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Effects of forkhead Box protein A1 on cell proliferation regulating and EMT of cervical carcinoma

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Abstract. – OBJECTIVE: Cervical cancer is a common tumor in gynecological malignancies. However, the patients are often in an advanced stage when diagnosed. It was found that forkhead box protein A1 (FOXA1) is abnormally expressed in various tumors, such as breast cancer, ovarian cancer, and is closely related to tumorigenesis. This study aimed to investigate the expression and the related roles of FOXA1 in cervical cancer.

PATIENTS AND METHODS: Real Time-PCR (RT-PCR) and Western blot were used to analyze expression of FOXA1 in cervical cancer and adjacent tissue. The small-interfere RNA (siRNA) was adopted to down-regulate FOXA1 expression in HeLa cells. The effect of FOXA1 on apoptosis of HeLa cells was detected by using thiazolyl blue tetrazolium bromide (MTT) method. The apoptosis rate of HeLa cells was detected by using flow cytometry. The Western blot was selected to evaluate the epithelial mesenchymal transition (EMT) related protein, vimentin, E-cadherin, and vascular endothelial growth factor (VEGF) changes.

RESULTS: Compared with adjacent tissues, FOXA1 mRNA and protein expressions significantly increased in cervical cancer ($p<0.05$). SiRNA significantly reduced FOXA1 expression in Hela cells compared with the control group and siRNA-NC group, thus inhibiting tumor cell proliferation and enhancing cell apoptosis rate ($p<0.05$). E-cadherin elevated, Vimentin decreased, and VEGF reduced after FOXA1 siRNA treatment.

CONCLUSIONS: FOXA1 expression increased in cervical cancer. Inhibition of FOXA1 expression blocked the proliferation of cervical cancer, promoted tumor cell apoptosis, suppressed the occurrence of EMT and VEGF production, and can regulate cervical cancer metastasis. FOXA1 can be used as a new molecular biological target for cervical cancer diagnosis and treatment.

Key Words:
FOXA1, Cervical cancer, Proliferation, EMT, VEGF.

Introduction

The incidence of cervical cancer has been consistently high, especially in developing countries and economically underdeveloped regions. It has become one of the most serious malignant tumors in the world[1,2]. Among gynecologic malignancies, cervical cancer is the second most common malignancy and one of the most frequently occurring gynecologic cancers in women. It ranks fourth in all types of malignancies and poses a serious threat to women's health and lives[3,4]. In China, cervical cancer screening is popularized, and prevention measures are backward. As a result, the incidence of cervical cancer remained high. Moreover, improper sexual behavior and human papillomavirus (HPV) infection lead to the onset of a younger trend, so the incidence and mortality of cervical cancer in China has always been relatively high in gynecological tumors[5,6]. Due to the lack of early interventions for cervical cancer patients, most patients are advanced when diagnosed, leading to poor prognosis and quality of life. It brings heavy mental and economic burden to patients and their families, which is a serious threat to global health security[7,8]. At present, the molecular targeted therapy of cervical cancer has become a hot spot, but the molecular mechanism

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involved in the occurrence and development of cervical cancer has not been fully elucidated. Forkhead family is one of the transcription factors and widely found in tissues and organs. It contains up to 100 family members and all have highly conserved DNA domains. The domain is approximately 110 amino acids[9]. As transcription factor, forkhead protein can play a role in a variety of pathophysiological activities, including apoptosis, epithelial mesenchymal transition (EMT), tumorigenesis, and angiogenesis[10,11]. Forkhead box protein A1 (FOXA1), a member of the forkhead family, is found to be abnormally expressed in a variety of tumors and performs a variety of biological functions[12]. FOXA1, also known as hepatocyte nuclear factor 3α, is a transcription factor with a wing-shaped helix and has been identified as a hepatocyte-enriched transcription factor[13,14]. It has been confirmed that FOXA1 is abnormally expressed in various tumors such as breast cancer and ovarian cancer, and is closely related to tumorigenesis[15,16]. However, the expression of FOXA1 in cervical cancer and related mechanisms remain to be studied.

Patients and Methods

Research Subjects' Selection

A total of 52 patients diagnosed with cervical cancer in The Affiliated Hospital of Qingdao University (Qingdao, Shandong, China) by pathological examination from May 2017 to October 2017 were selected. All selected patients with mean age of 57.8 ± 9.5 (48-72) years old were treated with surgery. Inclusion criteria: all patients were first diagnosed with cervical cancer and were treated by surgery for the first time. No chemotherapy, radiotherapy, or other treatment was performed before surgery. Exclusion criteria: recurrent cervical cancer, previous surgical treatment; previous radiotherapy or chemotherapy, combined with other diseases such as infectious diseases, malignant tumors, severe diabetes, other organ failure diseases, systemic immune diseases, and malignant cancer complications[5]. Tumor tissues and paracancerous tissues were collected during surgery and frozen in liquid nitrogen.

This study was approved by the Ethics Committee of The Affiliated Hospital of Qingdao University (Qingdao, Shandong, China). All of the selected subjects or patients signed the informed consent and approved this study.

Main Reagents and Instruments

RNA extraction kit and reverse transcription kit were purchased from RD Systems (Minneapolis, MN, USA). Other commonly used reagents were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). Real Time-PCR (RT-PCR) reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's Modified Eagle Medium (DMEM) medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from HyClone (South Logan, UT, USA). Dimethyl sulfoxide (DMSO) and thiazolyl blue tetrazolium bromide (MTT) powder were purchased from Gibco (Grand Island, NY, USA). Trypsin-ethylene diamine tetraacetic acid (EDTA) digest was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Covina, CA, USA). EDTA was purchased from HyClone (South Logan, UT, USA). Western blot related chemical reagents were purchased from Beyotime Biotechnology (Shanghai, China). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-human nuclear factor κB (NF-κB), FOXA1, Vimentin, E-cadherin, and vascular endothelial growth factor (VEGF) monoclonal antibodies, mouse anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Annexin V-FITC apoptosis detection kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). FOXA1 small interfere (siRNA) was designed and synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China). The Real Time-PCR instrument was purchased from ABI (Foster City, CA, USA). PCR System 2400 DNA Amplification Apparatus was purchased from PE Gene Applied Biosystems (Foster City, CA, USA). The SpectraMax iD5 microplate reader was purchased from MD (Foster City, CA, USA). The Accuri™ C6 flow cytometer was purchased from BD Biosciences (San Jose, CA, USA).

HeLa Cell Culture and Grouping

The Hela cell line preserved in liquid nitrogen was sub-cultured after recovery. The cells in the 3rd to 8th generation logarithmic growth phase were used for experiments. The cultured HeLa cells were randomly divided into 3 groups, including a control group: normal cell culture; siRNA-NC group, HeLa cells transfected with FOXA1 negative control; and FOXA1 siRNA group, HeLa cells transfected with FOXA1 siRNA.

FOXA1 siRNA Transfection

FOXA1 siRNA and siRNA-NC were transfected into HeLa cells, respectively. The FOXA1 siRNA sequence was 5'-GCGUGGAUUCGGUGUGGAAUCA-3'. The siRNA-NC sequence was 5'-UCUGAGAGGAUUCUAGGU-3'. In a 6-well plate, the cell density was fused to 70-80%. FOXA1 siRNA and negative control liposomes were added to 200 µl of serum-free DMEM medium, respectively. After incubating at room temperature for 15 min, they were mixed with lipo2000 and incubated at room temperature for 30 min. The mixture was added to each well together with 1.6 ml of serum-free DMEM medium and incubated in a 5% CO_2 incubator at 37°C for 6 h. After continued incubation for 48 h, the cells were used for experiments.

Real Time-PCR

TRIzol reagent was used to extract RNA from cervical cancer, paracancerous tissues, and HeLa cells. DNA reverse transcription synthesis was performed according to the kit instructions. The primers were designed by PrimerPremier 6.0 software (Palo Alto, CA, USA) and synthesized by Invitrogen/Life Technologies (Carlsbad, CA, USA) (Table I). The real-time PCR reaction condition contained 55°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C-60°C for 45 s, and 72°C for 35 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference. Based on fluorescence quantification, the initial cycle number (CT) of all samples and standards was calculated. The relative expression level was calculated based on $2^{-\cdot Ct}$ method.

MTT Assay

HeLa cells in logarithmic growth phase were inoculated in 96-well culture plate and cultured in DMEM medium containing 10% fetal bovine serum at the density of 5×10^3 cells. After 24 h, the well was added with 20 µl sterile MTT for 4 h. Then the supernatant was completely removed, and 150 µl/well DMSO was added for 10 min until the purple crystals were completely dissolved. At last, the microplate reader was measured at a wavelength of 570 nm.

Flow Cytometry

HeLa cells were digested and inoculated in a 50 ml flask at a concentration of 5×10^5/ml. The cells were randomly divided into 3 groups as above. After treatment, the cells were fixed with pre-chilled 75% ethanol at 4°C overnight. After resuspension in a mixture of 800 µl 1× phosphate buffered saline (PBS) and 1% bovine serum albumin (BSA), the cells were incubated in 100 µg/ml propidium iodide (PI) dye solution (3.8% Sodium Citrate, pH 7.0) and 100 RNase (RnaseA, 10 mg/ml) 37°C avoid of light for 30 min. The cells were detected on flow cytometry and analyzed using FCSExpress software (version: 3.0, De Novo Software, Los Angeles, CA, USA).

Western Blot

HeLa cell protein was extracted from each group. The cells were treated by lysates and the protein was quantified and stored at -20°C. The isolated proteins were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the protein was transferred to PVDF membrane at 160 mA for 1.5 h. After blocking in 5% skim milk at room temperature for 2 h, the membrane was incubated in FOXA1, Vimentin, E-cadherin, and VEGF diluted primary antibodies (1:1500, 1:1500, 1:1000, 1:1500, and 1:2000) at 4°C overnight. Next, the membrane was incubated in 1:2000 goat anti-rabbit secondary antibody avoiding of light at room temperature for 30 min. After washing with PBST, the membrane was treated by chemiluminescence reagents for 1 min and imagined. The result was scanned using protein image processing system software and analyzed on Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). The experiment was repeated four times (n=4).

Statistical Analysis

All data analyses were performed on SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between the two groups. The Tukey's post-hoc test was used to validate the ANOVA for comparing measurement

Table I. Primer sequences.

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	AGTAGTCACCTGTTGCTGG	TAATACGGAGACCTGTCTGGT
FOXA1	ATACTCCATGGAGATTGG	TCACCG CTCGAAGCTGGTA

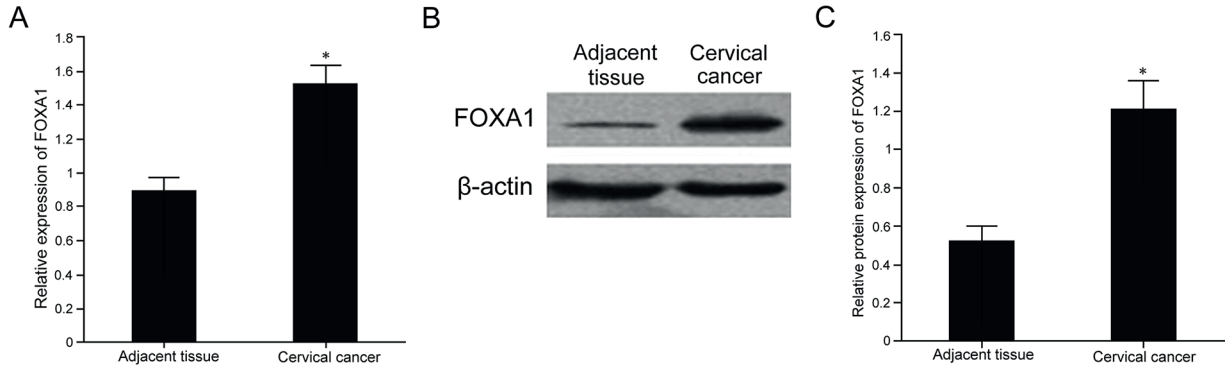

Figure 1. FOXA1 expression in cervical cancer. ***A,*** FOXA1 mRNA expression in cervical cancer. ***B,*** FOXA1 protein expression in cervical cancer. ***C,*** FOXA1 protein expression analysis. $^{*}p<0.05$, compared with adjacent tissues.

data among groups. $p<0.05$ represents a significant difference.

Results

FOXA1 Expression in Cervical Cancer

Real-time PCR and Western blot were used to detect the expression of FOXA1 mRNA and protein in cervical cancer. The results showed that FOXA1 mRNA and protein expressions were significantly higher in cervical cancer than in adjacent tissues ($p<0.05$, Figure 1).

The Impact of FOXA1 siRNA on HeLa Cells

After FOXA1 siRNA was transfected into HeLa cells, the expression of FOXA1 was detected. The expressions of FOXA1 mRNA and protein in HeLa cells were markedly reduced as compared with the control group and the siRNA-NC group ($p<0.05$, Figure 2).

The Influence of FOXA1 siRNA on HeLa Cell Proliferation

After FOXA1 siRNA knockdown the expression of FOXA1 in HeLa cells, MTT assay was adopted to analyze cell proliferation. The results showed that transfection of FOXA1 siRNA for 48 h markedly inhibited the proliferation of HeLa cells compared with the control group ($p<0.05$, Figure 3), suggesting that the regulation of FOXA1 has a significant inhibitory effect on the proliferation of HeLa cells.

The Effect of FOXA1 siRNA on HeLa Cell Apoptosis

After FOXA1 siRNA knockdown the expression of FOXA1 in HeLa cells, flow cytometry was selected to test its effect on cell apoptosis. The results demonstrated that transfection of FOXA1 siRNA for 48 h apparently promoted the apoptosis rate of HeLa cells compared with the control group ($p<0.05$, Figure 4), revealing that the regulation of FOXA1 had a significant role in promoting on HeLa cells apoptosis.

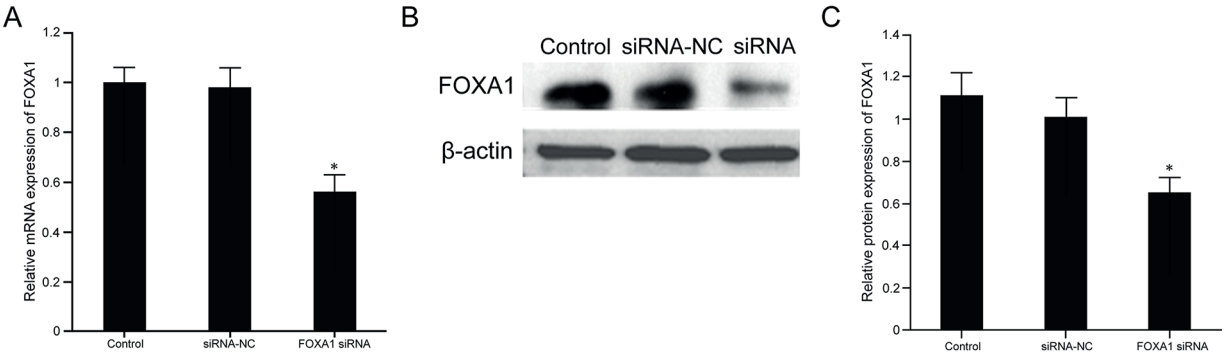

Figure 2. The impact of FOXA1 siRNA on HeLa cells. ***A,*** FOXA1 mRNA expression in cervical cancer. ***B,*** FOXA1 protein expression in cervical cancer. ***C,*** FOXA1 protein expression analysis. $^{*}p<0.05$, compared with control.

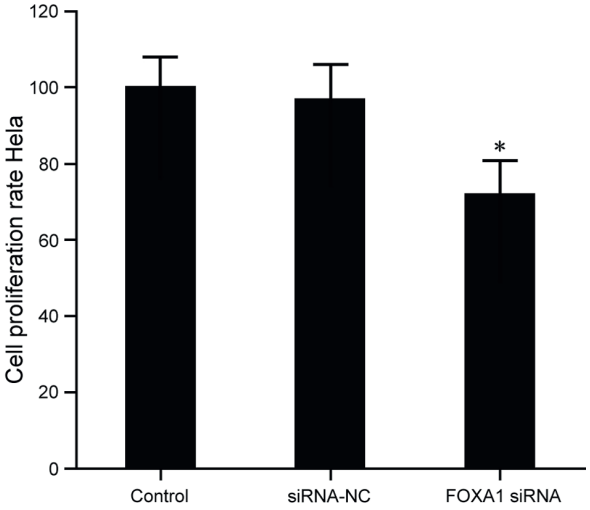

Figure 3. The influence of FOXA1 siRNA on HeLa cell proliferation. $^{*}p<0.05$, compared with control.

The Impact of FOXA1 siRNA on EMT in HeLa Cells

The effect of FOXA1 knockdown on EMT in HeLa cells was analyzed by Western blot. The expression of EMT-related proteins Vimentin and E-cadherin was analyzed. The results exhibited that transfection of FOXA1 siRNA for 48 h significantly increased expression of E-cadherin and reduced expression of vimentin in HeLa cells compared with the control group ($p<0.05$, Figure 5). It indicated that regulation of FOXA1 has an inhibitory effect on the occurrence of EMT in HeLa cells.

The Influence of FOXA1 siRNA on VEGF Expression in HeLa Cells

The influence of FOXA1 knockdown on VEGF expression in HeLa cells was analyzed by Western blot. The results exhibited that transfection of FOXA1 siRNA for 48 h obviously declined VEGF expression in HeLa cells compared with the control group ($p<0.05$, Figure 6). It showed that FOXA1 may have a regulatory role in inhibiting neovascularization.

Discussion

The occurrence and development of cervical cancer involve multiple factors, steps, and genes.

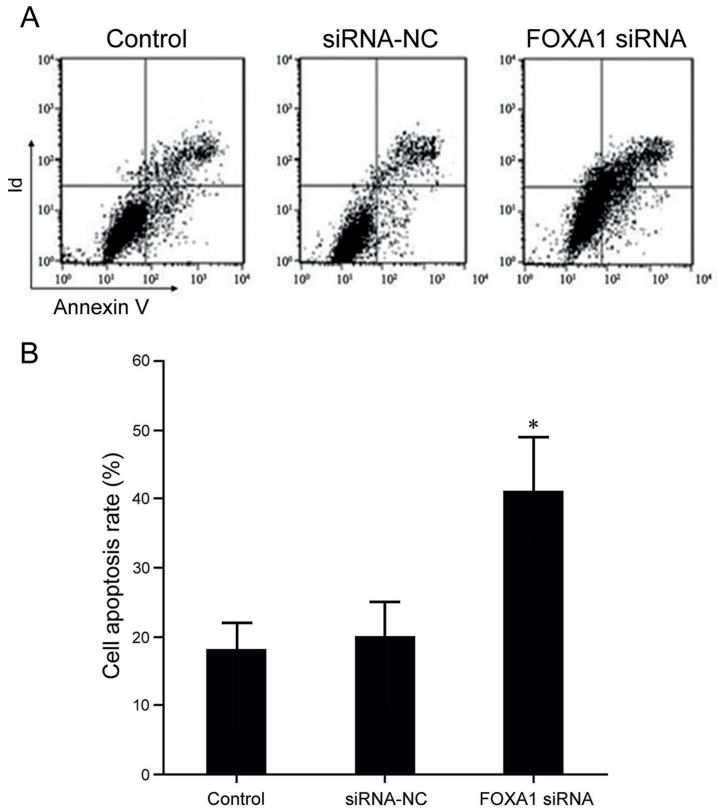

Figure 4. The effect of FOXA1 siRNA on HeLa cell apoptosis. **A,** Flow cytometry detection of cell apoptosis. **B,** HeLa cell apoptosis rate analysis. $^{*}p<0.05$, compared with control.

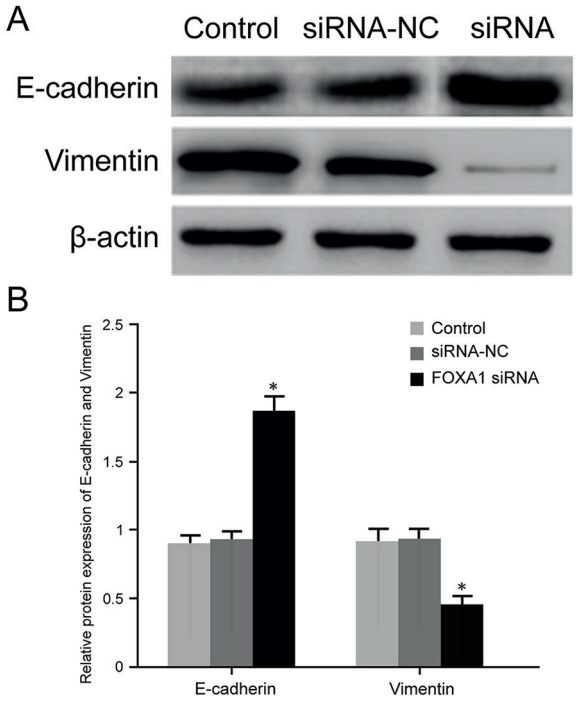

Figure 5. The impact of FOXA1 siRNA on EMT in HeLa cells. ***A,*** Western blot detection of protein expression. ***B,*** Protein expression analysis. $^{*}p<0.05$, compared with control.

HPV infection is an important factor in its pathogenesis. Although the conventional treatment of cervical cancer includes surgery combined with chemotherapy and radiotherapy, and various treatment methods appeared following the improvement of techniques, it has not achieved the desired therapeutic effect up to now. The prognosis of advanced cervical cancer patients is still poor[17,18]. Therefore, finding biomarkers for assisted cervical cancer screening may reveal new therapeutic targets for cervical cancer and provide potential screening targets. The FOX protein is a large family that can be divided into 17 subgroups from A-Q based on the conserved amino acid sequence of the forkhead domain[19]. FOX is associated with embryonic development, cell cycle regulation, cell proliferation, transformation, immune regulation, differentiation, longevity, and many other biological processes. Gene mutations and expression abnormalities in the FOX protein family may be related to developmental abnormalities, metabolic diseases, and tumorigenesis[20,21]. FOXA1 is a member of the FOXA subfamily that mediates the nuclear hormone receptor signaling pathway, which in turn regulates the activities of the estrogen receptor and the androgen receptor[22]. FOXA1 regulates nuclear hormone receptor activity in breast and prostate cancers, which may be related to the tumorigenic phenotype of FOXA1[23]. In breast cancer, FOXA1 expression was correlated with improved prognosis in breast cancer patients[24]. In this study, we analyzed the expression of FOXA1 in cervical cancer. Firstly, we detected the expression of FOXA1 in cervical cancer tissues. The results confirmed that both FOXA1 mRNA and protein expressions in cervical cancer were significantly increased. Therefore, we transfected FOXA1 siRNA into Hela cells to reduce FOXA1 expression. The results confirmed it markedly inhibited tumor cell proliferation and promoted tumor cell apoptosis. The main molecular features of EMT include the expression and functional loss of epithelial markers such as E-cadherin, and overexpression of interstitial cell markers such as vimentin[25]. This work demonstrated for the first time that targeting FOXA1 can increase the expression of E-cadherin and reduce the expression of vimentin, suggesting that it can inhibit the occurrence of EMT. Furthermore, it was found that FOXA1 inhibited the expression of VEGF, indicating that targeting FOXA1 may restrain the angiogenesis of cervical cancer. However, its related mechanism still needs further study.

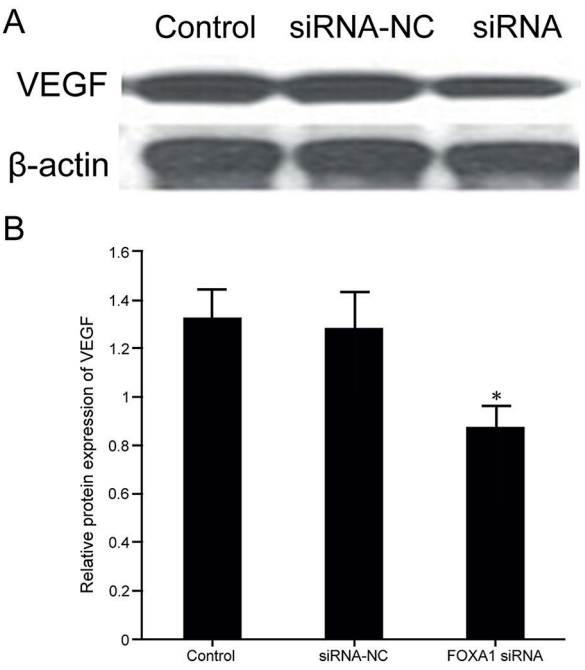

Figure 6. The influence of FOXA1 siRNA on VEGF expression in HeLa cells. ***A,*** Western blot detection of protein expression. ***B,*** VEGF protein expression analysis. $^{*}p<0.05$, compared with control.

Conclusions

We demonstrated that FOXA1 expression increased in cervical cancer. Inhibition of FOXA1 expression blocked the proliferation of cervical cancer, promoted tumor cell apoptosis, suppressed the occurrence of EMT and VEGF production, and can regulate cervical cancer metastasis. FOXA1 can be used as a new molecular biological target for cervical cancer diagnosis and treatment.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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