Original Article
FOXC1 promotes proliferation and epithelial-mesenchymal transition in cervical carcinoma through the PI3K-AKT signal pathway

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Abstract: Recently, Forkhead box C1 (FOXC1) has been identified to play important roles in human cancers. However, the clinical significance and biological role of FOXC1 in cervical cancer remains unclear. Here, we showed that FOXC1 was frequently overexpressed in cervical cancer versus adjacent non-tumor tissues. Overexpression of FOXC1 was significantly correlated with tumor stage (P=0.011), tumor size (P=0.034), stromal invasion (P=0.001), and lymph nodes metastasis (P=0.008). Survival analysis further suggested that high FOXC1 expression was significantly correlated with poor overall survival (P=0.007) and recurrence-free survival (P=0.003) in cervical cancer patients. Moreover, we found that knock-down of FOXC1 by short hairpin RNAi significantly suppressed cervical cancer cells proliferation, migration, and invasion in vitro. Mechanistic studies showed that the FOXC1 requires PI3K/AKT signaling for its regulation of cell proliferation, migration and invasion. Our findings indicate that FOXC1 plays an important oncogenic role in cervical cancer progression.

Keywords: FOXC1, cervical cancer, proliferation, EMT, progression, prognosis

Introduction

Cervical cancer has been ranked as the third leading cause of cancer deaths among gynecological malignancies in the world, with about 529,00 new cases and 275,000 deaths every year [1]. Despite the improvement in diagnostic and therapeutic strategies, the 5-year survival rate for patients with advanced stage cervical cancer remains poor [2]. Hence, comprehensive understanding of the molecular mechanisms underlying cervical cancer progression may allow the identification of effective treatment modalities for cervical cancer patients.

Forkhead box C1 (FOXC1), a member of the forkhead transcription factor family, is characterized by a distinct DNA-binding fork head domain [3]. The FOXC1 gene is located in chromosome 6p25 and encodes transcriptional factor to regulate a wide array of biological processes including cell differentiation, ocular growth, and embryonic development. It has been identified as a specific regulator in the maintenance of the mesenchymal niches for haematopoietic stem and progenitor cells [4]. Similarly, it maintains the hair follicle stem cell niche and governs stem cell quiescence to preserve long-term tissue-regenerating potential [5]. Mutation in FOXC1 results in anterior segment dysgenesis disorders and miliaria sweat retention disorder [6, 7]. Recent studies have shown that FOXC1 was involved in the development of several human cancers. For example, high expression level of FOXC1 was detected in nasopharyngeal carcinoma, and FOXC1 has a pivotal role in EMT through the upregulation of Vimentin, fibronectin and N-cadherin expression [8]. Significantly up-regulated expression of FOXC1, which is a critical mediator of EGFR function in basal-like breast cancer (BLBC), has been identified to control cancer stem cell properties enriched BLBC cells via activation of Smoothened-independent Hedgehog signaling pathway [9]. In hepatocellular carcinoma, FOXC1 contributes to microvascular invasion via regulating the epithelial-mesenchymal transition (EMT) [10]. These investigations suggest that FOXC1 has a close relationship with several types of cancer. However, the biological func-

tions of FOXC1 in cervical cancer has not been investigated.

In this study, we examined the role of FOXC1 in human cervical cancer. We demonstrated that FOXC1, whose high expression correlates with tumor progression and poor prognosis, promoted cervical cancer cell proliferation and EMT through activating PI3K/AKT signaling pathway. Our study implied that inactivation of FOXC1 might be helpful for clinic treatments of cervical cancer.

Materials and methods

Patients and clinical samples

This study was conducted on a total of 219 paraffin-embedded cervical cancer samples, which were histopathologically and clinically diagnosed at Guangzhou Women and Children's Medical Center of Guangzhou Medical University between January 2004 and December 2010. None of these patients had received chemo-radiotherapy before surgery. In addition, 56 pairs of snap-frozen cervical cancer and normal cervical samples were collected for Real-time PCR. The tissue specimens were immediately frozen in liquid nitrogen after surgical removal and stored at -80 degree until analysis. The present study was approved by the Institutional Review Board of Guangzhou Women and Children's Medical Center. Written informed consent was obtained from all the members who participated in this study.

Cell lines

Primary normal cervical epithelial cells (NCEC), obtained from fresh specimens of patients without cervical lesion, were cultured in Ham's F-12 (Gibco, NY) supplemented with 20% fetal calf serum (Gibco, NY) and antibiotics (120 mg/ml streptomycin and 120 mg/ml penicillin). Cervical cancer cell lines (HeLa, ME-180, SiHa, C33A, CaSKi, and MS751) were grown in the RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and and 1% penicillin/streptomycin (Invitrogen).

Quantitative real-time reverse transcriptase-PCR

Total RNA was extracted from cultured cells and frozen cervical cancer tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions as previously described [11]. All of the manipulations of the RNA were carried out under RNase-free conditions. The concentration and purity of RNA were measured spectrophotometrically at 260 and 280 nm. cDNA were generated from 1 ul total RNA using One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's instructions. Then, quantitative real-time PCR was performed using SYBR RT-PCR kit (Takara) on an ABI 7500 Real-Time PCR System (Applied Biosystems, USA) with the following primers: FOXC1, forward, 5'-CATCCGCCACAACCTCTCG-CT-3', reverse, 5'-GTGCAGCCTGTC CTTCTCCT-CC-3'. GAPDH, forward: 5'-AGCCACATCGCTCA-GACAC-3' and reverse: 5'-GAATTTGCCATGGG-TGGA-3'. GAPDH was used as an internal control.

Western blot

Total protein from cervical cancer cells and tumor specimens were extracted and separated in 10% SDS-PAGE, and then electro-transferred onto PVDF membranes. After blocked in Blocking Buffer (Beyotime, China) for 1 hour, the membranes were incubated with primary antibody against FOXC1, CyclinD1, E-cadherin, N-cadherin, Vimentin (1:1000; Abcam, USA), p-PI3K, PI3K, p-AKT, AKT, p-GSK-3β, GSK-3β, Zeb1, Snail, Twist (1:1000; Cell signaling, Boston, MA, USA), and GAPDH (1:2000; Santa Cruz, USA), and secondary antibody according to protocols of the manufacturer. Finally, membrane was detected with an enhanced chemiluminescence kit.

Immunohistochemistry

Briefly, 4-μm-thick paraffin sections were baked for 30 min at 65°C, deparaffinized in xylene, rehydrated in gradually varied alcohol, and then the sections were managed with 1% H_2O_2 to neutralize endogenous peroxidase for 30 min. The antigen retrieval was processed with 10 mmol/L citrate buffer (pH=6.0) in a microwave oven. After inhibition of endogenous peroxidase activities by 3% hydrogen peroxide in methanol, the sections were incubated with primary antibody FOXC1 (1:200; Abcam, USA), and secondary antibody. The sections were then stained with DAB (3,3-diaminobenzidine) and terminated in PBS, and then counter-
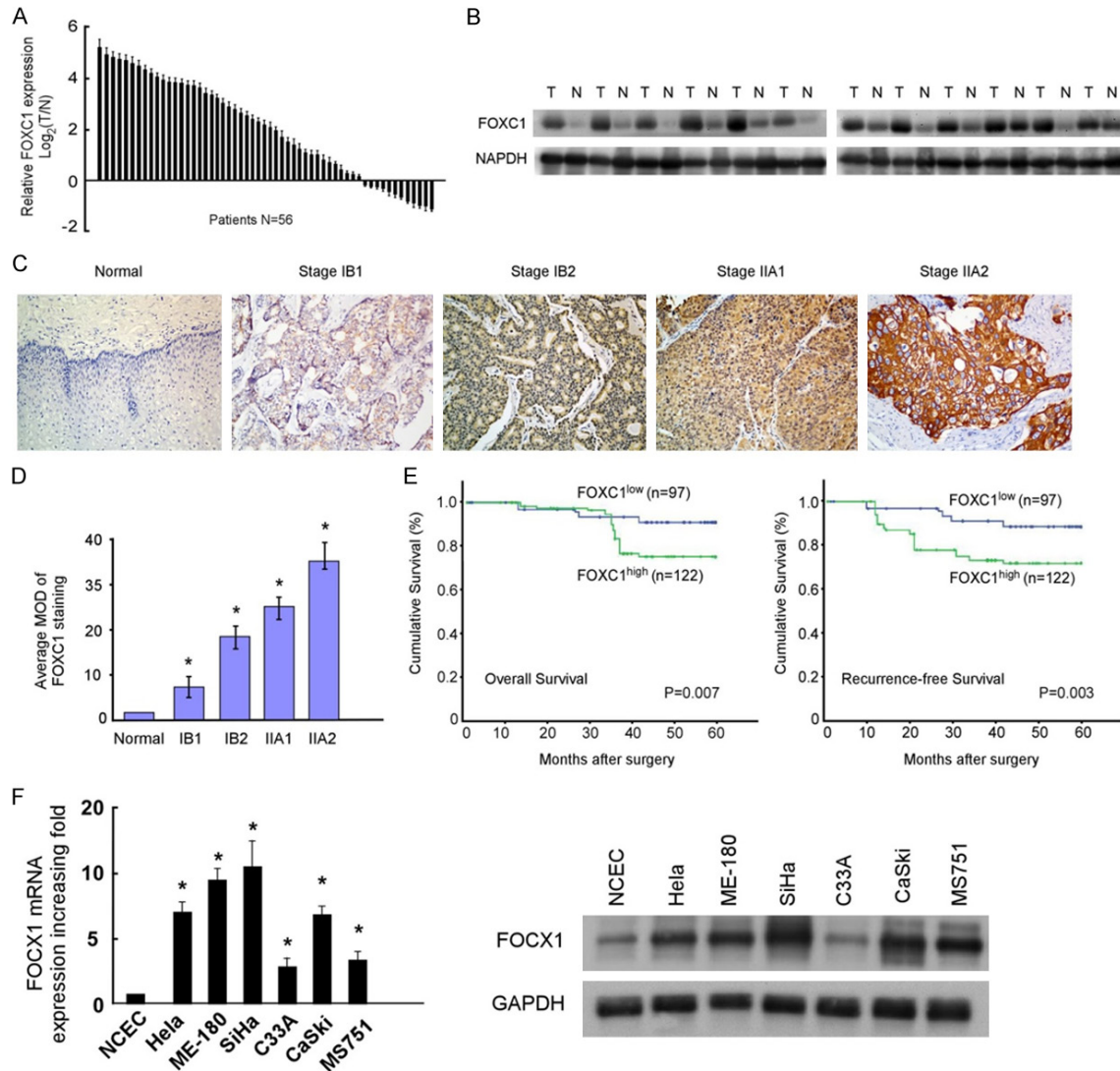
Figure 1. Expression pattern and clinical implications of FOXC1 in cervical cancer. A. FOXC1 mRNA expression in 56 pairs of cervical cancer and adjacent non-tumor tissues. B. The protein expression of FOXC1 in 12 pairs of randomly selected tumor (T) and adjacent non-tumor tissues (N). C. Representative images of IHC analysis of FOXC1 in cervical cancer specimens (clinical stage IB1-IIA2) compared with normal esophageal tissue (400 ×). D. Statistical analysis of the mean optical density (MOD) of FOXC1 staining in normal cervical tissue and cervical cancer specimens at different clinical stages. E. Kaplan-Meier curves for overall survival (OS) and recurrence-free survival (RFS) based on FOXC1 expression in 219 patients with cervical cancer. F. FOXC1 mRNA and protein expression in normal cervical epithelial cells (NCECs) and cervical cancer cell lines (HeLa, ME-180, SiHa, C33A, CaSKi, and MS751).

stained with hematoxylin. Based on the staining intensity of FOXC1 in each case, the standard for score was as follows: 0, negative; 1, weak; 2, moderate; 3, strong. Tumor cell proportions were scored as follows: 0 (no positive tumor cells); 1 (<10% positive tumor cells); 2 (10-35% positive tumor cells); 3 (35-75% positive tumor cells) and 4 (>75% positive tumor cells). Two observers graded the score of staining intensity independently. The staining index was calculated as the product of the staining intensity score and the proportion of positive tumor cells. Using this method of assessment, the expression of FOXC1 was evaluated by the staining index (scored as 0, 1, 2, 3, 4, 6, 8, 9 or 12). Staining index score ≥6 was identified as high expression, while score <6 was low expression.

MTT assay

Cell viability was measured using MTT assay. Cells (2×10^3/well) were seeded in triplicate wells of 96-well plates. At each time point, cells

Table 1. Relationship between FOXC1 expression and clinicopathological parameters of cervical cancer patients

Clinicopathological parameters	Group	Patient number	FOXC1		P value
			Low	High	
Age (years)	≤50	148	67	81	0.674
	>50	71	30	41	
SCC level	≤1.5 ng/ml	45	21	24	0.719
	>1.5 ng/ml	174	76	98	
Tumor stage	≤IB	167	82	85	0.011
	>IB	52	15	37	
Tumor size	≤4 cm	156	77	80	0.034
	>4 cm	63	21	42	
Differentiation	1/2	94	43	51	0.707
	3	125	54	71	
Histological type	SCC	191	78	103	0.436
Stromal invasion	AC	38	19	19	
	≤1/2	115	64	53	0.001
	>1/2	104	33	71	
LN Metastasis	No	174	85	89	0.008
	Yes	45	12	33	

SCC: squamous cell cancer; AC: Adenocarcinoma; LN: lymph nodes.

were stained with 100 μl sterile MTT dye (0.5 mg/ml, Sigma) for 4 h at 37°C, followed by removal of the culture medium and addition of 150 μl of DMSO (Sigma). The absorbance was measured at 570 nm, with 655 nm as the reference wavelength. All experiments were performed in triplicates.

Colony formation assay

Cells (1 × 10³/well) were plated on a 6-well plate and observed two weeks according to the size of cell colony. The colonies were fixed with 4% paraformaldehyde for 30 min and stained with 1.0% crystal violet for 15 min. All experiments were performed in triplicate.

Bromodeoxyuridine (BrdUrd) labeling

Cells were plated on coverslips (Fisher, Pittsburgh, PA) and allowed to settle for 24 hours, incubated with bromodeoxyuridine (BrdUrd) for 1 h and stained with anti-BrdUrd antibody (Sigma), according to the manufacturer's instruction. Gray level images were acquired under a laser scanning microscope (Nikon Co., Tokyo, Japan).

Cell invasion and migration assay

Cell invasion and migration ability was detected with a transwell system based on a transwell 24-well chambers (Millipore). For migration assay, 1 × 10⁴ cells in 200 ul serum-free DMEM were seeded in the upper chamber of a transwell, and the bottom of the chamber was filled with 500 μl of DMEM containing 30% FBS. For invasion assay, 50 μl BD Matrigel (diluted 1:5 with RPMI Medium 1640) was added to the transwell chambers previously and incubated at 37°C for 3 h. Cells on the upper side of the filter were removed after 24 h. The filter membrane was stained with crystal violet, and the number of the cells that remained adherent to the underside of the membrane were counted using an inverted microscope (Olympus Corp).

Small RNA transfection

For siRNA transfection, double-stranded, siRNAs targeting FOXC1 were designed and synthesized by Santa Cruz Biotechnology. In vitro transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After incubation for 48 h, cells were subjected to proliferation, migration and invasion assay.

Statistical analysis

All statistical analyses were conducted using SPSS 18.0 software. Each experiment was repeated three times, unless otherwise indicated. Student's t-test was used to calculate differences between the various study groups. Pearson Chi-square test was performed to evaluate the relationship between FOXC1 expression with clinicopathologic parameters. Survival analyses were performed using the Kaplan-Meier plots, and differences between curves were compared by the log-rank test. A two-tailed P<0.05 was considered significant.

Results

Upregulation of FOXC1 in human cervical cancer

To investigate the potential role of FOXC1 in cervical cancer, mRNA expression was evaluated in 56 paired cervical cancer and adjacent non-tumor tissues. The result showed that upregulation of FOXC1 was detected in 78.6% (44/56) of the patients. In addition, in randomly selected 12 pairs of cancer and adjacent non-

Table 2. Multivariate analyses of various prognostic variables in cervical cancer patients

Prognostic variables	Overall Survival		Recurrence-free Survival	
	Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Age (>50 y vs ≤50 y)	2.091 (0.485-4.976)	0.246	2.072 (0.404-5.080)	0.307
SCC level (>1.5 ng/ml vs ≤1.5 ng/ml)	1.540 (0.115-2.217)	0.409	1.242 (0.562-2.946)	0.356
Tumorstage (>IB vs IB)	2.837 (0.303-4.319)	0.024	2.346 (0.715-6.130)	0.077
Tumor size (>4 cm vs ≤4 cm)	2.185 (0.749-4.679)	0.270	2.110 (0.158-4.605)	0.156
Differentiation (Grade 3 vs 1/2)	1.306 (0.899-2.480)	0.436	1.315 (0.763-3.085)	0.456
Histological type (SCC vs AC)	2.796 (1.091-5.016)	0.183	2.101 (0.956-5.276)	0.145
LN Metastasis (+ vs -)	3.160 (0.641-8.625)	0.006	3.990 (1.061-8.202)	0.019
Stromal invasion (>1/2 vs ≤1/2)	2.192 (0.094-5.538)	0.043	2.056 (0.399-5.709)	0.067
FOXC1 expression (high vs low)	2.928 (0.508-6.585)	0.021	2.776 (0.207-7.538)	0.035

tumor tissues, western blot analysis showed obviously higher FOXC1 expression in cervical cancer than that in normal human cervical tissues (**Figure 1A** and **1B**). Moreover, both real-time RT-PCR and western blot analyses showed that FOXC1 was markedly up-regulated to various levels in 6 cervical cancer cell lines compared with NCEC (**Figure 1F**). The IHC assay showed that FOXC1 was localized both in the nucleus and cytoplasm.

Relationship between FOXC1 expression and clinicopathological characteristics and prognosis of cervical cancer patients

To explore the relationship between FOXC1 expression and cervical cancer progression, 219 paraffin-embedded archived clinical cervical cancer specimens were examined by IHC assay (**Figure 1C**). The correlation between FOXC1 expression and the clinicopathological characteristics of cervical cancer was explored. The results showed a positive correlation between FOXC1 expression and tumor stage (P=0.011), tumor size (P=0.034), stromal invasion (P=0.001), and lymph nodes metastasis (P=0.008) (**Table 1**). However, there were no significant correlations between FOXC1 expression and other clinicopathologic features, such as age, SCC level, histological type, or grade. Furthermore, the correlation between the tumor stage and FOXC1 expression was analyzed based on the computer-generated MOD. The results showed that FOXC1 expression in cervical cancer increased with advanced clinical stage (**Figure 1D**). Moreover, high expression of FOXC1 was significantly associated with decreased survival and increased risk of recurrence in cervical cancer patients (**Figure 1E**).

Patients in high FOXC1 expression group had obviously worse overall survival (OS) and shorter time to recurrence (RFS) than those in low expression group. MultivariateCox regression analysis revealed that FOXC1 expression remained to be an independent prognostic factor for both OS and RFS (P=0.021 and P=0.035, respectively) (**Table 2**). Taken together, these results strongly indicate that increased FOXC1 expression is correlated to cervical cancer progression.

FOXC1 promotes cervical cancer cells proliferation and migration in vitro

To investigate the role of FOXC1 in cervical cancer cells, we first knocked down the expression of FOXC1 using its specific siRNA(s) in Hela and C33A cell lines. We also confirmed effective knockdown activities in both cells (**Figure 2A**). Compared with control cells, MTT and Brdu assays found that the decreased expression of FOXC1 effectively suppressed proliferation of cancer cells (**Figure 2B** and **2C**). Using transwell assay, we found that FOXC1 silencing significantly decreased cell migration and invasion in both Hela and C33A cells (**Figure 3**) (P<0.005). These results consisted with the analysis results above, indicating that FOXC1 was able to promote the proliferation and migration of cervical cancer.

FOXC1 regulates cervical cancer cell proliferation and EMT via PI3K/AKT signaling pathway

To investigate the underlying mechanism of FOXC1-associated promotion of cervical cancer progression, we tested the effect of FOXC1 knockdown on the expression of EMT related genes and PI3K/AKT signaling molecules.
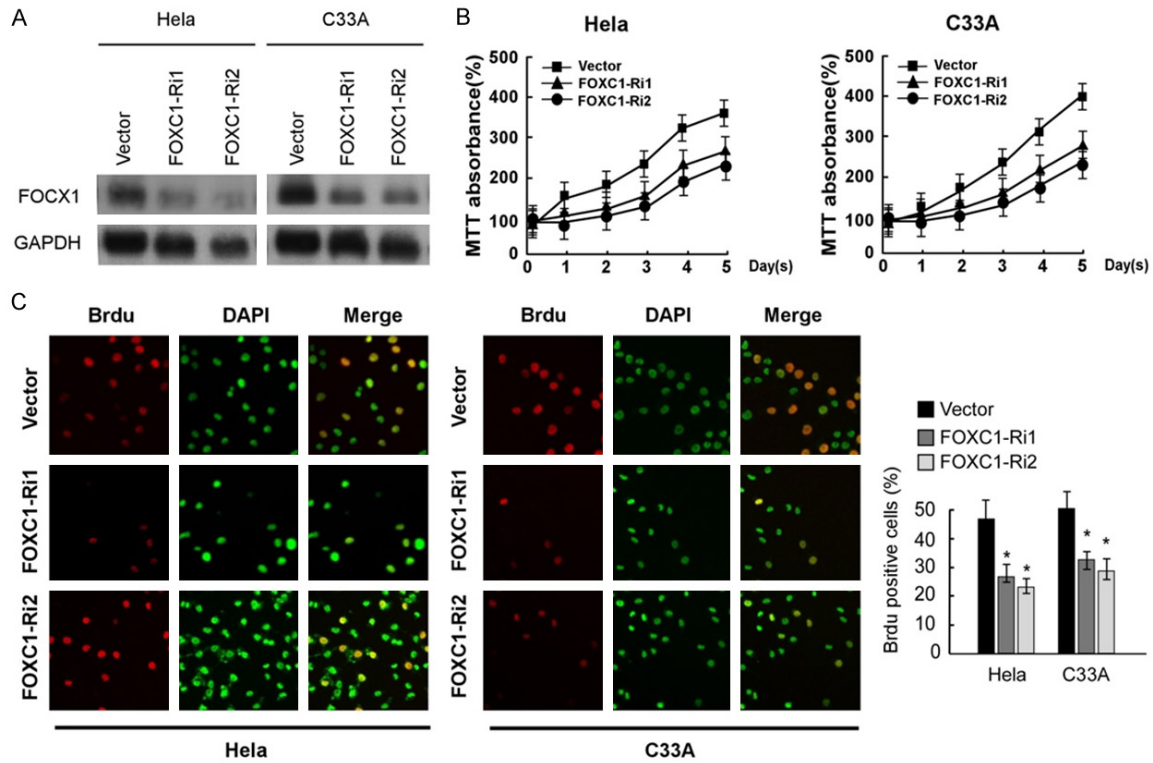
Figure 2. Knock down of FOXC1 inhibits cervical cancer cell proliferation in vitro. A. Western blotting analysis of FOXC1 expression in cervical cancer cell lines stably silencing FOXC1. B. Inhibition of FOXC1 reduces growth rate of cervical cancer cells, determined by MTT assay. C. Representative micrographs (left) and quantification (right) of the BrdUrd incorporation assay in Hela and C33A cell lines. Bars represent the mean ± SD of three independent experiments. *P<0.05.
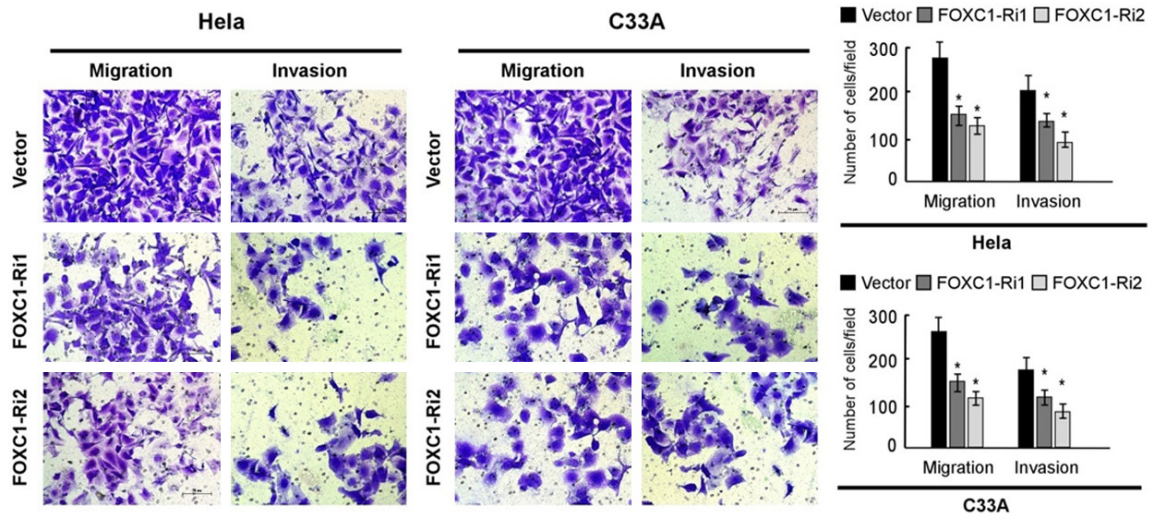

Figure 3. Downregulation of FOXC1 suppresses cell migration and invasion of cervical cancer cells in vitro. Representative micrographs (left) and quantification (right) of transwell assay in Hela and C33A cell lines. Bars represent the mean ± SD of three independent experiments. *P<0.05.

Consistently, downregulation of FOXC1 in Hela and C33A cells caused a significant decrease in AKT and GSK3β phosphorylation, and cell-cycle regulator CyclinD1 levels, as compared
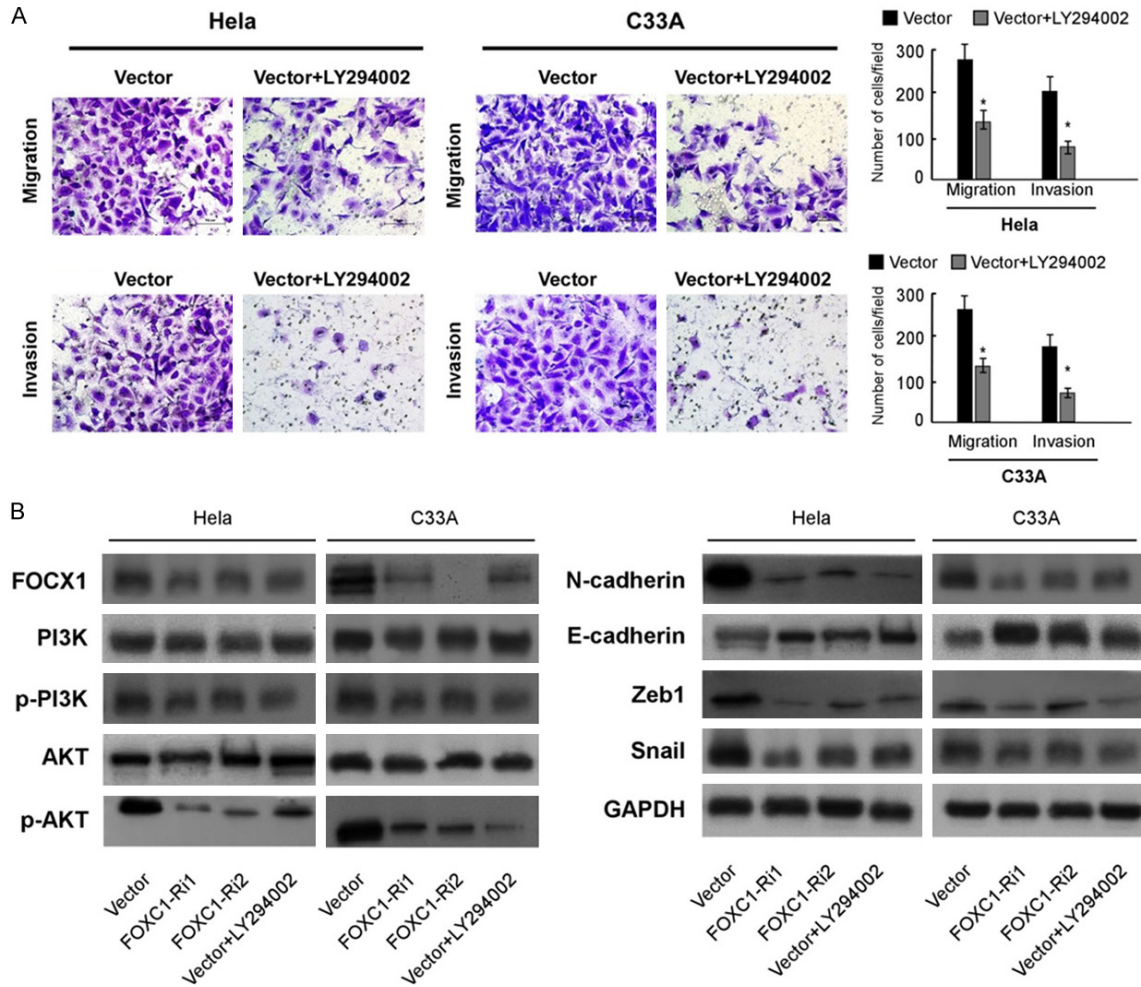
Figure 5. FOXC1 regulates EMT process through PI3K/AKT signaling pathway. A. Cervical cancer cells were treated with or without the PI3K inhibitor LY294002, and applied to migration and invasion assays. Representative micrographs (left) and quantification (right) were shown in each group. Bars represent the mean ± SD of three independent experiments. *$P<0.05$. B. Protein levels of PI3K, p-PI3K, AKT, p-AKT, epithelial and mesenchymal makers, and transcription factors were compared in indicated cells. GAPDH was used as loading control.

expression of FOXC1 via the phosphoinositide 3-kinase signaling to AKT and hypoxia-inducible factor 1α in hepatocellular carcinoma cells [15]. Xu et al. unraveled that microRNA-495 downregulates FOXC1 expression to suppress cell growth and migration in endometrial cancer [16]. Wang et al. reported that miR-133 inhibits pituitary tumor cell migration and invasion via down-regulating FOXC1 expression [17]. We present evidence that knockdown of FOXC1 reduced tumorigenesis and tumor progression of cervical cancer cells. Together, these studies indicate that FOXC1 could be a potential therapeutic target for treatment of cancers.

An oncogenic role for FOXC1 has been previously reported in BLBC, hepatocellular carcinoma, non-small cell lung cancer, pancreatic ductal adenocarcinoma, gastric cancer, and melanoma [18]; however, contrary tumor suppressive roles of FOXC1 has been reported in breast cancer cell lines by inhibiting migration and invasion of cancer cells in vitro and reduced the pulmonary metastasis in vivo [19]. Our study showed that FOXC1 activated PI3K/AKT pathway and exerted function via the pathway in cervical cancer. Our findings highlighted the FOXC1/PI3K/AKT axis in cervical cancer and may partially explain the different roles for FOXC1 among different cancer types.

Interestingly, FOXC1 expression was shown to be associated with progress and prognosis of human cancers. Xu et al. demonstrated that overexpression of FOXC1 correlates with progression and poor prognosis of gastric cancer [20]. Wei et al. revealed that high-level FOXC1 expression was correlated with poor tumor differentiation, tumor-node-metastasis stage, and lymph node metastasis in patients with non-small cell lung cancer [21]. Wang et al. reported that FOXC1 regulates the functions in human BLBC cells by modulating NF-κB signaling [22]. In the present study, we found that FOXC1 expression was significantly correlated with tumor stage, tumor invasion, and lymph node status, and the prognosis of cervical cancer patients, further suggesting that FOXC1 expression was well correlated with cancer progression.

In conclusion, we elucidated the linkage of FOXC1 with cervical cancer progression. FOXC1 plays an oncogenic role in cervical cancer development and progression by directly targeting and activating the PI3K/AKT pathway to promote cancer cell viability, proliferation, and EMT. In addition, FOXC1 may function as an independent prognostic marker of cervical cancer. The clinical relevance and functional significance of FOXC1 in cervical cancer make it a promising therapeutic target for future drug development.

Disclosure of conflict of interest

None.

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