Am J Cancer Res 2017;7(8):1642-1653
www.ajcr.us /ISSN:2156-6976/ajcr0057537

Original Article

FoxC1 promotes epithelial-mesenchymal transition through PBX1 dependent transactivation of ZEB2 in esophageal cancer

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Received May 16, 2017; Accepted June 2, 2017; Epub August 1, 2017; Published August 15, 2017

Abstract: Esophageal cancer (EC) was one of the most lethal malignancies worldwide with intricate mechanisms. Here we reported that Forkhead box C1 (FoxC1), a member of the forkhead family transcription factors, was up-regulated in EC tissues and cell lines in comparison with controls. FoxC1 levels were negatively correlated with tumor stage, lymph node metastasis and survival status of EC patients. Knockdown of FoxC1 inhibited the proliferation, colony formation and epithelial-mesenchymal transition (EMT) of EC cells, while overexpression of FoxC1 promoted these biological behaviors. Mechanically, serial deletion and chromatin immunoprecipitation assays showed that ZEB2, a well-reported transcriptional suppressor of E-cadherin, was a direct transcriptional target of FoxC1. Moreover, FoxC1 was recruited to the ZEB2 promoter by its interaction with the pioneer transcription factor pre-B-cell leukemia homeobox 1 (PBX1). Importantly, significant correlation between levels of FoxC1 and ZEB2 was observed in EC tissues and the two proteins could be used as prognostic biomarkers together. Hence, our results revealed a critical role of FoxC1 in the EMT process of EC and uncovered a novel mechanism for the regulation of ZEB2-E-cadherin axis in EC.

Keywords: Forkhead Box C1, ZEB2, esophageal cancer, epithelial-mesenchymal transition

Introduction

Esophageal cancer (EC) is one of the most common malignancies, which is widely distributed internationally [1]. As in China, EC occurs at a very high frequency in specific regions including Linzhou and the nearby counties of Henan province [3]. Esophageal tumor cells are found to invade the submucosal layer or transfer to distant tissues in early stage, which contribute to limited treatment methods, poor prognosis and low 5-year survival rate [2]. Esophageal squamous cell carcinoma (ESCC) accounts for most of the EC cases and occurs with intricate etiologies, such as environmental factors, genetic changes and epigenetic alterations of certain genes [4, 5]. Thus, identifying key genes involved in ESCC carcinogenesis and clarifying the underlying biological mechanisms will contribute to the development of more effective diagnostic and treatment modalities for this lethal disease.

Human Forkhead-box (FOX) gene family, which shares an evolutionary conserved 'forkhead' or 'winged-helix' DNA-binding domain, consists more than 40 evolutionarily conserved transcriptional regulators [6-8]. FOX proteins are involved in multiple physiological processes including cell proliferation, differentiation and apoptosis [7]. Meanwhile, deregulation of FOX proteins has been reported to induce developmental disorders or carcinogenesis [9]. FoxC1, a member of the FOX transcription factor family, has been revealed as an emerging marker and therapeutic target for human tumors recently. Previous researches in breast cancer demonstrated FoxC1 as an adverse prognostic marker of basal-like breast cancer (BLBC) subtype and promoted cell invasion via targeting matrix metalloprotease 7 (MMP7) [10, 11]. Accumulating evidences suggested that FoxC1 had clinical and/or biological roles in digestive system tumors, such as hepatocellular carcinoma (HCC), pancreatic cancer and gastric cancer

Table 1. Primer sequences used in the current study

Name	Sequeces (5'-3')
Real-time PCR	
GAPDH-F	5'-GCACCGTCAAGGCTGAGAAC-3'
GAPDH-R	5'-TGGTGAAGACGCCAGTGGA-3'
FoxC1-F	5'-CAGCATCCGCCACAACCTCT-3'
FoxC1-R	5'-GCAGCCTGTCCTTCTCCTCCT-3'
Vimentin-F	5'-TTGAACGCAAAGTGGAATC-3'
Vimentin-R	5'-AGGTCAGGCTTGGAAACA-3'
Fibronectin-F	5'-TGTTATGGAGGAAGCCGAGGTT-3'
Fibronectin-R	5'-GCAGCGGTTTGCGATGGT-3'
E-cadherin-F	5'-CTGAGAACGAGGCTAACG-3'
E-cadherin-R	5'-TTCACATCCAGCACATCC-3'
Snail-F	5'-TTACCTTCCAGCAGCCCTAC-3'
Snail-R	5'-AGCCTTTCCCACTGTCCTC-3'
Slug-F	5'-CCTCCATCTGACACCTCC-3'
Slug-R	5'-CCCAGGCTCACATATTCC-3'
ZEB1-F	5'-AAGTGGCGGTAGATGGTA-3'
ZEB1-R	5'-TTGTAGCGACTGGATTTT-3'
ZEB2-F	5'-TTCTGCGACATAAATACG-3'
ZEB2-R	5'-GAGTGAAGCCTTGAGTGC-3'
Twist-F	5'-CGACGACAGCCTGAGCAACA-3'
Twist-R	5'-CCACAGCCCGCAGACTTCTT-3'
ChIP primers	
ZEB2-F	5'-TAAAGAATGCCCCTATGC-3'
ZEB2-R	5'-GGGACACCCTGACTAAAA-3'

[12-14]. However, the involvement of FoxC1 in EC, especially in ESCC, was still unknown.

In this study, we figured out the expression of FoxC1 in ESCC tissues as well as cell lines. Our results revealed that knockdown of FoxC1 inhibited the epithelial-mesenchymal transition (EMT) of ESCC cells. Furthermore, we demonstrated that FoxC1 could transcriptionally activate ZEB2 expression through a PBX1 dependent manner, which might greatly contribute to clarifying the progression of ESCC.

Materials and methods

Clinical data

The study was approved by the research ethics committee of Henan Provincial People's Hospital, and written informed consent was obtained from each patient. From 2010 to 2012, a total of 147 esophageal cancer patients who underwent curative resection were collected in Henan Provincial People's Hospital. Patients who had pre-operative anti-cancer treatment or distant metastasis or tumors of other organs or unavailable data during the follow-up were excluded of the study. Additionally, another cohort of 112 paired frozen fresh tumor tissues and non-tumor tissues were collected from 2014 to 2016 for analyzing the mRNA and protein level of FoxC1.

Immunohistochemical (IHC) staining and scoring

147 esophageal cancer tissues and paired non-tumor tissues were constructed to a tissue microarray, which was stained for FoxC1 (ab5079, Abcam) and ZEB2 (ab53519, Abcam) expression. IHC staining and scoring were performed according to a previous protocol [15]. Briefly, staining intensity of each cases were scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong) and positive rate were scored as 0 (no expression), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). The final score of each sample was determined by adding the score of staining intensity and the positive rate. The cases scored 0-3 were considered to be low expression and 4-7 were considered to be high expression.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with Trizol reagent (Qiagen), and *qRT-PCR* was performed as described previously [15]. The primers used in the present study were listed in **Table 1**.

Cell culture

Human normal esophageal epithelial cell Het-1A immortalized by introducing plasmid pRSV-T with RSV-LTR promoter and SV40 T antigen was obtained from the American Type Culture Collection (Manassas, VA, USA) [16]. ESCC cell lines TE-1 and Kyse150 were obtained from the cell bank of type culture collection of Chinese academy of sciences (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China). ESCC cell lines Kyse30, Kyse140, Kyse180, Kyse410, Kyse510 and Kyse520, were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), the German Resource Center for Biological Material [17]. ESCC cells were cultured in the DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA).

Transient transfection

siRNAs targeting FoxC1, ZEB2 or the scrRNA were purchased from Genepharma (China). FoxC1 and ZEB2 expressing plasmids (pcDNA3.1-FoxC1, pcDNA3.1-ZEB2) were purchased from GeneChem (China). Lipofectamine 2000 (Invitrogen) was used for transient transfection according to the manufacturers' protocol. All functional experiments were conducted 12 hours after transfection.

Cell viability and colony formation assay

For cell viability assay, cells in early log phase were seeded at 1×10^3 per well in 96-well plates. Cell viability was measured by using cell counting kit-8 (CCK-8) (Beyotime, China) following the manufacturer's instructions. The absorbance value (OD 450 nm) of each sample was measured at the same time point of each day. As to colony formation analysis, cells in early log phase were seeded at 1×10^3 per well in six-well plates for colony formation analysis. After incubated for 15 days, the supernatant was discarded, and the cloning was fixed with 4% formaldehyde then stained with crystal violet dye for counting.

Cell migration and invasion assay

Cell invasion assay was performed using the transwell chamber (Corning) with a matrigel-coated filter. A total of 1×10^5 cells to be tested were starved in serum free medium for 12 h and then plated on the top chamber as indicated for 24 h. As to cell migration assay, 5×10^4 cells were plated into transwell chamber. Then the invasive/migrated cells on the lower side were fixed, stained with 0.1% crystal violet and the cells inside the upper chamber were removed by cotton swabs.

Western blot and Co-Immunoprecipitation assay

Western blot assay was performed as described in our previous study [15]. For Co-Immunoprecipitation assay, cells were lysed by buffer (20 mM Tris/HCL, pH 7.6, 100 mM NaCl, 20 mM KCl, 1.5 mM $MgCl_2$, 0.5% NP-40) containing Protease Inhibitor Cocktail (Selleck). Cell lysate was incubated with anti-FoxC1 or anti-ZEB2 antibody overnight and followed by incubation with protein A/G agarose for 4 hours. Then the immunoprecipitates were washed by lysis buffer and boiled for Western blot assay.

Chromatin immunoprecipitation assay (ChIP)

Cells (2×10^6) in a 10 cm culture dish were treated with 1% formaldehyde to cross-link proteins and genomic DNA. Then cell lysates were sonicated to shear DNA to sizes of 400-1000 bp. Equal aliquots of chromatin supernatants were incubated with 5 μg of anti-FoxC1 or anti-rabbit IgG (Santa Cruz, Dallas, TX, USA) antibody. After the reverse cross-link process, precipitated DNA was amplified by PCR using ZEB2-promoter region primers. Non-immunoprecipitated chromatin fragments were used as an input control. The primers were listed in **Table 1**.

Luciferase assay

For luciferase assay, 2×10^3 cells were plated in triplicate in 48-well plates. The pcDNA3.1-FoxC1, FoxC1 siRNA or control siRNA/plasmid were co-transfected with luciferase reporter plasmids into cultured cells. Each sample was co-transfected with the pRL-TK renilla plasmid (Promega), which was used to monitor transfection efficiency. The relative luciferase activity was normalized by renilla luciferase activity.

Statistical analysis

All statistical analyses were performed using SPSS 18.0 for windows (SPSS Inc., Chicago, IL, USA). Associations between protein expression and clinicopathological parameters were analyzed by χ^2 test. Kaplan-Meier survival curves and log-rank test were used for survival analyses. The significances of differences between two groups were assessed by two-tailed Student's *t*-test. One-way ANOVA analysis was used to compare the significances of differences between three groups. Differences were considered significant when the *P*-value less than 0.05.

Results

Overexpression of FoxC1 was correlated with progression and poor prognosis in ESCC

To study the clinical significance of FoxC1 in ESCC, we evaluated the transcriptional levels of FoxC1 in 42 paired ESCC tissues as well as

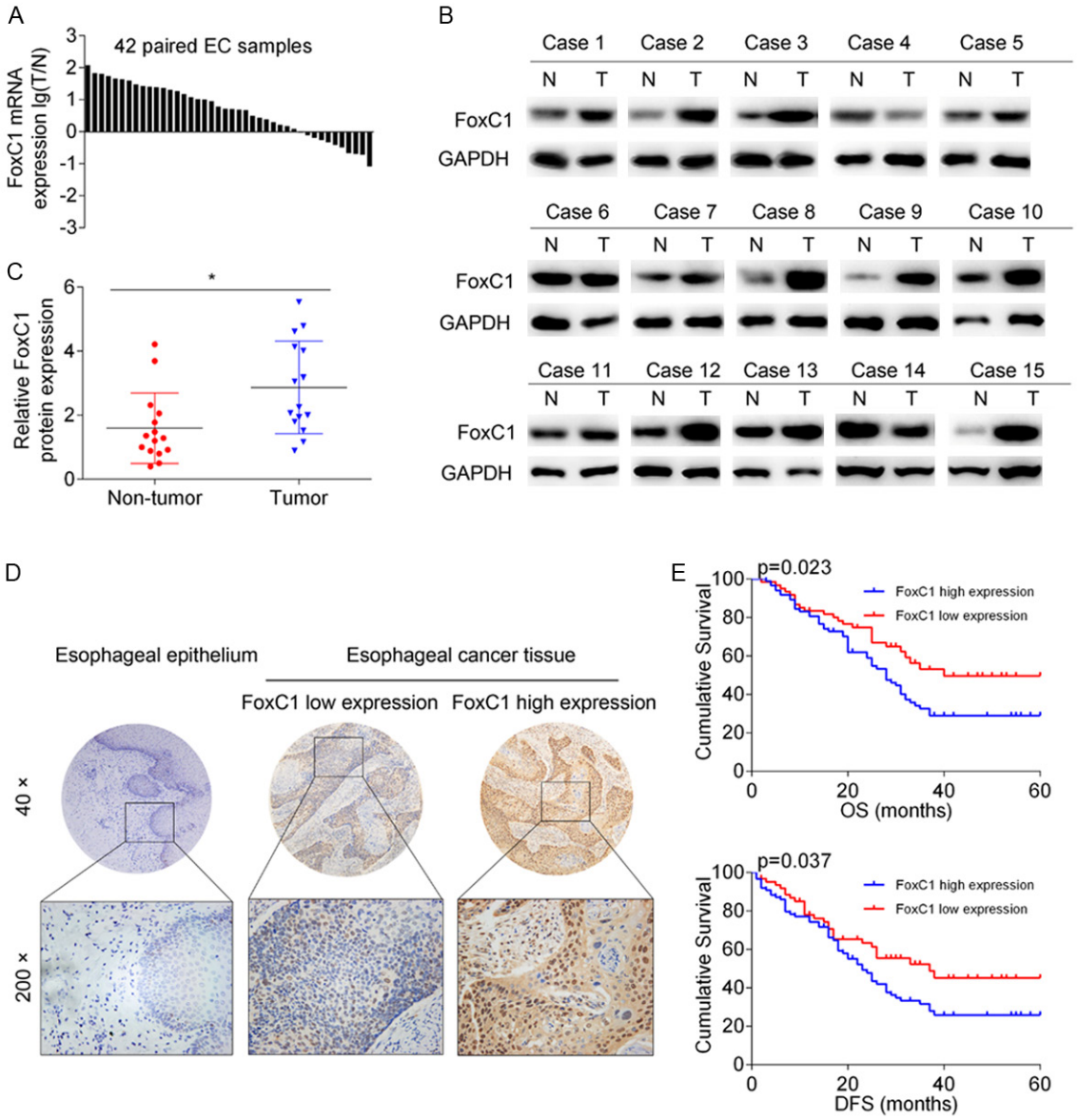

Figure 1. FoxC1 expression was positively correlated with tumor stage, lymph node status and poor prognosis of ESCC. A: The mRNA levels of FoxC1 in 42 paired esophageal cancer tissues were evaluated by qRT-PCR assay. B, C: The protein levels of FoxC1 in 15 paired esophageal cancer tissues were evaluated by western blot assay and the relative gray values were presented. D: Representative IHC staining images of FoxC1 expression in non-tumor tissues and tumor tissues. E: Kaplan-Meier curve showing a correlation between FoxC1 expression and OS/DFS of esophageal cancer patients. (*P<0.05).

non-tumor tissues firstly. We found that FoxC1 was expressed in all ESCC and normal tissues. Besides, FoxC1 transcript levels were significantly increased in ESCC samples compared to paired normal tissues (**Figure 1A**). We then randomly chose 15 paired ESCC tissues and non-tumor tissues for western blot analysis. The result confirmed the consistency of FoxC1 transcriptional levels and protein levels (**Figure 1B** and **1C**). We also found that FoxC1 was differentially upregulated, at both mRNA and protein levels, in the majority of ESCC cell lines (6/8) (**Figure 2A** and **2B**).

We further investigated the relationship between FoxC1 expression and clinicopathological parameters of ESCC patients by a tissue microarray stained with anti-FoxC1 antibody. In

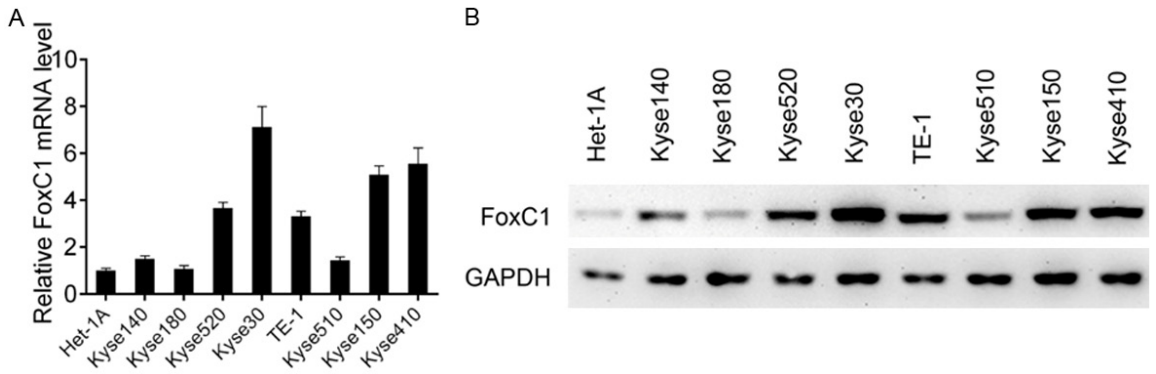

Figure 2. The mRNA level (A) and protein level (B) of FoxC1 in a couple of ESCC cell lines and the normal esophageal epithelial cell line Het-1A.

Table 2. Relationship between FoxC1 expression and clinicopathologic parameters of ESCC patients

Parameters	n	FoxC1 expression		*p*
		Low	High	
Total	147	61	86	
Age (years)				
<55	59	24	35	0.869
≥55	88	37	51	
Gender				
Male	106	47	59	0.261
Female	41	14	27	
Differentiation				
Well	44	23	21	0.083
Moderate/Poor	103	38	65	
LNM				
Negative	97	32	65	0.004
Positive	50	29	21	
AJCC7 stage				
I/IIA	63	32	31	0.048
IIB/III	84	29	55	
Family History				
Yes	51	19	32	0.447
No	96	42	54	

Abbreviations: AJCC7, American Joint Committee on Cancer; LNM, lymph node metastasis.

the 147 ESCCs, high FoxC1 expression was observed in 86 (62.6%) tumors. Additionally, high expression of FoxC1 was significantly associated with higher tumor stage ($P = 0.048$) and lymph node metastasis ($P = 0.004$; **Table 2**). Kaplan-Meier analysis revealed that patients with high FoxC1 expression had poorer overall survival (OS) and disease-free survival (DFS) when compared to patients with low FoxC1 expression (OS: $P = 0.023$, DFS: $P = 0.037$, **Figure 1D** and **1E**).

FoxC1 regulated ESCC cell growth, migration and invasion

We transfected siRNAs into Kyse30 and Kyse410 ESCC cells, which showed higher FoxC1 expression, to evaluate the cell proliferation and colony formation ability. As shown in **Figure 3**, ESCC cell lines transfected with siFoxC1 (Kyse30/siFoxC1 and Kyse410/siFoxC1) showed reduced cell viability in comparison with the control groups (**Figure 3A**, **3B**, **3D** and **3E**). Furthermore, the result of plate colony formation assay showed that the ability of colony formation was also associated with the expression level of FoxC1 (**Figure 3C** and **3F**). Given that FoxC1 overexpression predicted higher risk of lymph node metastasis, we next explored the role of FoxC1 in ESCC cell motility. As the result, knockdown of FoxC1 by siRNA significantly inhibited the migration and invasion ability of Kyse30 (**Figure 3G**) and Kyse410 (**Figure 3H**) ESCC cells compared with the negative control groups.

The impact of FoxC1 on ESCC cell biological functions was further evaluated in Kyse510 cells by ectopic expressing FoxC1. As the result of CCK-8 and colony formation assay, FoxC1-overexpressing ESCC cells Kyse510/pcDNA3.1-FoxC1 displayed higher growth rate and stronger colony formation ability compared with control groups (**Figure 4A-C**). Additionally, the result of transwell assay indicated overexpres-

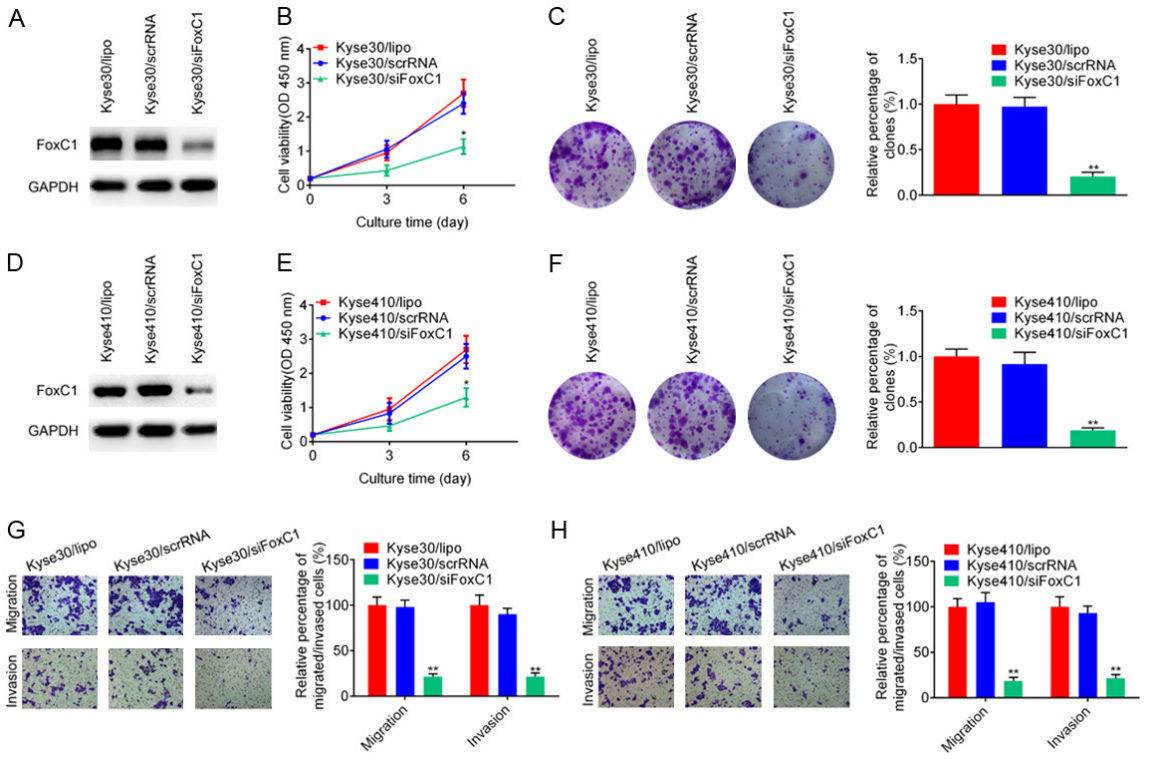

Figure 3. Knockdown of FoxC1 inhibited cell proliferation, colony formation, migration and invasion ability of ESCC cells. A, D: The knockdown efficiency of FoxC1 in Kyse30 and Kyse410 cells were confirmed by western blot assay. B, E: Cell proliferation ability was evaluated by CCK-8 assay. C, F: The colony formation ability was evaluated and representative images were presented. G, H: Knockdown of FoxC1 in Kyse30 cells suppressed cell migration and invasion ability. Representative images and quantitative data of transwell assays were presented, respectively. (Data were presented with mean ± SD of three independent experiments, **P*<0.05, ***P*<0.01).

sion of FoxC1 promoted the migration/invasion ability of Kyse510 cells (**Figure 4D**). These results indicated that the expression levels of FoxC1 were positively correlated with ESCC cell growth and metastasis, collectively.

FoxC1 induced EMT through the transactivation of ZEB2

Recently, EMT had gained much attention due to its importance in the acquisition metastatic potential during tumor progression. Given the fact that FoxC1 was strongly correlated with the migration and invasion ability of ESCC cells, we then asked whether FoxC1 could regulate the EMT system in ESCC. The epithelial marker E-cadherin was significantly up-regulated, while the mesenchymal markers including Vimentin and fibronectin were significantly down-regulated in both cell lines after knockdown of FoxC1, as evidenced by qRT-PCR and Western blot assay (**Figure 5A**).

Down-regulation of E-cadherin was considered to be a main hallmark of EMT. The expression of E-cadherin was mainly regulated by several well-reported transcription factors, such as Snail, Slug, ZEB1, ZEB2 and Twist. Here, we observed that both the mRNA and protein level of ZEB2 were down-regulated after knockdown of FoxC1, while the level of Snail, Slug, ZEB1 and Twist showed no significant change (**Figure 5B**). To determine the mechanisms through which FoxC1 regulated ZEB2 transcription, reporter constructs containing serial 5' deletions of the ZEB2 promoter (pGL3-1389, pGL3-925, pGL3-680, pGL3-398 and pGL3-201) were co-transfected with pcDNA3.1-FoxC1 or control plasmids. Our results showed that a deletion from nt-1389 to nt-680 had no significant influence on FoxC1-induced ZEB2 promoter activity. However, the activity of the fragment in pGL3-398 was markedly decreased compared with pGL3-680, indicating that the sequence between nt-680 and -398 was the

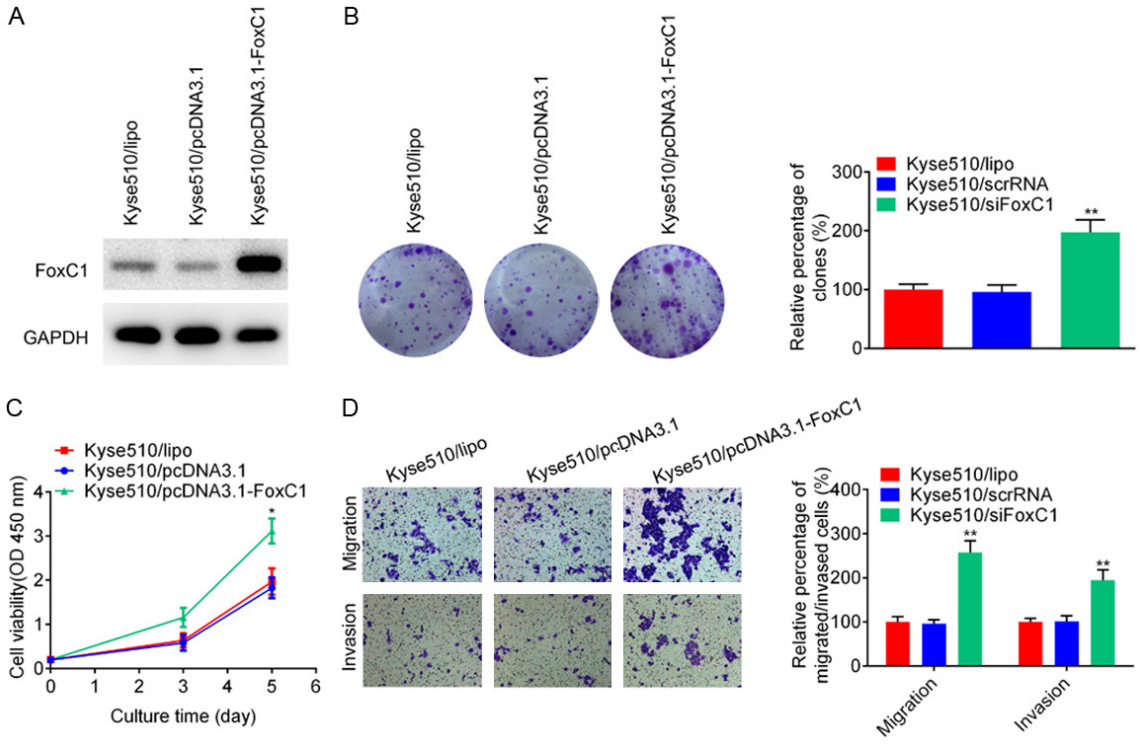

Figure 4. Overexpression of FoxC1 promoted proliferation, colony formation, migration and invasion ability of Kyse510 cells. A: Overexpression of FoxC1 in Kyse510 cells was measured by western blot assay. B: Representative images (left) and quantitative analysis (right) of colony formation ability of FoxC1 overexpressed Kyse510 cells. C: Overexpression of FoxC1 promoted Kyse510 cell proliferation ability measured by CCK-8 assay. D: Overexpression of FoxC1 promoted migration/invasion ability of Kyse510 cells. Representative images (left) and quantitative analysis (right) of transwell assays. (Data were presented with mean ± SD of three independent experiments, **P*<0.05, ***P*<0.01).

core promoter region regulated by FoxC1 (**Figure 5C**). Additionally, ChIP assay confirmed that FoxC1 was capable of binding to this region in Kyse30 cells (**Figure 5D**).

Analysis of the ZEB2 promoter region using the JASPAR database (http://jaspar.genereg.net/) identified a potential PBX1-specific binding site. IP assays showed that FoxC1 could interact with PBX1 (**Figure 5E**), which was coincided with previous reports [18]. Then we explored whether knockdown or overexpression of PBX1 could influence the DNA binding efficiency of FoxC1. Surprisingly, knockdown of PBX1 could inhibit the DNA binding of FoxC1 within the promoter of ZEB2 in Kyse30 cells (**Figure 5F**). This result was different from a previous study in which the authors observed that the transcriptional activity of FoxC1 could be impaired by PBX1. Considering the different context of melanoma cells and ESCC cells, we guess that PBX1 might regulate the transcriptional activity of FoxC1 through a cell line specific manner, which still need further studies.

FoxC1 regulated motility of ESCC cells through ZEB2

Our results revealed a potential FoxC1-ZEB2 axis in ESCC, while whether ZEB2 was essential for FoxC1-mediated ESCC metastasis/invasion still need to be confirmed. To this end, we co-transfected siFoxC1 with ZEB2 expression plasmid pcDNA3.1-ZEB2 or the control plasmid pcDNA3.1 in Kyse30 and Kyse410 to explore whether re-expression of ZEB2 could rescue the migration/invasion ability. As shown in **Figure 6A** and **6B**, restoring ZEB2 expression could partly reverse the effects of FoxC1 down-regulation, resulting in a significant promotion of cell migration/invasion ability. In addition, Kyse510 cells co-transfected with pcDNA3.1-FoxC1 and siZEB2 (Kyse510/pcDNA3.1-FoxC1 + siZEB2 group) showed decreased migration/

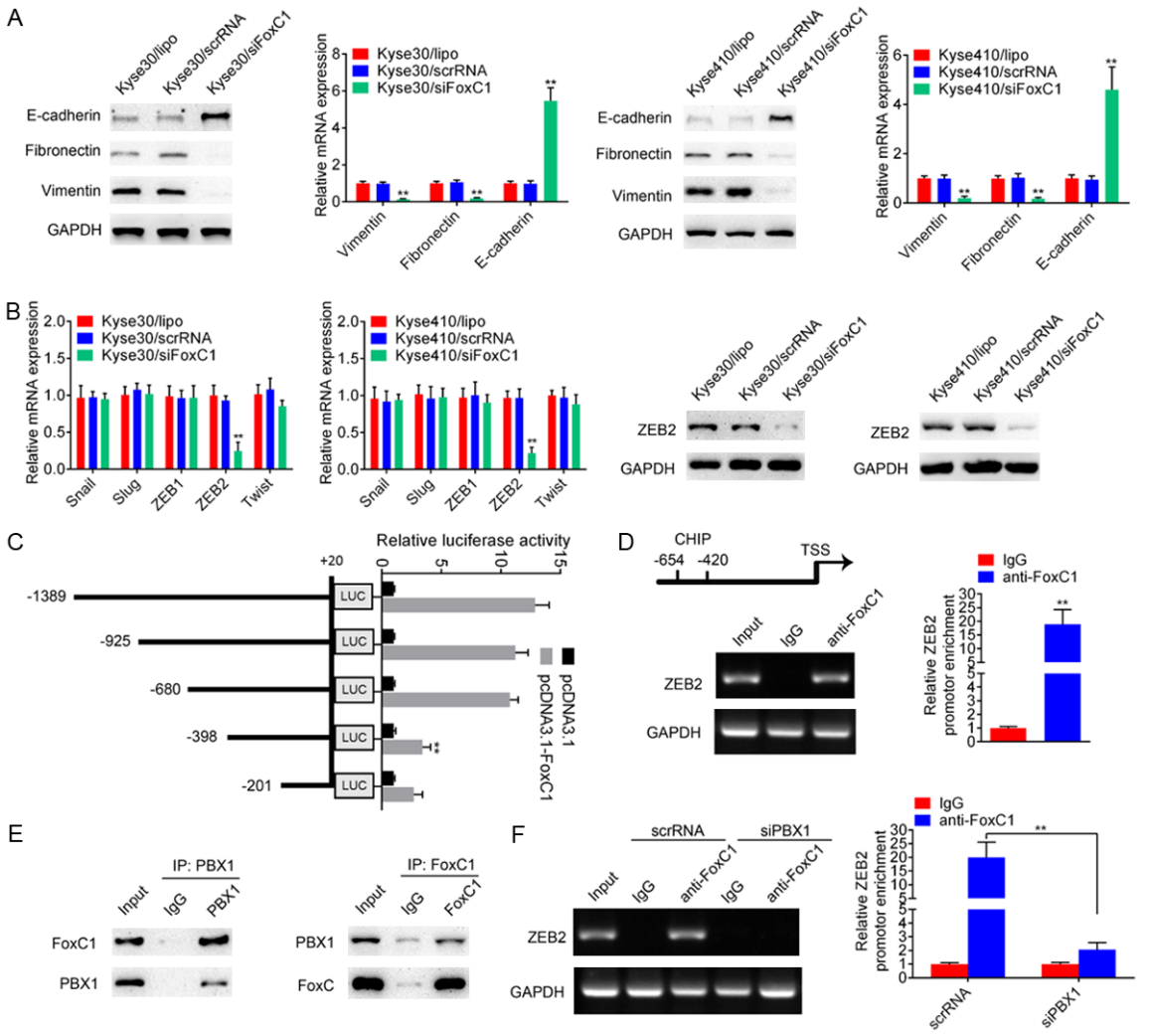

Figure 5. FoxC1 regulated EMT of ESCC cells and ZEB2 was a direct transcriptional target of FoxC1. A: Knockdown of FoxC1 in Kyse30 and Kyse410 cells promoted the mRNA and protein levels of epithelial marker E-cadherin, while decreased the mRNA and protein levels of mesenchymal markers like fibronectin and Vimentin. B: The mRNA levels of Snail, Slug, ZEB1, ZEB2 and Twist were evaluated by qRT-PCR and confirmed by Western blot assay. C: Progressive truncation analysis identified FoxC1-responsive regions in the ZEB2 promoter. D: ChIP analysis was done with FoxC1 antibody and the result revealed a physical association between FoxC1 protein and ZEB2 promoter. E: IP assays were performed using FoxC1/PBX1 antibody to confirm the association of the two proteins. F: Knockdown of PBX1 abrogated the binding efficiency of FoxC1 within ZEB2 promoter. (Data were presented with mean ± SD of three independent experiments, $**P<0.01$).

invasion ability compared with the Kyse510/pcDNA3.1-FoxC1 or Kyse510/pcDNA3.1-FoxC1 + scrRNA group (**Figure 6C**). Altogether, these results provided evidences that FoxC1 regulated motility of ESCC through ZEB2, at least partly if not all.

FoxC1 and ZEB2 could be used as prognostic biomarkers together

Given that FoxC1 promoted EMT of ESCC through ZEB2, we then asked that whether the combination of FoxC1 and ZEB2 could better predict survival than either protein. To this end, we analyzed the relationship between FoxC1 and ZEB2 expression in our cohort. The respresentative pictures of FoxC1/ZEB2 staining were provided in **Figure 7A**. FoxC1 or ZEB2 overexpression was strongly associated with poor survival (both OS and DFS) of ESCC patients. Moreover, patients with high expression of both FoxC1 and ZEB2 had a worse prognosis than patients with only high expression of either protein, indicating that FoxC1 and ZEB2 could be

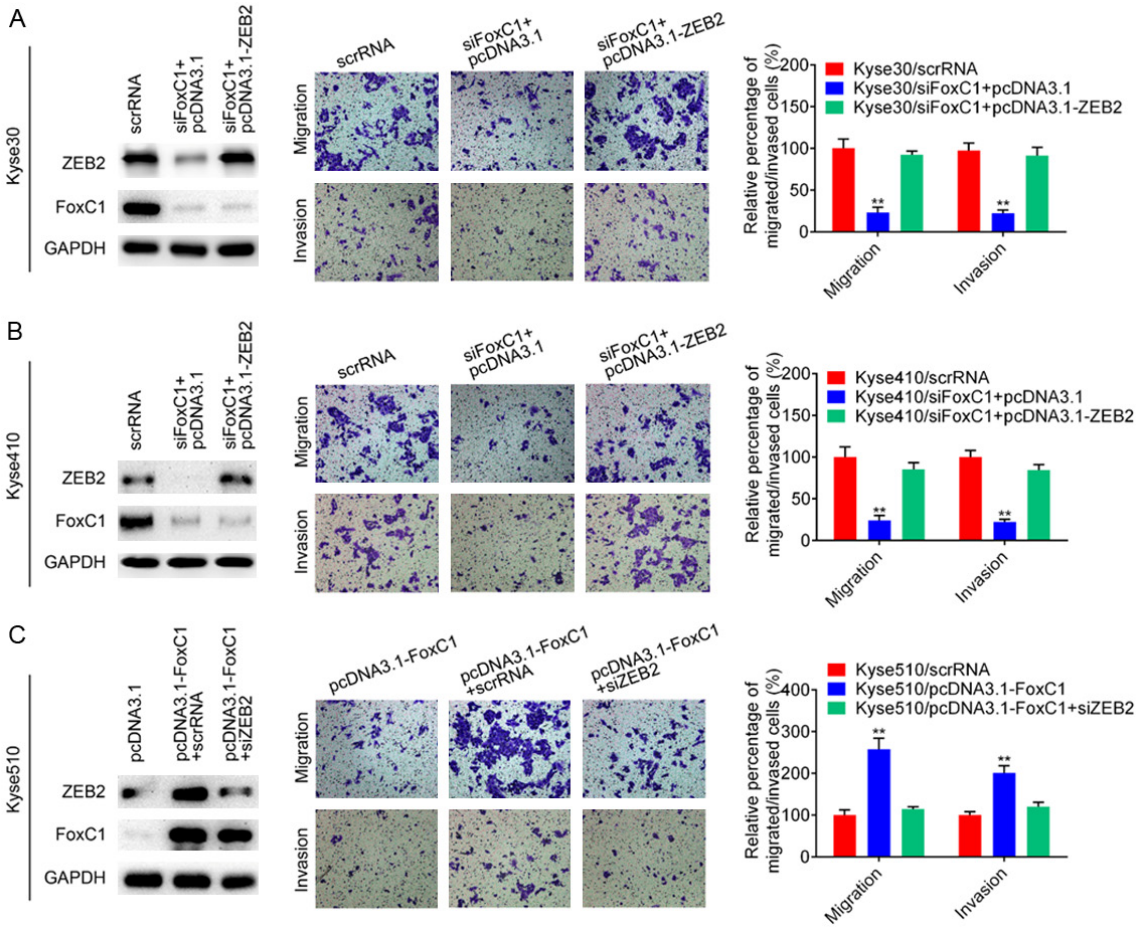

Figure 6. ZEB2 was responsible for FoxC1-mediated cell migration/invasion. A, B: Knockdown of FoxC1 inhibited cell migration/invasion of Kyse30 and Kyse410 cells while re-expression of ZEB2 rescued the migration/invasion ability. C: Overexpression of FoxC1 promoted the migration/invasion ability of Kyse510 cells and promoted the level of ZEB2, while knockdown of ZEB2 decreased the migration/invasion ability. (Data were presented with mean ± SD of three independent experiments, $^{**}P<0.01$).

used as prognostic biomarkers together (**Figure 7B**).

Discussion

In current study, we provided first evidence that FoxC1 was frequently up-regulated in EC tissues and that higher FoxC1 expression in tumor tissues was correlated with unfavorable clinicopathological parameters and poorer survival status, which strongly suggested that FoxC1 might be served as a prognostic biomarker.

EMT, a process characterized by the transdifferentiation of epithelial cells and mesenchymal cells, was involved in development, wound healing, fibrosis and tumor progression [19]. FOX proteins were involved in EMT process of multiple human tumors. FoxQ1 could directly bind to the promoter region of the core EMT regulator E-cadherin and promoted the migration and invasion of breast cancer [20]. FoxM1 overexpression promoted ZEB2 mediated EMT and metastasis in HCC [21]. A recent study provided direct evidence that FoxC1 could regulate the EMT of HCC through transactivating Snail [12]. In this study, we explored the biological role of FoxC1 in EC progression. We observed that knockdown of FoxC1 markedly reduce, whereas overexpression of FoxC1 enhanced the proliferation, colony formation, migration and invasion of EC cells. On the other hand, knockdown of FoxC1 reduced the level of epithelial marker E-cadherin and promoted the level of mesenchymal markers including Vimentin and fibronectin. Thus, overexpression of FoxC1 promoted the aggressive behaviors of EC cells though EMT process.

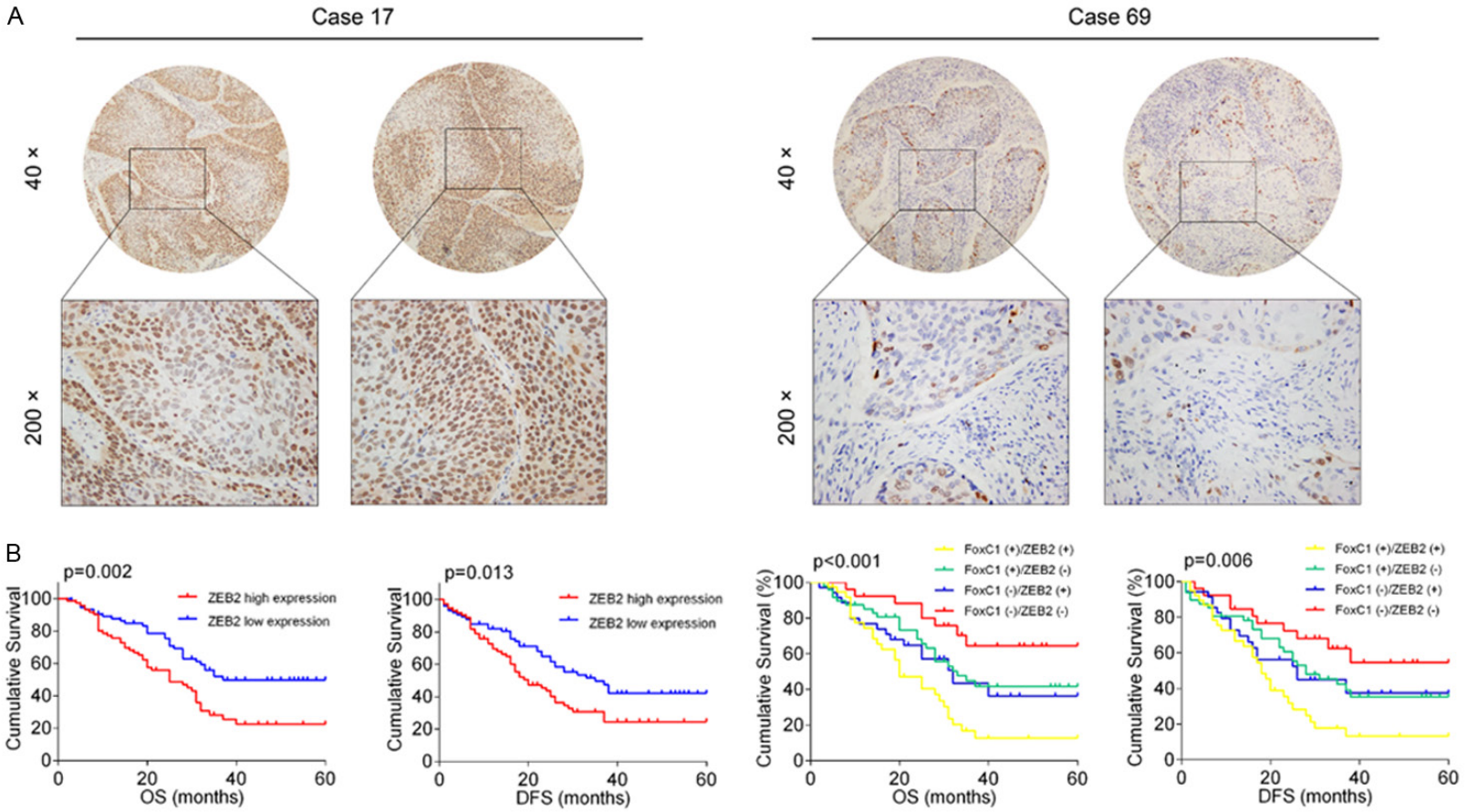

Figure 7. FoxC1 and ZEB2 could be used as prognostic biomarkers together. A: Representative images of IHC staining of FoxC1 and ZEB2 in ESCC tissues as well as non-tumor tissues. B: Prognostic values of FoxC1 combined with ZEB2 by Kaplan-Meier test.

Disruption of E-cadherin mediated adhesion system was a key hallmark for EMT process. Several literatures had reported that FoxC1 was involved in the regulation of E-cadherin. However, the mechanisms through which FoxC1 regulated E-cadherin expression were still elusive. The study in mammary epithelial cells revealed that overexpression of FoxC1 decr-e ased the level of E-cadherin while another study indicated that FoxC1 could transcriptionally activate Snail, a well-reported transcriptional suppressor of E-cadherin [12, 22]. Our current study revealed a novel mechanism of EMT process in which ZEB2 might be served as a direct target of FoxC1. Then we observed that knockdown of FoxC1 attenuated the luciferase activity and inhibited the transcriptional level of ZEB2. The binding efficiency of FoxC1 within the ZEB2 promoter was further confirmed by ChIP assay. Moreover, re-expression of ZEB2 in EC cells partly rescued the migrated/invaded cells through transwell chamber. These results suggested that FoxC1 regulated EMT of EC cells through ZEB2.

Given the clinical significance of FoxC1 in EC patients and the biological consistency between FoxC1 and ZEB2, we then asked whether FoxC1 and ZEB2 could be used as prognostic biomarkers together. Interestingly, EC patients who had high levels of FoxC1 and ZEB2 (FoxC1+/ZEB2+) exhibited worst prognosis, while those had low levels of FoxC1 and ZEB2 (FoxC1-/ZEB2-) showed better prognoses compared with FoxC1+/ZEB2- or FoxC1-/ZEB2+ cases. Taken together, FoxC1-ZEB2 axis played an important role in EC progression and provided useful information for diagnosis and prognosis of ESCC.

Pioneer factors are proteins that can recognize specific DNA targets prior to gene activation, and thus promoted the binding efficiency of specific transcription factors [23, 24]. Several recent literatures reported that Pre-B-cell leukemia homeobox 1 (PBX1), a member of the three Amino acid Loop Extension (TALE)-class homeodomain family, had transcriptional pioneer functions. In the process of skeletal muscle differentiation, PBX1 could interact with the pro-myogenic transcription factor MYOD and contribute myogenic differentiation [25, 26]. As to human malignancies, Magnani et al. reported that PBX1 served as a pioneer factor of ERα-signaling in breast cancer [27]. The study in prostate cancer revealed that PBX1-HoxC8 complex could induce androgen-independent cell proliferation, suggesting a potent relationship between PBX1 and Hox family proteins [28]. Our current work provided new findings that PBX1 could interact with FoxC1 in EC cells. We also found that knockdown of PBX1 impaired the FoxC1-ZEB2 transaction axis through abating the binding ability of FoxC1 at ZEB2 promoter region, raising a possibly transcriptional complex for ZEB2. In conclusion, our results from clinical to biological studies suggested that FoxC1 promoted EMT of EC through PBX1 dependent transactivation of ZEB2.

Disclosure of conflict of interest

None.

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