Int J Clin Exp Pathol 2014;7(4):1534-1543
www.ijcep.com /ISSN:1936-2625/IJCEP1401053

Original Article

FOXL2 suppresses proliferation, invasion and promotes apoptosis of cervical cancer cells

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Received January 17, 2014; Accepted February 26, 2014; Epub March 15, 2014; Published April 1, 2014

Abstract: FOXL2 is a transcription factor that is essential for ovarian function and maintenance, the germline mutations of which give rise to the blepharophimosis ptosis epicanthus inversus syndrome (BPES), often associated with premature ovarian failure. Recently, its mutations have been found in ovarian granulosa cell tumors (OGCTs). In this study, we measured the expression of FOXL2 in cervical cancer by immunohistochemistry and its mRNA level in cervical cancer cell lines Hela and Siha by RT-PCR. Then we overexpressed FOXL2 in Hela cells and silenced it in Siha cells by plasmid transfection and verified using western blotting. When FOXL2 was overexpressed or silenced, cells proliferation and apoptosis were determined by Brdu assay and Annexin V/PI detection kit, respectively. In addition, we investigated the effects of FOXL2 on the adhesion and invasion of Hela and Siha cells. Finally, we analyzed the influences of FOXL2 on Ki67, PCNA and FasL by flow cytometry. The results showed that FOXL2 was highly expressed in cervical squamous cancer. Overexpressing FOXL2 suppressed Hela proliferation and facilitated its apoptosis. Silencing FOXL2 enhanced Siha proliferation and inhibited its apoptosis. Meanwhile, silencing FOXL2 promoted Siha invasion, but it had no effect on cells adhesion. In addition, overexpressing FOXL2 decreased the expression of Ki67 in Hela and Siha cells. Therefore, our results suggested that FOXL2 restrained cells proliferation and enhanced cells apoptosis mainly through decreasing Ki67 expression.

Keywords: FOXL2, cervical cancer, proliferation, apoptosis, invasiveness

Introduction

FOXL2 is a single-exon gene encoding a forkhead transcription factor, whose germline mutations are responsible for the blepharophimosis ptosis epicanthus inversus syndrome (BPES; MIM 110100) [1]. This genetic disorder is characterized by malformation of the external eye with blepharophimosis, ptosis and a variable degree of epicanthus inversus. There are two clinical subtypes of this disease characterized by the clinical presence or absence of premature ovarian failure (POF). In females, BPES is divided into two types: Type I with POF and Type II with normal fertility [1, 2]. Truncating mutations of FOXL2 generally lead to Type I disease whereas frame shifts or duplications downstream of the forkhead domain lead to Type II disease [3]. The protein sequence of FOXL2 is highly conserved and contains a characteristic forkhead DNA-binding domain and a polyalanine tract of 14 residues of unknown function, which is strictly conserved among eutherian mammals [4, 5]. Other members of the large family of winged helix/forkhead transcription factors are involved in different developmental and metabolic processes and some of them are responsible for genetic, developmental diseases [6]. In mice, Foxl2 mRNA is expressed in embryonic eyelids and in embryonic and adult ovary [1, 7], and the murine Foxl2 gene is essential for differentiation of granulosa cells and maintenance of ovarian structure and function [7, 8]. Furthermore, two independent Foxl2$^{-/-}$ knock-out mice models have been generated and have confirmed the key role of Foxl2 in ovarian determination and granulose cell physiology [8, 9].

FOXL2 is expressed in ovarian granulosa cells throughout female life [5]. A recent study revealed that a markedly reduced expression of

FOXL2 in a series of juvenile ovarian granulose cell tumors (OGCTs) [10]. Moreover, Shah et al discovered a recurring somatic mutation 402C>G in the gene Foxl2 in 2009 [10]. This mutation was confirmed to be present in 97% adult ovarian granulose cell tumors (OGCTs) and specific to this tumor type [11-15]. However, inactivation of the Foxl2 locus by somatic hypermethylation was found in colorectal cancer [16]. Most interestingly, this mutation was not found in any other sex-cord stromal tumors, nor in any unrelated ovarian or breast tumors [17, 18]. In addition, Wegman P found that FOXL2 was expressed in breast cancer and correlated with aromatase as well as with clinical outcome [19].

To the best of our knowledge, FOXL2 has not been previously studied in cervical cancer and we therefore aimed to investigate its expression and the effect on cervical cancer cells.

Materials and methods

Tissue collection

All tissue samples were obtained with informed consent in accordance with the requirements of the Research Ethics Committee in the Affiliated Hospital of Weifang Medical University. Samples from 25 patients in International Federation of Gynecology and Obstetrics (FIGO) stages of cervical cancer were obtained from women age 25-55 years. Among them, 5 (20%) patients had FIGO Stage Ib2 disease, 4 (16%) had Stage IIa, 6 (24%) had Stage IIb, 8 (32%) had Stage IIIb, and 3 (8%) had Stage IVa disease. Moreover, specimens from 8 patients of cervical intraepithelial neoplasia (CIN) were acquired from women age 23-50 years. Among them, 3 (37.5%) patients had low-grade squamous intraepithelial lesion (LSIL), and 5 (62.5%) patients had high-grade squamous intraepithelial lesion (HSIL). Besides, cervical tissues from cervicitis (n=10; age 22-48 years) were collected as control. All the samples were confirmed histologically according to established criteria. In cancer group, squamous cell carcinoma was found in 23 (92%) patients and two with adenocarcinoma.

Immunohistochemistry

Paraffin sections (5 μm) of the cervical tissues from cervical cancer (n=25), CIN (n=8) or cervicitis (n=10) were rehydrated in phosphate buffered saline (PBS) and incubated with 3% hydrogen peroxide to block endogenous peroxidase. The samples were then incubated with anti-human Foxl2 antibody (1:400 dilution, Beijing Biosynthesis Biotechnology Co., Ltd., China) or goat IgG isotype antibody overnight at 4°C in a humid chamber. After washing three times with PBS, the sections were overlaid with peroxidase-conjugated anti-goat IgG antibody (Golden Bridge International, China), and the reaction was developed with 3, 3- diaminobenzidine (DAB), and counterstained with hematoxylin. The experiments were repeated three times.

Cell culture

Cervical cancer cell lines Hela and Siha were obtained from American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% antibiotic-antimycotic. These cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

FOXL2 overexpression or silence shRNA plasmids transfection

Hela and Siha cells were grown in culture medium with 10% FBS. When cells had reached confluency, Lipofectamine 2000 (Invitrogen; USA), OPTI-MEM (Gibco, USA) and plasmid pCDNA3.1-FOXL2 or H1-FOXL2 shRNA (short hairpin RNA) (GeneChem, Shanghai, China) were mixed and incubated for 20 min and added to Hela and Siha cells at room temperature respectively. After 6 h of incubation, these cells were then incubated in RPMI-1640 containing 10% FBS in 5% CO_2 at 37°C. After 48 h transfection, cells were subjected to western blotting analysis. Untransfected cells were thought to be blank control, and cells transfected with pcDNA3.1 (empty vector) and H1 (empty vector) were considered as negative control.

RT-PCR

The total RNA was extracted from Hela and Siha cells with Trizol reagent (Invitrogen, USA). The complementary DNA (cDNA) was generated with oligo (dT) 18 primers by using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas Life Science, USA). The 50 μl PCR amplification of the single strand cDNA was

performed by 28 cycles of 5 min precycle at 95°C, then denaturation (94°C) for 45 s, annealing (59°C) for 45 s, and elongation (72°C) for 45 s using 2.5 U Taq polymerase (Fermentas Life Science, USA). The primer sequences are: for *Foxl2* (97 bp), sense: 5'-GAG TTT TTG TTG GGC CTT CA-3'; antisense: 5'-GAG GGT GAA ACT TCC CCA AT-3'; for *GAPDH* (258 bp), sense: 5'-AGA AGG CTG GGG CTC ATT TG-3', antisense: 5'-AGG GGC CAT CCA CAG TCT TC-3'. The amplified DNA was fractionated by 2% agarose gel (Oxiod, UK) electrophoresis, and ethidium bromide-stained bands were photographed. The experiments were repeated three times.

Western blotting

The cells were harvested and lysed in RIPA buffer. Protein concentration was determined with BCA Protein Assay Kit (Beyotime, China). Samples of 30 μg total protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes using the Bio-Rad electrotransfer system. After blocked by 5% w/v nonfat dried milk for 1 h, the membranes were probed with primary antibodies to Foxl2 (1:400 dilution, Beijing Biosynthesis Biotechnology Co., Ltd., China). The specific reaction for horseradish peroxidase-conjugated secondary antibody was visualized using ECL western blotting detection reagent (Thermo Scientific Pierce) and luminescent images were captured by an LAS-3000 (Fujifilm, Tokyo, Japan). GAPDH (Golden Bridge International, China) was used as internal control for protein loading and analysis.

Cell proliferation assay

The ability of Hela and Siha cells was detected by Brdu cell proliferation assay (Millipore, USA) according to the manufacturer's instructions. Briefly, the control and transfected cells were seeded at a density of 1×10^4 cells/well in 96-well flat-bottom and cultured for 48 h. The proliferation assay was performed 12 h following the addition of BrdU reagent (10 ng/ml). The absorbance values measured at 450 nm wavelength represented the rate of DNA synthesis and corresponded to the number of proliferating cells. Each experiment was performed in triplicate and repeated five times.

Cell apoptosis assay

Phosphatidylserine externalization was quantified by flow cytometry with a commercially available annexin V-FITC apoptosis detection kit (Invitrogen, USA) according to the manufacturer's guideline. In brief, the control and transfected cells were seeded at a density of 2×10^5 cells/well in 24-well flat-bottom and cultured for 48 h. The cells were harvested and resuspended in 100 μl annexin-binding buffer with 5 μl FITC-annexin V and 1 μl PI working solution, and then were incubated in the dark for 15 min at room temperature. Next additional 400 μl binding buffer was added, and Hela and Siha cells were analyzed immediately by flow cytometry (BD Biosciences). Each experiment was carried out in triplicate, and repeated five times.

Cell adhesion assay

Cell adhesion assay was performed with the Cell Adhesion Assay Kit (Cell Biolabs; San Diego, CA, USA) according to the manufacturer's protocol. After being transfected with plasmid or shRNA for 48 h, the control or transfected cells (1×10^5/well) were seeded in the 48-well plates coated with fibronectin. Following incubation at 37°C for 60 min and the removal of unattached cells by PBS washings, cell stain solution and extraction solution were added to the wells. Finally, the extracted samples were transferred to a 96-well plate and detected the optical density at 560 nm in DigiScan Microplate Reader.

Matrigel invasion assay

The invasion of Hela and Siha cells were analyzed using the Matrigel invasion assay.

The cells inserts (8 μm pore size, 6.5 mm diameter, Corning, USA) coated with 25 μl matrigel were placed in a 24-well plate. The control and transfected cells at a density of 1×10^5 cells/well were plated in the upper chamber, while the lower chamber were added with RMPI-1640 containing 1% FBS. The cells attached to the upper surface of filter were removed by scrubbing with cotton swab, and cells on the underside of the membrane were fixed, stained with hematoxylin, and counted (five random fields) by two independent investigators. The results

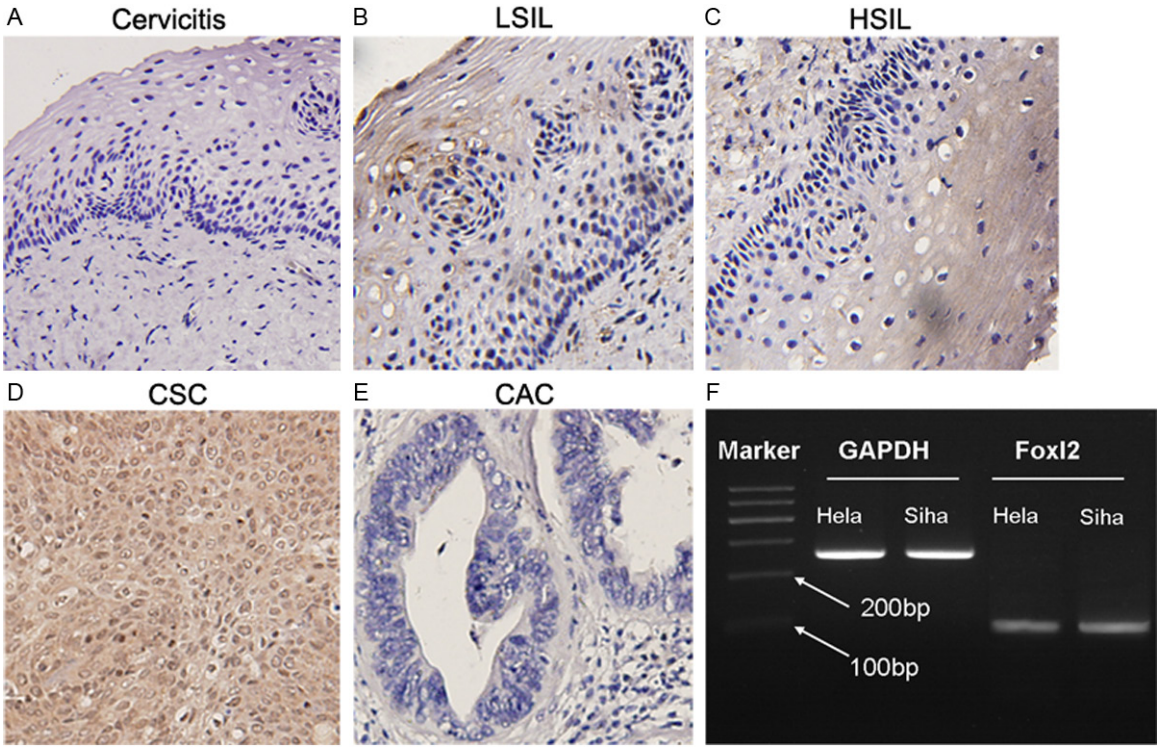

Figure 1. *FOXL2 is expressed in cervical squamous cancer and cervical cancer cells.* Representative photomicrographs for FOXL2 immunostaining in cervicitis (A), low-grade squamous intraepithelial lesion (LSIL) (B), high-grade squamous intraepithelial (HSIL) (C), cervical squamous cancer (CSC) (D) and cervical adenocarcinoma (CAC) (E), respectively. Slides are counterstained with hematoxylin. Original magnification: × 200. (F) Foxl2 mRNA was measured in cervical cancer cell lines Hela and Siha by RT-PCR.

were expressed as a percentage of the controls.

Flow cytometry

The control and transfected cells at a density of 1×10^5 cells/well were seeded in a 24-well plate, and flow cytometry was performed to analyze the expression of Ki67 (Biolegend, San Diego, USA), PCNA (BD, USA) and FasL (Biolegend, San Diego, USA) in Hela and Siha cells. Samples were analyzed in a FACS Calibur flow cytometer (FACSCalibur, BD). Statistical analysis was conducted by using isotype matched controls. The same sample was measured by flow cytometry three times, and the experiments were repeated at least 3 times.

Statistical analysis

All values are shown as the mean ± SD. Data were analyzed by t test or one-way ANOVA analysis with SPSS 15.0. Differences were considered as statistically significant at $P<0.05$.

Results

FOXL2 is expressed in cervical cancer tissues

We measured FOXL2 protein levels in 25 cervical carcinoma, 8 cervical intraepithelial neoplasia (CIN) and 10 cervicitis samples by immunohistochemistry. Representative sections of FOXL2 expression in all tissues were shown in **Figure 1**. FOXL2 was expressed at a high level in cervical squamous carcinoma (CSC) tissues, but not expressed in cervical adenocarcinoma (CAC), CIN and cervicitis tissues. Thereafter we investigated the mRNA level of Foxl2 in cervical cancer cell lines Hela and Siha (**Figure 1**), and found that Hela and Siha cells could transcribe Foxl2. These findings showed that FOXL2 was expressed highly in cervical squamous carcinoma and cervical cancer cells.

FOXL2 suppresses growth and promotes apoptosis of Hela and Siha cells

To investigate the effect of Foxl2 on cervical cancer cells, we overexpressed Foxl2 in Hela

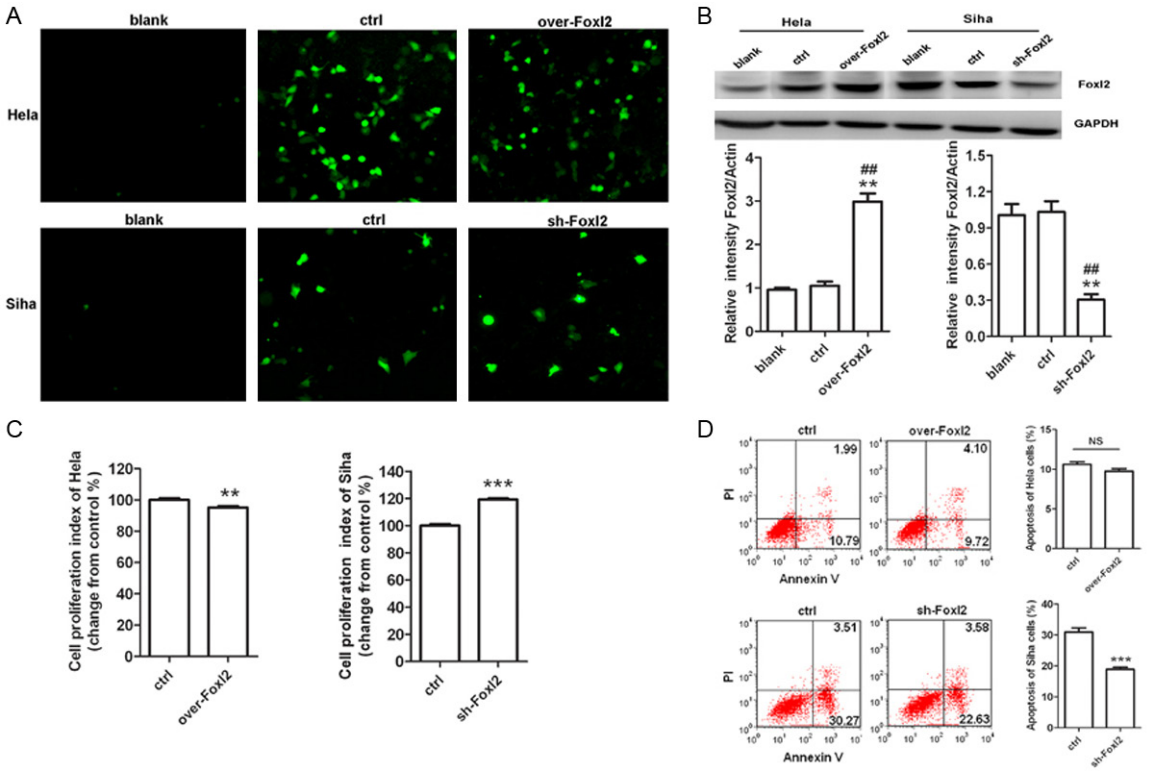

Figure 2. *FOXL2 suppresses proliferation and promotes apoptosis of cervical cancer cells.* Hela cells were transfected with pCDNA3.1-FOXL2, pcDNA3.1 empty vector (control) and no plasmid (blank), and Siha cells were transfected with H1-FOXL2 shRNA, H1 (control) and no shRNA (blank) for 48 h. A. The photographs of Hela and Siha cells transfected with plasmid or shRNA. Original magnification: × 200. B. The expression of FOXL2 after that Hela and Siha cells transfected with plasmid or shRNA for 48 h was measured by western blotting. C. The effects of overexpressing FOXL2 or silencing FOXL2 on the proliferation of Hela and Siha cells were detected by Brdu assay. D. The influences of overexpressing FOXL2 or silencing FOXL2 on the apoptosis of Hela and Siha cells were detected by flow cytometry. $^{**}P<0.01$, $^{***}P<0.001$ compared to the control group; $^{\#\#}P<0.01$ compared to the blank group.

cells by plasmid transfection, and silenced its expression in Siha cells by shRNA transfection ($P<0.01$) (**Figure 2A**, **2B**). The results showed that overexpressing FOXL2 in Hela cells inhibited cell growth levels ($P<0.01$) (**Figure 2C**). Meanwhile, compared to the control, silencing Foxl2 in Siha cells enhanced the proliferation ratio and restrained its apoptosis ($P<0.001$) (**Figure 2C**, **2D**). Therefore, these results indicated that Foxl2 suppressed the growth of cervical cancer cells.

FOXL2 restrains adhesion of Hela and Siha cells

Based on the above findings, experiments were then designed to explore whether FOXL2 played a role in regulating adhesion of Hela and Siha cells. In the control group, Siha cells were found to have a significantly lower adhesion ability to attach to fibronectin than FOXL2-silenced Siha cells ($P<0.05$) (**Figure 3A**). However, there was no significant difference between the control group and FOXL2-overexpressed Hela cells. Thus we believe that Foxl2 controls the adhesion of cervical cancer cells.

FOXL2 suppresses invasion of cervical caner cells

To further explore the influence of Foxl2 on cervical cancer cells, we also overexpressed Foxl2 in Hela cells and silenced its expression in Siha cells, and then a matrigel-based transwell assay was carried out. The overexpressing FOXL2 Hela cells and silencing FOXL2 Siha cells were added to the upper chamber, and the number of cells migrating to the lower surface was counted in 48 h of incubation. The invasion index of each group was calculated as the ratio of the number of cells migrating to the lower surfaces to the control group. As shown in

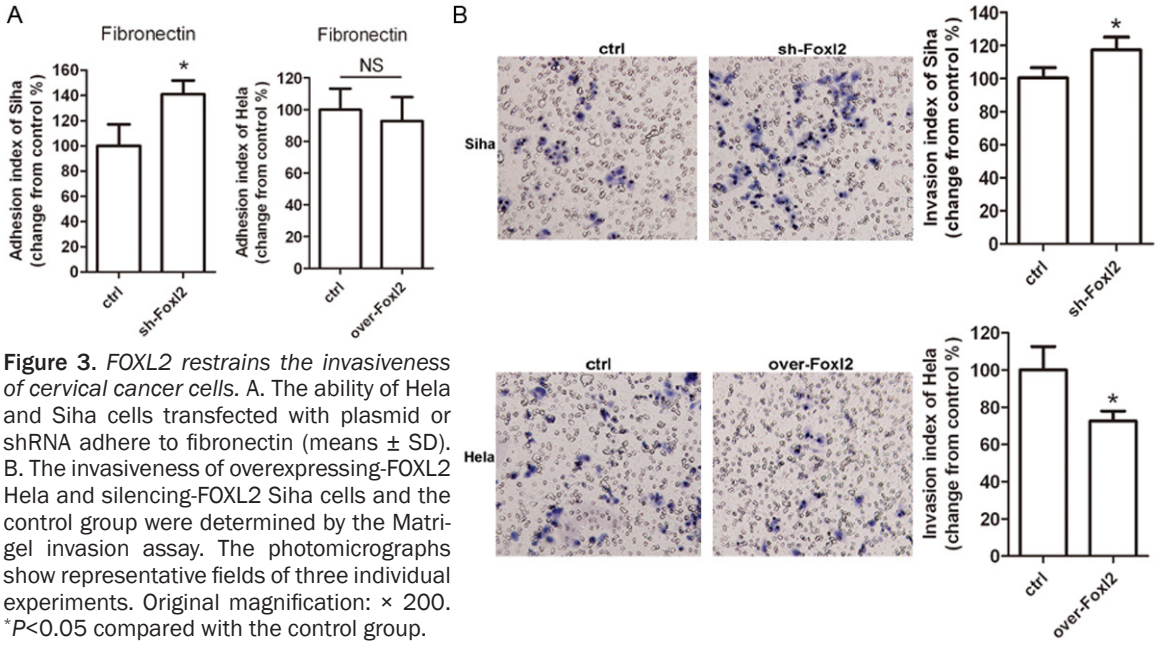

Figure 3. *FOXL2 restrains the invasiveness of cervical cancer cells.* A. The ability of Hela and Siha cells transfected with plasmid or shRNA adhere to fibronectin (means ± SD). B. The invasiveness of overexpressing-FOXL2 Hela and silencing-FOXL2 Siha cells and the control group were determined by the Matrigel invasion assay. The photomicrographs show representative fields of three individual experiments. Original magnification: × 200. *P<0.05 compared with the control group.

Figure 3B, overexpressing FOXL2 restrained the invasiveness of Hela cells (P<0.05). Meanwhile, silencing FOXL2 could significantly enhance the invasiveness of Siha cells compared with the control group (P<0.05).

FOXL2 down-regulates Ki67 and up-regulates FasL expression in Hela and Siha cells

To determine whether Foxl2 regulates the proliferation and apoptosis-related molecules, we treated Hela and Siha cells with plasmids transfection, and then analyzed the expression of Ki67, PCNA, and FasL in them. It was shown in **Figure 4** that overexpressing Foxl2 diminished Ki67 expression (P<0.001) (**Figure 4A**). In contrast, silencing Foxl2 markedly promoted Ki67 expression and inhibited FasL expression (P<0.001, P<0.01) (**Figure 4A**, **4C**). However, these treatments had no effect on the PCNA translation (P>0.05) (**Figure 4B**). The results above indicate that Foxl2 restrains Ki67 expression and promotes the level of FasL, which may further modulate the downstream signaling molecules and control the proliferation and apoptosis of cervical caner cells.

Discussion

Cervical cancer is ranked as the second leading cause of female cancer mortality worldwide, with an annual incidence of approximately 200,000 deaths and more than 500,000 new cases diagnosed [20-22]. The incidence of cervical cancer is high in developing countries [23].

The present work has first found that cervical cancer cells express FOXL2. As we have known, FOXL2 has been identified as one of the earliest markers of ovarian differentiation, and its expression persists into adulthood. FOXL2 is required for the normal development of granulosa cells [5, 8] and shows strong expression in granulosa cells and moderate expression in stromal cells; no expression has been detected in oocytes [5, 24]. In addition, FOXL2 gene mutations of different origin result in premature ovarian failure (POF) and/or craniofacial malformation in BPES patients [1, 5]. Moreover, Shah SP et al [11] found that a somatic FOXL2 missense mutation 402C>G (p.C134W) was present in four OGCTs. Recently, Benayoun et al [25] suggested that this mutation might interfere with the capacity of FOXL2 to modulate the cell cycle. And the previous studies have illustrated that Foxl2 is able to form functional complexes with the TGF-β effector transcription factor Smad3 [26, 27]. Interestingly, Smad3 has been shown to be a crucial mediator of the TGF-β cytostatic program [28]. Indeed, an increase of Smad3 expression alone can induce accumulation of cells in G0/G1 arrest through upregulation of the cell cycle progression inhibitors p15/INK4b, p16/INK4a, p19/ARF and p21/CIP1 [29].

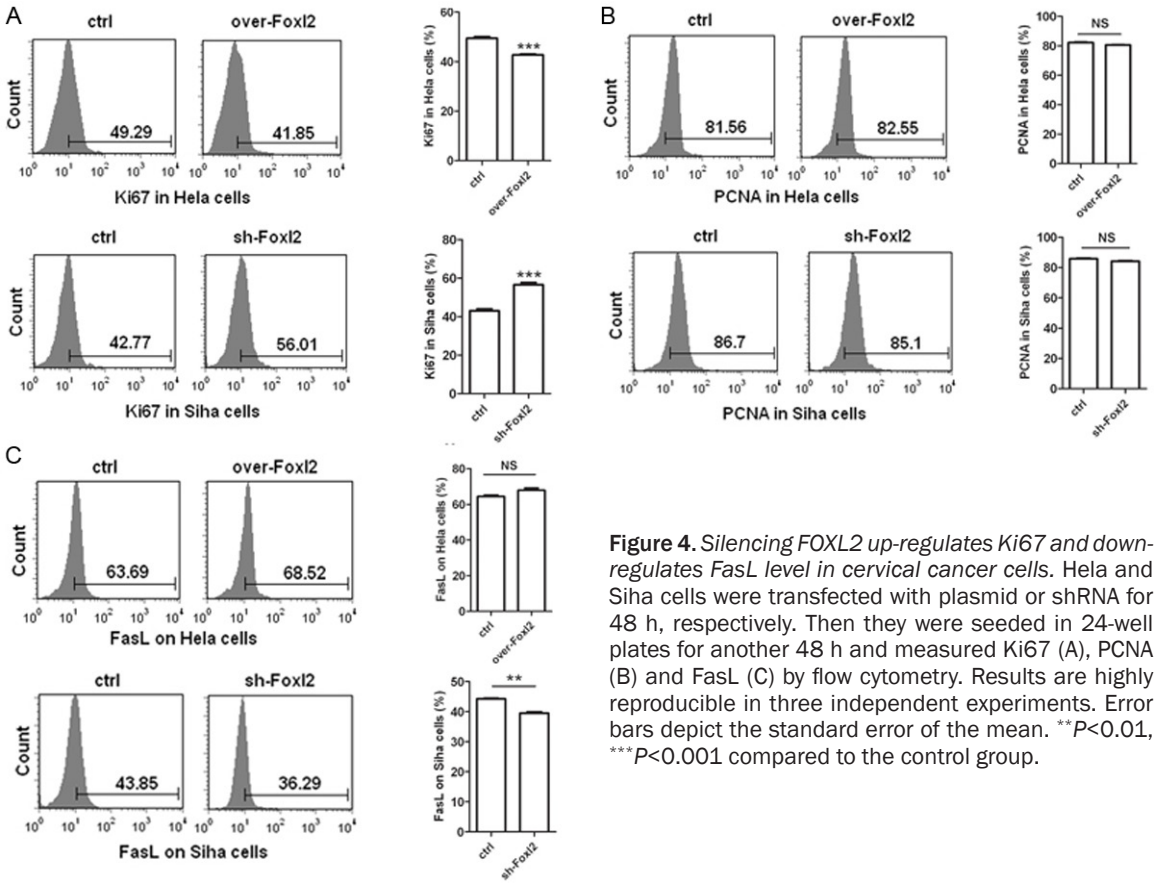

Figure 4. *Silencing FOXL2 up-regulates Ki67 and down-regulates FasL level in cervical cancer cells.* Hela and Siha cells were transfected with plasmid or shRNA for 48 h, respectively. Then they were seeded in 24-well plates for another 48 h and measured Ki67 (A), PCNA (B) and FasL (C) by flow cytometry. Results are highly reproducible in three independent experiments. Error bars depict the standard error of the mean. $^{**}P<0.01$, $^{***}P<0.001$ compared to the control group.

The Ki-67 protein (also known as MKI67) is a cellular marker for proliferation [30]. Ki67 is expressed during late G1, S, G2 and M phases of the cell cycle. Ki67 shows a good correlation with the number of mitotic cells [31]. The fraction of Ki-67-positive tumor cells is often correlated with the clinical course of cancer. Moreover, the proliferating cell nuclear antigen (PCNA) is a nuclear polypeptide essential for DNA synthesis and replication of mammalian cells. Its expression reaches a maximum point in the S-phase of the cell cycle. And it is commonly used as a proliferation marker [31]. In addition, Apoptosis is an important physiological process and the dysfunction of apoptosis may result in the development of common cancers [32, 33]. Fas ligand (FasL or CD95L) is a type II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. FasL mainly cooperates with its receptor Fas to trigger programmed cell death, which plays an important role in the regulation of immune system and the progression of cancer [34]. Thus we investigated the effects of FOXL2 on these molecules such as Ki67, PCNA and FasL, and the results showed that silencing FOXL2 up-regulated Ki67 expression and down-regulated the FasL level in cervical cancer cells (**Figure 4**). Accordingly, the results indicated that FOXL2 suppressed cervical cancer growth and promoted its apoptosis.

Cell adhesion is a complex mechanism involved in a variety of processes including cell migration or invasion, embryogenesis, wound healing and tissue remodeling. Cell adhesion molecules (CAMs) are proteins located on the cell surface which are involved in binding with other cells or with the extracellular matrix (ECM). In essence, cell adhesion molecules help cells stick to each other and to their surroundings. The CAMs include members of the immunoglobulin, calcium-dependent cadherin families and integrins [35]. For instance, these cell adhesion molecules VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1) and ELAM-1 (endothelial leukocyte adhesion molecule-1) have been

studied in several tumors [36]. Furthermore, recent studies have demonstrated that matrix metalloproteases (MMPs) and their tissue inhibitors tissue inhibitors of metalloproteinases (TIMPS) play a vital role in the degradation of connective tissue which is associated with the development of tumor metastasis [37]. In particular, MMP-2 and MMP-9 are involved in the systemic dissemination of tumors. The importance of MMPs during tumor metastasis may be related to their proteolytic activity against type IV collagen, which is a major component of epithelial basement membranes [37]. In addition, Benayoun BA et al found [38] that FOXL2 inhibited the expression of MMP23 which regulated the cells adhesion. Moreover, Kalfa et al [10] have reported a decreased expression of FOXL2 in juvenile ovarian granulosa cell tumors with an aggressive pattern of progression. Consistent with the literature, we found that silencing FOXL2 could obviously accelerate the invasiveness of cervical cancer cells. However, the specific mechanism remains unclear. Therefore, Foxl2 may regulate the adhesion and metastasis of cervical cancer cells via these above-mentioned adhesion and metastasis related molecules, and it needs further investigation.

In summary, our study demonstrated that FOXL2 was expressed in cervical squamous cancer. On the one hand, FOXL2 suppressed the proliferation and promoted apoptosis of cervical cancer cells mainly through decreasing Ki67 expression and increasing FasL expression; on the other hand, FOXL2 restrained the invasiveness of cervical cancer cells. Hence, FOXL2 might be a novel tumor suppressor, at least in cervical cancer. It may be valuable for estimating the metastasis and recurrence of cervical cancer, and it needs further investigation.

Acknowledgements

This work was partly supported by National Natural Science Foundation of China No. 81272122 (to S-J Tang); Science and Technology Projects of Shandong Province No. jk67 (to S-J Tang); Science and Technology Plan Projects of Colleges and Universities in Shandong Province No. J09LF20 (To B-B Yang); Science and Technology Innovation Fund of Weifang Medical University No. WY20110 (To B-B Yang).

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