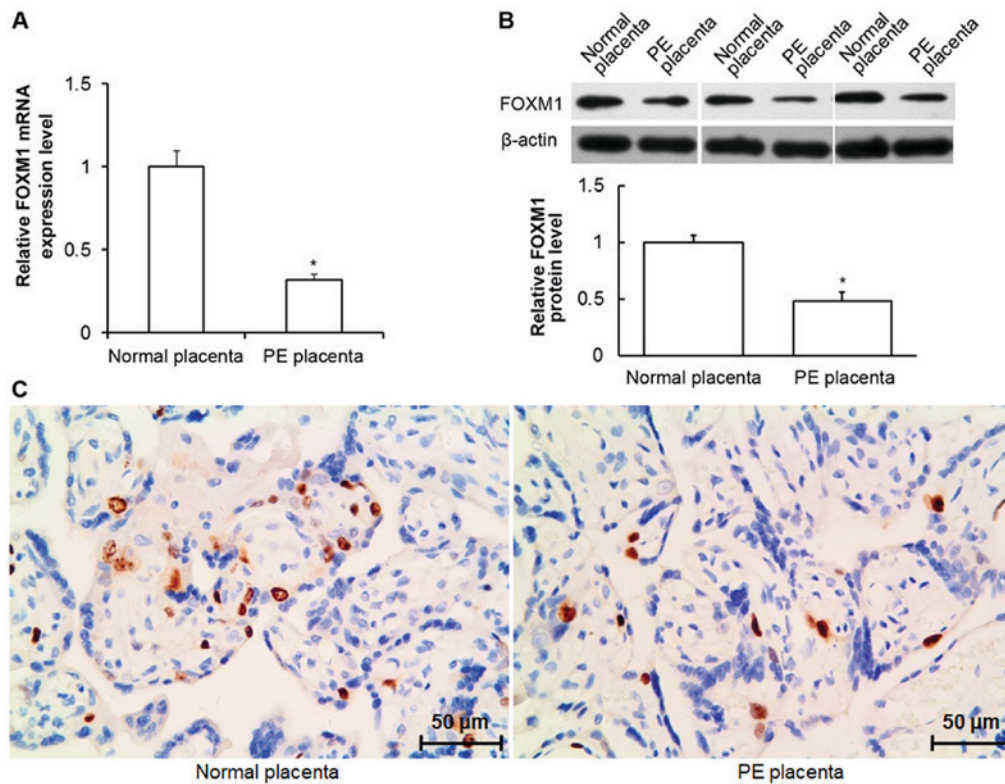


Figure 1. FOXM1 expression is decreased in the placentas of patients with PE. The expression of FOXM1 (A) mRNA and (B) protein was decreased in PE placenta compared with normal placenta. (C) Immunohistochemical staining of FOXM1 expression in placental tissues. * $P < 0.05$ vs. normal placenta. PE, preeclampsia; FOXM1, forkhead box protein M1. Tamhane's T2 was used for statistical analysis.

Dulbecco's Modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS). Cells were plated in 24-well plates at 3×10^5 cells/well 1 day prior to transfection and cultured in 10% FBS in F12/DMEM without antibiotics. Transfection was performed when cells reached 70% confluence. A total of 50 nM FOX1 small interfering (si)RNA (designed by Sangon Biotech Co., Ltd.) was transfected into cells using Lipofectamine[®] 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (20). The sequence of FOX1 siRNA was forward, 5'-ACCCAAACCAGCUAUGAUGdTdT-3' and reverse, 5'-CAUCAUAGCUGGUUUGGGUdTdT-3'. The sequences of the scramble siRNA were as follows: Forward, 5'-UUCUCCGAACGUGUCACG UdTdT-3' and reverse, 5'-ACGUGACACGUUCGGAGAAdTdT-3'. Levels of FOXM1 mRNA and protein were measured 48 h following transfection.

HTR8/SVneo cell proliferation following transfection. Cells were plated in triplicate in 96-well plates at a density of 2×10^3 cells/well. Subsequently, 20 μ l 5 g/l MTT solution was added at 24, 48 and 72 h. Purple crystals were solubilized by adding 150 μ l dimethyl sulfoxide and plates were then incubated in a humidified atmosphere at 37°C for 4 h. The cell proliferation curve was constructed by measuring the spectrophotometric absorbance of the samples at a wavelength of 490 nm.

Flow cytometric detection of HTR8/SVneo cell cycle and apoptosis post-transfection. At 48 h post-transfection, cells were collected. Cells (2×10^5) were fixed with 75% alcohol

overnight at 4°C and incubated with 1 mg/ml RNaseA for 30 min at 37°C. Cells were stained with propidium iodide (PI) at room temperature for 15 min. Cells were also incubated with Annexin V at room temperature for 15 min for apoptosis analysis. The cell cycle and cell apoptosis were analyzed using a flow cytometer.

Statistical analysis. All data were analyzed using SPSS statistical software (version 18.0; SPSS, Inc., Chicago, IL, USA) and were tested for normal distribution. Data are expressed as mean \pm standard deviation. Multiple measurement data were tested by one-way analysis of variance followed by the Least Significant Difference and Student-Newman-Keuls method or Tamhane's T2, where appropriate. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

FOXM1 mRNA and protein expression is decreased in the placentas of patients with preeclampsia. The results of RT-qPCR demonstrated that the expression of FOXM1 mRNA in the placental tissue of patients with preeclampsia was significantly decreased compared with the control group ($P < 0.05$; Fig. 1A). The expression of FOXM1 protein in the placental tissue of patients with preeclampsia was also significantly decreased ($P < 0.05$; Fig. 1B). This was supported by the results of immunohistochemical staining, which exhibited decreased staining for FOXM1 in the placental tissues of patients with preeclampsia (Fig. 1C). The results of the current study indicate that the expression of FOXM1 mRNA and protein is

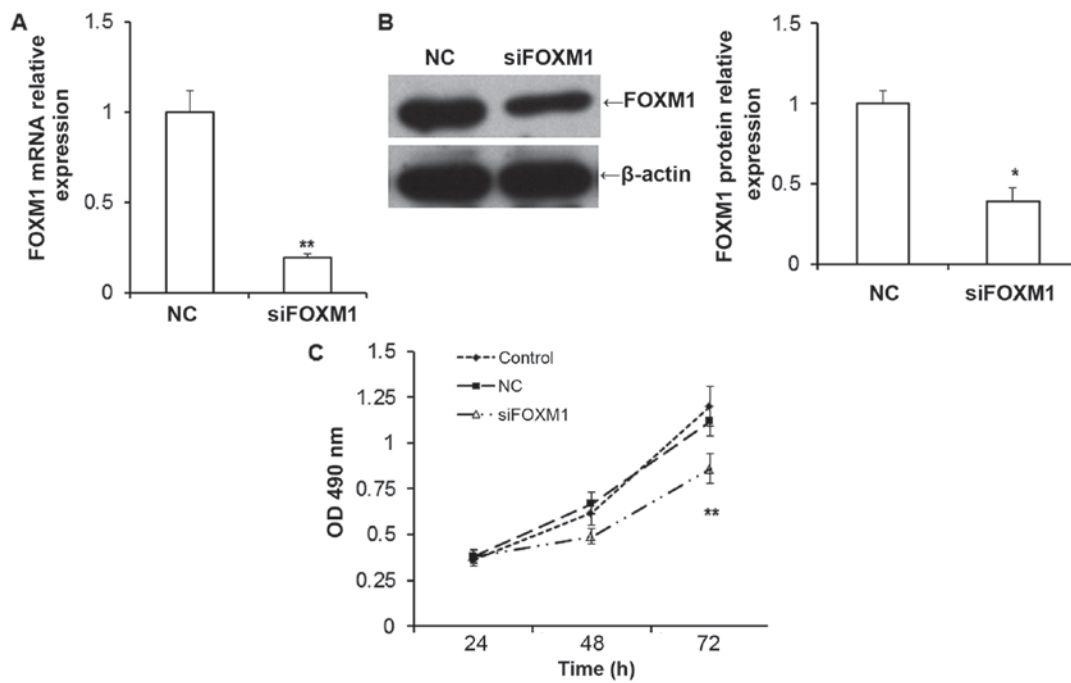


Figure 2. FOXM1 silencing decreases the proliferation of trophoblasts. FOXM1 (A) mRNA and (B) protein expression in HTR8/SVneo cells following transfection with siFOXM1. (C) MTT assay measuring the proliferation of HTR8/SVneo cells following transfection with siFOXM1 or NC. * $P < 0.05$ and ** $P < 0.01$ vs. NC. NC, negative control; FOXM1, forkhead box protein M1; si, small interfering RNA; OD, optical density. Student-Newman-Keuls method was used for statistical analysis.

downregulated in patients with preeclampsia, suggesting that FOXM1 may serve a regulatory role in preeclampsia.

FOXM1 silencing decreases the proliferation of HTR8/SVneo cells. To examine the function of FOXM1 in the placenta, *in vitro* evaluations were conducted by silencing FOXM1 expression in HTR8/SVneo cells. The expression of FOXM1 mRNA ($P < 0.01$; Fig. 2A) and protein ($P < 0.05$; Fig. 2B) were significantly downregulated in HTR8/SVneo cells transfected with FOXM1 siRNA compared with negative control cells. The results of the MTT assay demonstrated that the proliferation of HTR8/SVneo cells was inhibited following siFOXM1 transfection compared with cells transfected with the negative control (NC; $P < 0.01$; Fig. 2C). This suggests that the downregulation of FOXM1 inhibits the proliferation of HTR8/SVneo cells.

FOXM1 silencing increases the proportion of cells in the G0/G1 phase. Flow cytometry was used to assess the proportion of cells in each stage of the cell cycle (Fig. 3A). The results demonstrated that the proportion of HTR8/SVneo cells in the G0/G1 phase was significantly increased in cells transfected with FOXM1 siRNA ($P < 0.05$), whereas the proportion of cells in the G2 and S-phases was decreased (all $P < 0.05$; Fig. 3B). These results indicate that decreased FOXM1 expression may attenuate the cell cycle in HTR8/SVneo cells.

FOXM1 silencing increases the proportion of apoptotic cells and decreases BCL-2 protein expression. The proportion of early apoptotic cells and late apoptotic cells in HTR8/SVneo cells was examined using flow cytometry (Fig. 4A). The results revealed that transfection with FOXM1 siRNA significantly increased the proportion of apoptotic cells compared with cells transfected

with NC siRNA ($P < 0.01$; Fig. 4B), suggesting that decreased FOXM1 expression may increase apoptosis in HTR8/SVneo cells. The results also revealed that BCL-2 protein expression was significantly decreased following transfection with FOXM1 siRNA ($P < 0.05$; Fig. 4C), indicating that FOXM1 is associated with BCL-2 expression.

FOXM1 silencing decreases VEGF expression. VEGF is one of the most potent angiogenic factors and promotes angiogenesis, which, in turn increases blood supply (21). The reduction in VEGF expression leads to the premenstrual onset of preeclampsia, therefore the current study examined the association between FOXM1 and VEGF. The expression of VEGF mRNA and protein in HTR8/SVneo cells was determined by RT-qPCR and western blotting, respectively. The results demonstrated that the decrease of FOXM1 expression significantly downregulated the expression of VEGF mRNA and protein (both $P < 0.05$; Fig. 5A and B), suggesting that FOXM1 and VEGF may function in concert and that changes in the expression of either protein may induce vascular dysfunction. VEGF receptor (VEGFR) expression in placental tissue samples were also examined in a preliminary experiment by our group, however, the results revealed no significant changes in patients with preeclampsia compared with controls (data not shown).

Discussion

In the present study, the role of placental FOXM1 expression in the pathogenesis of preeclampsia was investigated. The expression of FOXM1 mRNA and protein was examined in the placenta tissues of patients with or without preeclampsia that underwent cesarean sections. The association between

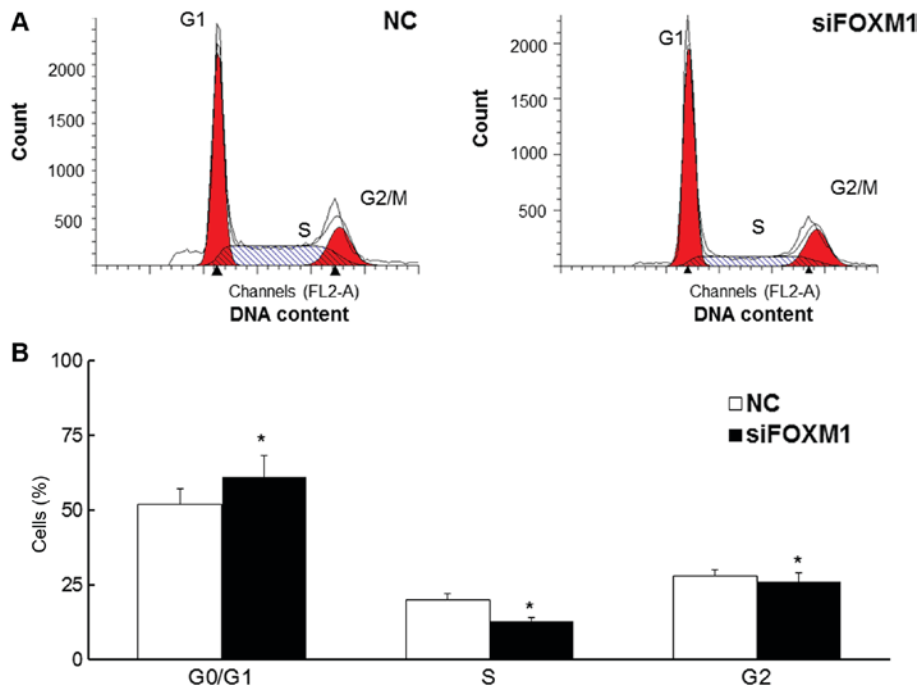


Figure 3. FOXM1 silencing increases the proportion of cells in the G0/G1 phase. (A) Cell cycle distribution and (B) quantification of cell cycle distribution of HTR8/Svneo cells following FOXM1 siRNA transfection. *P<0.05 vs. NC. NC, negative control; FOXM1, forkhead box protein M1; si, small interfering. Student-Newman-Keuls method was used for statistical analysis.

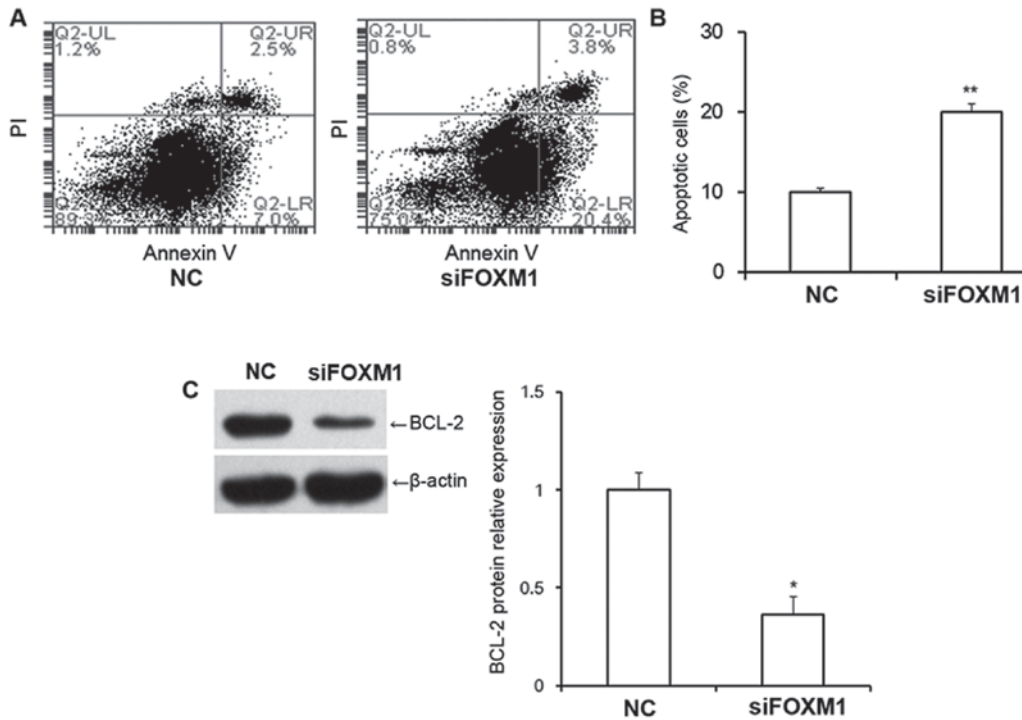


Figure 4. FOXM1 silencing increases the proportion of apoptotic cells and decreases BCL-2 protein expression. (A) Flow cytometric analysis identifying the (B) proportion of apoptotic cells in HTR8/SVneo cells following transfection with FOXM1 siRNA. (C) BCL-2 protein expression in transfected cells. *P<0.05 and **P<0.01 vs. NC. NC, negative control; FOXM1, forkhead box protein M1; si, small interfering RNA; BCL-2, apoptosis regulator BCL-2; PI, propidium iodide. Student-Newman-Keuls method was used for statistical analysis.

FOXM1 and VEGF was further investigated by performing *in vitro* HTR8/SVneo trophoblast cell cycle studies.

The primary manifestations of preeclampsia include the shallow invasion of trophoblasts into the endometrium,

cessation of vascular remodeling in the uterine spiral arteries and the decreased surface area and density of the villi. Patients with preeclampsia often exhibit disordered vascular remodeling (22,23). Placental hypoperfusion causes placental

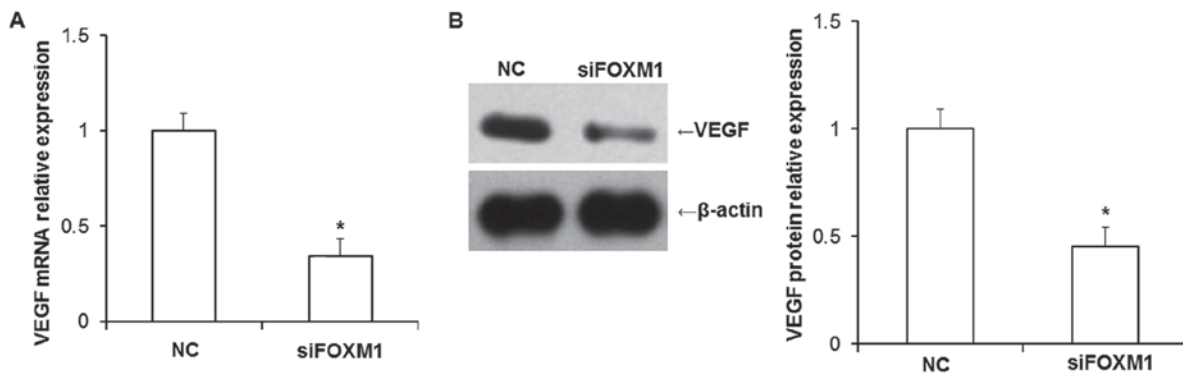


Figure 5. FOXM1 silencing decreases VEGF expression. The expression of VEGF (A) mRNA and (B) protein was decreased in HTR8/Svneo cells following transfection with FOXM1 siRNA. * $P < 0.05$ vs. NC. NC, negative control; FOXM1, forkhead box protein M1; si, small interfering RNA; VEGF, vascular endothelial growth factor. Student-Newman-Keuls method was used for statistical analysis.

hypoxia, metabolism disorders, placental sourced toxicity, vascular endothelial injury and an imbalance of vasoactive substances, which can eventually lead to the development of preeclampsia (24). It is widely accepted that preeclampsia develops in two different stages (25,26). During the first stage, vascular remodeling disorders cause a decrease in villous trophoblast invasion and induce placental hypoxia. In the second stage, multiple peptides produced by the placenta are secreted into the blood circulation of the mother and reactive oxygen species (ROS) build up in the vascular endothelium. The imbalance between ROS production and the antioxidant defense system exacerbates systemic small artery injury, leading to ROS accumulation, hypertension and proteinuria (27). Previous studies have suggested that preeclampsia may be associated with decreased placental trophoblast invasion, ischemia and hypoxia, increased apoptosis, abnormal lipid metabolism and other placental dysfunctions during pregnancy (28-30). It has been reported that the extravillous trophoblasts serve an important role in the process of recoil in spiral arteries; the diminished invasion of extravillous trophoblast cells leads to abnormal vascular remodeling and shallow embryo implantation and ultimately, the development of preeclampsia (22,31).

FOXM1 is highly expressed in a number of malignancies and is involved in tumor cell proliferation, apoptosis, invasion and metastasis; it is therefore a marker of tumor development (32-35). The biological characteristics of trophoblasts in embryos are very similar to those of cancer cells and a number of factors involved in cancer invasion, including human leukocyte antigen and DNA methyltransferase 3A, also serve a role in trophoblast invasion (36,37). The primary difference is that trophoblast invasion is a tightly regulated process (38). FOXM1 is expressed in the cytotrophoblast of villus and decidual tissue during early pregnancy (11,39). FOXM1 is also expressed in the maternal decidual cells and FOXM1 may serve as an indirect regulator of trophoblast invasion via paracrine signaling (40). These results suggest that FOXM1 is involved in the development and progression of preeclampsia.

In the present study, the expression of FOXM1 was measured in normal and preeclampsia placental tissues. The expression of FOXM1 mRNA and protein was significantly decreased in patients with preeclampsia. This suggests

that FOXM1 may indirectly regulate trophoblast invasion through paracrine signaling, as decreased FOXM1 expression decreases trophoblast invasion and attenuates the invasion of trophoblast cells into the endometrium (38,40). To the best of our knowledge, the present study is the first to report the abnormal expression of FOXM1 in preeclampsia placenta.

To further investigate the effect of FOXM1 on trophoblast cells, FOXM1 siRNA was transfected into human trophoblast cells, HTR8/Svneo. The results of the MTT assay demonstrated that FOXM1 silencing attenuates the proliferation of HTR8/Svneo cells. Additionally, the results of flow cytometry demonstrated that the decreased expression of FOXM1 blocks the cell cycle at the G0/G1 phase and increases the apoptosis of HTR8/Svneo cells. Furthermore, the results of RT-qPCR and western blotting revealed that decreased FOXM1 expression leads to the downregulation of VEGF mRNA, and VEGF and BCL-2 proteins. VEGF is one of the most effective angiogenic growth factors; it promotes angiogenesis and increases blood supply (41). It has been demonstrated that suppression of the VEGF signaling pathway is associated with the development of preeclampsia (42), which may partially explain the development of vascular remodeling disorder in patients with preeclampsia. Silencing FOXM1 decreases the expression of VEGF, suggesting that FOXM1 may serve its role via the VEGF signaling pathway. VEGFR expression in tissue samples was also examined in a preliminary experiment by our group, however the results revealed no significant changes in patients with preeclampsia compared with healthy patients, which may be a result of other biological functions.

In conclusion, the results of the present study demonstrate that the altered expression of FOXM1 may cause changes in the expression of associated proteins, resulting in decreased trophoblast cell proliferation, prolonged cell cycle and increased cellular apoptosis. This demonstrates that FOXM1 serves an important role in the development of preeclampsia by regulating the VEGF signaling pathway.

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