FOXD1 promotes proliferation, migration and epithelial-mesenchymal transformation of esophageal squamous cell carcinoma by targeting SNAI1

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Abstract

The transcription factor forkhead box D1 (FOXD1) is an important member of the FOX family, which is widely expressed in human embryonic cells and is thought to regulate organogenesis. It has been shown that FOXD1 could affect proliferation, migration, and angiogenesis of various tumors and its deletion and overexpression in organisms will undoubtedly have important influence on the change of cell fate and the occurrence of tumors. However, the underlying functions and molecular mechanisms of FOXD1 in esophageal squamous cell carcinoma (ESCC) have not been fully clarified. According to the present study, the expression levels and functional roles of FOXD1 were investigated, and its prognostic value and molecular mechanisms in tumorigenesis and progression of ESCC were clarified. The expression level of FOXD1 was significantly upregulated in ESCC tissues and cell lines, and correlated with TNM stage, pathological differentiation, depth of invasion, and LN metastasis. Moreover, FOXD1 promoted cells migration and invasion as well as participated in TGF-β1 induced epithelial-mesenchymal transition (EMT) process. Furthermore, a positive correlation between FOXD1 and SNAI1 was explored in ESCC. FOXD1 could directly bind to promoter regions of SNAI1 gene, leading to transcriptional promotion of SNAI1 in human esophageal cancer cells. Taken together, FOXD1 may play a tumor activator role in ESCC and may be applied as a new therapeutic target and prognostic marker for ESCC.

1 | INTRODUCTION

Esophageal cancer has become one of the most common malignant tumors in the world. According to the latest statistics of global cancer, the global incidence of esophageal cancer is increasing every year. There are significant regional differences in the incidence of esophageal cancer, and nearly half of the cases of esophageal cancer and death occurred in China, which seriously threatened the health of residents. Different from adenocarcinoma in Europe and America, more than 90% of esophageal cancers in China are esophageal squamous cell carcinoma (ESCC). For the advanced metastatic ESCC patients in high prevalence areas, the current first-line standard treatment is chemotherapy or combined immunotherapy. The median survival time is still about one year. The 5-year survival rate of advanced esophageal cancer treatment is less than 20%. It is very important to explore the etiology of esophageal cancer and find molecular targets for early screening, risk assessment and prognostic judgment of esophageal cancer to reduce the incidence and mortality of esophageal cancer. Therefore, it is urgent to explore more effective treatment means to prolong the survival time for Chinese patients with esophageal squamous cell carcinoma.

Forkhead box (FOX) protein family is a relatively conserved transcriptional regulator which mediates a variety of biological functions, such as DNA damage, embryonic development, cell cycle and metabolic balance regulation, suggesting that the FOX gene family may also be involved in multiple complex processes such as tumorigenesis and development. More increasing evidences show that many members of the FOX gene family are related to the development and progression of cancer. FOXD1 is one of the important transcription factors of the FOX family, which is mainly localized at 5q13.2,
encoding a DNA-binding protein containing 465 amino acids and involving in a variety of biological functions. Multiple studies have confirmed that FOXD1 is involved in tumorigenesis and development through different mechanisms of action in a variety of solid tumors. Zeng et al. found that FOXD1 could bind to the promoter of ZNF532 and activate its transcription, which targeted to regulate the process of laryngeal squamous cell carcinoma EMT and enhance the invasion ability of AMC-HN-8 and TU212 cell lines. Chen et al. showed that FOXD1 promoted EMT and stemness through transcriptional activation of SNAI2 in oral squamous cell carcinoma. In addition, FOXD1 can directly bind to CTGF promoter and regulate its expression level to promote melanoma dedifferentiation and drug resistance to targeted therapy. There is growing evidence showing that FOXD1 expression is decreased in various cancer tissues, suggesting that FOXD1 is a valuable prognostic marker for cancers. However, little is known about the function and mechanism of FOXD1 in ESCC.

Epithelial-mesenchymal transition (EMT) enhances the movement and invasion ability of tumor cells, and plays a key role in the process of tumor proliferation and metastasis. More and more studies have shown that EMT program is activated during the progression of malignant tumors. Transforming growth factor (TGF-β) is one of the important factors promoting the remodeling of tumor extracellular matrix in tumor microenvironment, which plays an important role in the induction of EMT process, tumor invasion and metastasis. However, whether FOXD1 participates in regulating EMT and stemness in ESCC remains unknown at present.

In this study, we found that FOXD1 expression was upregulated in ESCC and was associated with poorer clinical outcomes. We then demonstrated that TGF-β1 induced up-regulation of FOXD1 expression and promoted EMT and stemness in ESCC. Further studies showed that FOXD1 promoted the transcriptional activity of SNAI1, a key regulatory gene associated with EMT. This study reveals the role and mechanism of FOXD1 in regulating tumor progression and analyzed the possibility of FOXD1 as a potential target for the progression and prognosis of ESCC.

2 | MATERIALS AND METHODS

2.1 | Patients and specimens

The data samples used in the experiments were from patients who had ESCC surgery at the Fourth Affiliated Hospital of Hebei Medical University. The experiments generated 76 cases of ESCC tissues and paired adjacent non-tumor tissues during the period from January 2013 to December 2015. The patients included 29 females and 47 males who had not received chemotherapy or radiotherapy prior to the operation. Their consent were included as well. Based on the American Joint Committee on Cancer (AJCC) standards, TNM histological grading was determined. Table S1 presents the clinicopathological information retrieved from hospital records about patients suffering from ESCC. After surgery, the specimens were kept at the temperature of -80°C for RNA extraction. Patients were classified as having a family background of upper gastrointestinal cancer (UGIC) if they had a minimum of one immediate
family member or two family members in the second degree who had been diagnosed with esophageal or gastric cancer. The duration between the initiation of treatment and the conclusion of the follow-up period or death was used to assess survival, with the main outcome being overall survival. This study obtained the ethical approval from the hospital's Ethics Committee.

2.2 | Cell culture and treatment

The China Center for Type Culture Collection (located in Wuhan, China) provided the human ESCC cell lines ECA109, KYSE150, KYSE170, and TE1. In 2012, the ATCC Standards Development Organization (SDO) established the ANSI Standard (ASN-0002). According to this standard, Short Tandem Repeat (STR) analysis was employed to authenticate these cell lines. The cells were grown in a solution called RPMI 1640 (made by Gibco), which included 10% fetal bovine serum (FBS) (Invitrogen). They were exposed to 5% CO2 while maintained at a steady temperature of 37°C. The cells were then exposed to the recombinant TGF-β1 from R&D Systems (10 ng/mL). The culture medium RPMI 1640, which contained 10% FBS, was replaced every 48 hours and supplemented with TGF-β1 for a period of 21 days. A control group was established by culturing an equal volume of sodium citrate diluent under identical conditions.

2.3 | RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assay

Total RNA was extracted from ESCC cell lines, ESCC tissues, as well as their corresponding adjacent normal tissues by use of the TRlizol reagent following the instructions provided by Invitrogen. The RNA was then converted into cDNA through a process called reverse transcription by the Transcriptor First Strand cDNA Synthesis Kit from Roche. The resulting cDNA was used as a template for amplifying fluorescence quantitative PCR. The GoTaq® qPCR Master Mix from Promega was used for performing the qRT-PCR on the StepOne Real-Time PCR System by Applied Biosystems. With the aim of determining the relative expression level of RNAs, the 2−ΔΔCT method was adopted and the Human GAPDH gene was employed as a role of an internal control. 21 In this study, the primer sequences used can be found in Table S2, and each sample was tested three times.

2.4 | Immunohistochemistry assay

To determine the level of FOXD1 protein expression in ESCC tissues, an immunostaining method called avidin-biotin complex immunoperoxidase was used. This technique was applied to matching sections of tumor tissue and corresponding normal tissue, both of which were embedded in paraffin. The endogenous peroxidase and non-specific reactions were blocked before applying the primary antibody against FOXD1 (sc-293238, SCBT) to the sections. To ensure the accuracy of the results, a negative control was included, in which mouse IgG replaced the primary antibody.

At least two pathologists evaluated the degree of reactivity, and they were unaware of the tumors’ clinicopathological characteristics. The overall score for FOXD1 immunostaining was determined by taking into account both the intensity score and the proportion of tumor cells that exhibited a positive
result. In order to provide further details, the proportion of tumor cells was evaluated using a rating system that ranged from 0 to 4. A rating of 0 indicated that there was no evidence of positive staining, while a rating of 1 indicated that 1% to 25% of the tumor cells exhibited positive staining. A rating of 2 indicated that 26% to 50% of the tumor cells were positively stained, whereas a rating of 3 indicated that 51% to 75% of the tumor cells were positively stained. The highest rating of 4 indicated that 76% to 100% of the tumor cells were positively stained. Moreover, the intensity of staining was evaluated using a rating system ranging from 0 to 3. A score of 0 meant that there was no staining, a score of 1 meant there was a faint yellow staining, a score of 2 meant there was a strong yellow staining, and a score of 3 meant there was a dark brown staining\(^2\). To assess how FOXD1 expression correlates with clinicopathological factors, the experiment categorized patients into two groups using their immunoreactivity score (IRS) values, which were obtained by taking the product of the staining intensity score and the percentage of cells that tested positive. The patients with IRS scores between 0 and 4 were categorized as having a low level of expression, whereas those with IRS scores greater than 4 as having a high level of expression.

### 2.5 | Cell transfection

To study how FOXD1 overexpression influences the cells, an eGFP tag was used from Invitrogen to synthesize the FOXD1 sequence and inserted it into pcDNA3.1, creating pcDNA3.1-FOXD1. The ESCC cell lines were transfected using Lipofectamine 2000 Reagent (Invitrogen) with either an empty vector (pcDNA3.1-NC) or pcDNA3.1-FOXD1 at a concentration of 2 ug/uL as per the manufacturer's guidelines. G418 was used to select resistant cell clones, which were then expanded and characterized. GenePharma (Shanghai, China) designed and synthesized non-specific control siRNA (si-NC), \(SNAI1\) mRNA (si-SNAI1), and small interfering RNAs (siRNAs) targeting \(FOXD1\) mRNA (si-FOXD1-1, si-FOXD1-2, and si-FOXD1-3). The number of cells labeled with fluorescein was determined using fluorescence microscopy, while the effectiveness of the transfection was confirmed using the qRT-PCR technique. Table S3 provides the details of the constructs and siRNA sequences.

### 2.6 | Cell proliferation and colony formation assay

To determine cells' ability to proliferate, this experiment utilized the MTS and colony generation methods. In particular, the CellTiter96®AQueousOne Cell Proliferation Assay Kit (Promega) was employed in the implementation of the MTS assay and 96-well plates with \(1\times10^3\) cells per well were employed to inoculate the cells, with 6 replicates per set. A supplement of 20 \(\mu\)L of MTS reagent with a concentration of 500 \(\mu\)g/mL was introduced to each well after the cells adhered, at intervals of 0, 24, 48, 72, and 96 hours, and the optical density at 492 nm (OD490) was computed by employing a microplate analyzer. In the colony formation assay, cells that had been transfected were placed in 6-well plates with a seeding density of \(3\times10^3\) cells per well. After that, they were cultivated for one week with the cell density adjusted. After fixation with 4% paraformaldehyde, the cells were subjected to staining with 1% crystal violet solution for a duration of 30 minutes at ambient temperature. Using optical microscopy, the amount of clones was computed (considering a clone to be present if there were over 50 cells). As a result, the rate of clone formation can be measured.
2.7 | Cell migration and invasion assays

For the purpose of demonstrating cell transfer and invasion capacity, two methods, the transwell assay and the wound healing assay, were utilized. In the wound healing test, cells were grown to 80% confluence and then a 200 μL pipette was used to create a scratch on the surface of the cells after overnight starvation. The distance the cells moved into the wound was observed and recorded at 0 hr, 12 hr, 24 hr after scratching. For the transwell assays, the Matrigel-coated chambers (Corning) were used for the evaluation of cell invasion. To perform the experiment, the cells that had been transfected were placed into each well at a concentration of 1×10⁵ cells. The bottom compartment had 600 μL of complete medium, including 10% fetal bovine serum, while the top compartment contained 200 μL of medium without serum. After being incubated for 24 hours at 37°C, the lower chamber invasive cells were treated with 4% paraformaldehyde and stained for 20 minutes using 1% crystal violet while the upper chamber cells were removed. The amount of invasive cells in five random visual areas was then counted using a Leica microscope.

2.8 | Western blot analysis

As per the protocol guidelines, the total protein extracts were combined with PMSF (Solarbio) and a protease inhibitor (Promega) after being lysed with RIPA lysate. The BCA Protein Assay Kit (Multi Sciences) was utilized to measure the protein concentration. Additionally, the comparable proteins were examined through electrophoresis by utilizing 10 % SDS-PAGE gel and subsequently moved to polyvinylidene fluoride (PVDF) membranes (Millipore). Following this, at ambient temperature, the membranes that were transferred were left in 5% skimmed milk for a period of one hour. Thereafter, they were kept with a specific primary antibody at a temperature of 4°C for an entire night. The secondary antibody was introduced to the membranes at room temperature and left to incubate for one hour. After that, the enhanced ECL reagent (Multi Sciences) was utilized to visualize the membranes. The study utilized several primary antibodies such as anti-FOXD1 (sc-293238, SCBT), anti-β-Actin (AC026, ABclonal, Woburn, MA, USA) and a range of antibodies targeting EMT markers (Bioss). These EMT marker antibodies comprised anti-β-catenin (bs-1165R), anti-N-cadherin (bs-1172R), anti-SNAI1 (bs-1371R), anti-E-cadherin (bs-1519R), and anti-Vimentin (bs-0756R).

2.9 | Dual-luciferase reporter assay

Plasmids were generated to monitor the activity of the SNAI1 promoter, in order to investigate how FOXD1 affects the expression of the SNAI1 gene. The human SNAI1 promoter(-1200/+33, relative to the transcription start site (TSS)), as well as three truncations (-1154/+33, -907/+33, -709/+33 and -293/+33) were amplified by PCR. The products that were made more intense were placed into the XhoI and Hind restriction endonuclease sites within the pGL3-basic luciferase reporter vector, and verified through sequencing. A list of primers used for creating various fragments of the SNAI1 promoter can be found in Table S3. The experiment transfected pcDNA3.1-FOXD1 (TE1) or control samples together with SNAI1 promoter constructs (at a concentration of 200 ng per sample) and pRL-TK,
an internal control (at a concentration of 10 ng). The Dual-Luciferase Reporter Assay System (Promega) was adopted for quantifying the luciferase activity following 48 hours of transfection. The ratio of firefly to Renilla luciferase activity was employed to indicate the relative activation of the promoter.

2.10 | Chromatin immunoprecipitation (ChIP) assay

The experiment adopted ChIP assay to enrich the SNAIL1 promoter regions that contain FOXD1-binding components using an anti-FOXD1 antibody (sc-293238, SCBT). To achieve this, DNA was fragmented into 200 bp fragments via sonication. The enrichment of the samples was confirmed through PCR amplification and agarose electrophoresis gel with EB staining was utilized to visualize the amplified products. Table S2 shows the FOXD1 binding sites’s primer sequences in the SNAIL1 promoter.

2.11 | Statistical analysis

SPSS 25 programme was employed for the statistical analysis, and GraphPad Prism8.0 was used to plot figures. First of all, this analysis measured the mean ± standard deviation (SD) for all the data samples obtained from the experiments. Then, a comparison of two groups were performed based on the analysis results from the two independent samples t-test, and a comparison of multiple groups were conducted based on the analysis results from the Pearson's chi-square test. In addition, this analysis carried out the Kaplan-Meier assay to generate survival curves, and then the log-rank test to evaluate them. Furthermore, a Cox regression analysis was conducted with the aim of determining independent variables affecting the survival rate. The bivariate correlations among the variables investigated were assessed by Spearman correlation analysis. The analysis was carried out utilizing two-sided statistical tests, and any results indicating a difference with a significance level less than 0.05 was deemed as significant.

3 | RESULTS

3.1 | Upregulation of FOXD1 in ESCC cells and tumors

The expression levels of FOXD1 in 76 primary ESCC tissues, paired adjacent non-tumor tissues, as well as 4 different types of cancer cells were assessed by qRT-PCR. As shown in Fig.1A, mRNA levels of FOXD1 were markedly increased in ESCC tumor tissues, when comparing with corresponding healthy tissues (P < 0.05). Meanwhile, FOXD1 gene expression was also remarkably upregulated in four ESCC cell lines in comparison to the control group (Figure 1B). Furthermore, FOXD1 protein level was increased dramatically in ESCC tissues (72.4%, 55 cases), whereas only 27.6% (21 cases) of corresponding normal tissues were positive for FOXD1 (P=0.03), as evidenced by immunohistochemistry staining shown in Fig. 1C. To evaluate the clinical implication, we evaluated how FOXD1 expression levels correlates to the clinicopathological features of ESCC patients. Our results indicated that FOXD1 gene and protein expression levels were closely correlated with ESCC tumor tissue characteristics including: TNM stage, pathological differentiation, depth of invasion, and LN metastasis (Figure 1D and Table S1).

3.2 | Correlation between FOXD1 level and survival rate of ESCC patient
Given that FOXD1 is upregulated in tumor tissues, we next examined how FOXD1 levels correlates to patient survival. Patients with higher FOXD1 expression (>50% greater in tumor tissue than that of corresponding healthy tissues) showed a 5-year survival rate of 12.4% (median survival time of 22.9 months; P<0.01); however, the 5-year survival rate increased to 28.6 % (median survival time, 34.6 months) in patients with relatively lower expression levels of FOXD1 (Fig. 1E). In addition, integrative analysis of clinical stage and FOXD1 gene expression on patient prognosis showed that patients with high FOXD1 expression had poorer survival and tumor metastasis in stage III and IV disease (Figure 1F). We analyzed patients with stage I+II and stage III+IV separately as well. Our results showed that individuals with high FOXD1 levels had considerably shorter survival time compared to those with low FOXD1 levels, irrespective of the disease stage. (P < 0.05). Lastly, we performed multivariate analyses using the Cox proportional hazard regression model to identify independent predictors of survival for ESCC patients. Our results revealed that TNM stage (p=0.010), lymph node metastasis (p=0.021), and FOXD1 expression (p=0.003) were independently associated with the survival of ESCC patients (Figure 1G).

3.3 | FOXD1 promotes ESCC cell proliferation, migration, and invasion in vitro

In order to dissect the functional roles of FOXD1, we utilized both the pcDNA3.1-FOXD1 vector and small interfering RNAs (si-FOXD1-1/2/3) to overexpress or knockdown the expression of FOXD1 in TE1/KYSE150 cells, respectively. As shown in Fig. 2A, increased FOXD1 mRNA and protein levels indicated successful transfection of the vector into TE1 and KYSE150 cells. We also showed substantial FOXD1 knockdown using si-FOXD1-1 and si-FOXD1-2, which were chosen for the following experiments (Figure 2B). As shown by results from MTS and clone formation assays, upregulation of FOXD1 significantly enhanced TE1 cell proliferation (Figure 2C and 2D); similar results were observed in wound healing and transwell experiments, TE1 cells displayed increased migration and invasion functions (Figure 2E and 2F). In contrast, knocking down FOXD1 via transfecting cells with siRNA notably inhibited the proliferation, migration, and invasion function of KYSE150 cells (Figure 2C-2F). These findings suggested that FOXD1-induced proliferation and migration are responsible for promoting the growth of FOXD1-expressing tumor cells.

3.4 | Critical role of FOXD1 in TGFβ-mediated EMT process

Given that EMT process of epithelial tumor cells represents one of the major contributors for tumor metastasis, we hypothesize that FOXD1 could regulate EMT process to increase ESCC cell proliferation, migration, and invasion. We continuously treated the ESCC cells with TGF-β1 for 21 days to activate EMT process (Figure 3A). Interestingly, consistent TGF-β1 stimulation increased gene and protein expression levels of FOXD1 in these cells (Figure 3B-C and Fig. S1A). In addition, we detected lower abundance of E-cadherin but higher levels of mesenchymal cell markers in TE1 and KYSE150 cells after TGFβ administration (Figure 3B-C and Fig. S1B). In addition, knocking down of FOXD1 could reverse the expression level of EMT-related markers in TGF-β1-treated TE1 and KYSE150 cells at the protein levels. (Figure 3D and Fig. S1C)
3.5 | FOXD1 increases SNAI1 transcription by directly binding to its promoter

It has been demonstrated that TGF-β signaling plays an important role in regulating EMT-related cytokines, leading to tumor cell metastasis, immune evasion, and angiogenesis\textsuperscript{20,21}. Considering that SNAI1 is a significant mediator for TGF-β signaling transduction across various cancer types including ESCC\textsuperscript{22}, we next investigated the correlation between FOXD1 and SNAI1 to further dissect the molecular mechanisms involved in cancer progression. As shown in Fig. 4A, SNAI1 mRNA and protein levels were positively correlated to FOXD1 levels: increased SNAI1 levels in cells with FOXD1 overexpression, whereas FOXD1 knockdown led to downregulation of SNAI1. To identify how FOXD1 regulates SNAI1 expression, we used the target prediction program, HumanTFDB, and discovered three putative FOXD1-binding sites at the transcription start site (TSS) region of SNAI1 (Figure 4B and 4C). To validate these findings, we constructed several pGL3-SNAI1 vectors with luciferase reporter that contain these FOXD1 binding sites (Site1/2/3) in the promoter area of SNAI1. In accordance with our prediction, we detected markedly increased activities on SNAI1 promoter regions. Particularly, pGL3-SNAI1 (-907/+33) vector showed robust activity in transfected ESCC cells, when comparing to the control vector group (Figure 4D). Lastly, we performed the ChIP assay and validated that FOXD1 was highly enriched on SNAI1 promoters, particularly in the FOXD1-binding Site 2 spot (Figure 4E). Overall, these findings indicate that in TE1 cells, FOXD1 can enhance SNAI1 expression by activating its promoter that contain the predicted binding elements.

3.6 | FOXD1 upregulates SNAI1 to promote EMT and enhance ESCC cell growth, migration, and invasion

To further assess the necessity of SNAI1 in FOXD1-mediated effects, we co-transfected TE1 or KYSE150 cells with pcDNA3.1-FOXD1 and si-SNAI1. As shown in Fig. 5A-B and Fig. S2A, si-SNAI1 could markedly blunt SNAI1 expression mediated by FOXD1 overexpression. In addition, functional rescue experiments revealed that knockdown of SNAI1 could inhibit the biological effects of FOXD1 in promoting esophageal cancer cells proliferation as demonstrated by MTS and colony formation assays (Figure 5C-5D and Fig. S2B-S2C). Consistently, results from transwell assays showed reduced cell migration and invasion capabilities upon SNAI1 downregulation (Figure 5E-5F and Fig. S2D-S2E). Collectively, these findings indicate that SNAI1 is essential for FOXD1-triggered EMT process in ESCC.

3.7 | FOXD1 predicts unfavorable patients' outcomes combined with SNAI1

Lastly, we investigated the clinical relevance of FOXD1 and SNAI1 in ESCC progression. Our results displayed that SNAI1 expression level was elevated and positively correlated with FOXD1 in ESCC tissues (Fig. 5G). Furthermore, patients with high expression levels of FOXD1 and SNAI1 had markedly poorer overall survival when compared to individuals with low expression level of SNAI1 (Fig. 5H). Overall, our findings suggest that FOXD1 may promote ESCC progression by directly binding to SNAI1 promoter region, thus stimulating its transcription and subsequent activation of the downstream TGF-β signaling cascade.
4 | Discussion

Esophageal cancer remains a prevalent and deadly malignant tumor worldwide. Accumulating evidence have suggested the potential of transcription factors as molecular biomarkers to guide clinical diagnosis of various cancers, but the effectiveness of such approach in ESCC has not been well established\textsuperscript{23-24}. FOXD1, a transcriptional factor found on human chromosome 5q13.2, has been found to be upregulated in a variety of tumors, such as pancreatic cancer, gastric cancer, and breast cancer\textsuperscript{11-13}. Nonetheless, the functional roles of FOXD1 remain elusive in ESCC tumorigenesis and progression.

In current study, we demonstrated that FOXD1 expression was increased in ESCC tissues and cells. In addition, expression level of FOXD1 in ESCC tissues was found to be associated with clinicopathologic factors such as TNM stage, pathological differentiation, depth of invasion, and LN metastasis, which may have a significant impact on ESCC patient survival rate. Additionally, our findings showed that FOXD1 overexpression could enhance ESCC cell proliferation, migration, and invasion; whereas knocking down FOXD1 showed opposite effects. These evidences illustrate that FOXD1 is a critical contributor to esophageal cancer development.

Tumor cells utilize epithelial-mesenchymal transformation (EMT) process to detach from the primary tumor and disseminate into the bloodstream, enabling them to acquire an aggressive tumor phenotype\textsuperscript{25,26}. Previous publications showed that TGF-\(\beta\) is upregulated in advanced tumor cells; activating TGF-\(\beta\) signaling can promote tumor cell growth, invasion, and metastasis through various mechanisms such as facilitating EMT process, immune escape, and promoting tumor angiogenesis\textsuperscript{27,28}. Earlier research findings suggest that during TGF-\(\beta\)-induced EMT, many epithelium-related markers were downregulated, such as E-cadherin and cytokeratin, which is accompanied by increased expression of mesenchymal markers and transcription factors (\textit{i.e.} N-cadherin, vimentin, SNAI1, Slug, or TWIST1)\textsuperscript{29,30}. Here, we discovered that FOXD1 upregulation in TGF-\(\beta\)\textsubscript{1}-treated ESCC cells could promote EMT transition, as evidenced by significant upregulation of EMT-associated markers in these ESCC cells. Importantly, overexpression of FOXD1 could further enhance TGF-\(\beta\)\textsubscript{1}-mediated effects on cell migration and invasion.

To the best of our understanding, the function of FOXD1 in ESCC tumorigenesis has never been investigated. Through binding to the TGF-\(\beta\) receptor, TGF-\(\beta\)-induced EMT could lead to the activation of both classical (SMADs) pathway and non-classical pathways (\textit{i.e.} RhoA, JNK/p38, PI3K/Akt, and JNK/p38)\textsuperscript{31}. In current study, we treated TE1 cells with TGF-\(\beta\)\textsubscript{1}, the most effective stimulator for epithelial-mesenchymal transformation, which caused significant upregulation of FOXD1, N-cadherin, Vimentin, SNAI1, and \(\beta\)-catenin; yet this treatment led to noticeable suppression on E-cadherin levels, when comparing to untreated control group. The inhibitory effect of TGF-\(\beta\)\textsubscript{1} on E-cadherin expression was blunted in FOXD1-deficient TE1 cells, while both SNAI1 and \(\beta\)-catenin were significantly downregulated. These results suggested that knockdown of FOXD1 could partially reverse the process of epithelium-mesenchymal transformation in esophageal squamous cell carcinoma induced by TGF-\(\beta\)\textsubscript{1}. 
Previous reports have demonstrated that during EMT, increased levels of SNAI1 is able to promote the intracellular SMAD signal transduction mediated by TGF-β1, thereby stimulating the expression of various EMT-related genes, and acting as an oncogene during tumor development\textsuperscript{32}. Our study has shown that upregulating FOXD1 resulted in substantial increase in SNAI1 expression in ESCC cells. We also demonstrated that FOXD1 could transactivate SNAI1 expression through direct binding to its promoter regions. Importantly, our results further revealed that knocking down SNAI1 could inhibit the stimulatory effects of FOXD1 on ESCC cells, suggesting that FOXD1 may promote tumorigenesis and progression through transcriptional activation of SNAI1. In line with previous report showing a positive regulatory role of SNAI1 in esophageal cancer\textsuperscript{33}, our data also presented a negative correlation between expression levels of FOXD1/SNAI1 and the survival rate of ESCC patients. Thus, we have proposed a model based on current results, which highlights the functional roles of FOXD1 in regulating TGF-β\textsuperscript{3} signaling cascade in the event of tumor cell metastasis, indicating that TGF-β1/FOXD1/SNAI1 axis may be the underlying mechanism by which FOXD1 regulates the EMT process. However, the direct molecular mechanism for TGF-β\textsuperscript{1}-induced upregulation of FOXD1 remains to be further studied.

In conclusion, our study has shown for the first time that upregulated FOXD1 in ESCC could facilitate tumor cells proliferation, migration, and invasion. Furthermore, through directly binding to SNAI1 promoter and increasing its transcriptional activities, FOXD1 could also regulate the EMT process and activate TGF-β signaling. Hence, FOXD1 appears to be novel therapeutic target for ESCC; and with further investigation, its clinical implication might be extended to broader range of tumors.

**Declarations**

**ACKNOWLEDGEMENTS**

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**AUTHOR CONTRIBUTIONS**

Zhiyu Wang supervised the experiment and revised the manuscript. Zheng Wu performed the experiments, analyzed the data, and drafted the paper. Minghui Liu p and Hui Zhu performed the experiments. Xiaojin Guo prepared the tables and figures. Ya Liu and Lei Zhang performed the statistical analysis. Shenghu Guo and Yuehua Zhang collected the data and recruited the patients.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

Figure 1
Expression level of FOXD1 in ESCC and its clinical significance. A. The qRT-PCR method was employed to detect the relative expression of FOXD1 in ESCC tissues and corresponding normal tissues. B. Relative expression of FOXD1 in four ESCC cell lines (ECA109, KYSE150, KYSE170, and TE1). The control group demonstrated the mean expression of FOXD1 in the cDNA of 10 adjacent normal tissues and mixed well. C. Immunohistochemical analysis of FOXD1 expression in ESCC tissues and corresponding normal tissues (magnification, 20X, 40X). D. Relative expression of FOXD1 in different clinicopathologic subgroups. E. A Correlation between relative expression of FOXD1 and 5-year survival rate. F. Poor survival was detected for patients with high relative FOXD1 expression at stage III and IV ESCC. G. Multivariate Cox regression analysis for clinicopathological features associated with prognosis of ESCC patients in forest map. Log-rank p< 0.05, *p< 0.05.
Figure 2

FOXD1 promoted the proliferation, migration, and invasion in ESCC cells. A-B. The qRT-PCR and western blot methods were employed to detect FOXD1 overexpression (A) and knockdown (B) efficiency of transfection in KYSE150/TE1 cells. C-D. MTS (C) and colony formation (D) assays were used to detect the cell proliferation in FOXD1-overexpressing TE1 cells and FOXD1-depleting KYSE150 cells, respectively.
E-F. Wound healing (E) and transwell (F) assays were used to detect the cell migration or invasion in FOXD1-overexpressing TE1 cells and FOXD1-knockdown KYSE150 cells, respectively. *p< 0.05.

**Figure 3**

FOXD1 participated in the process of EMT induced by TGF-β1 in TE1 cells. A. Morphological change of ESCC cells with TGF-β1 for 21 days. B. The qRT-PCR method was used to examine and compare the
relative mRNA expression changes of EMT-related markers and FOXD1 treated by TGF-β1 and non-treated TE1 cells. C. The protein expression of EMT-related markers (E-cadherin, N-cadherin, Vimentin, SNAI1 and β-catenin) and FOXD1 were explored by western blot method in TE1 cells. D. The effect of FOXD1 on TGF-β1-mediated EMT-related markers at protein expression level in TE1 cells.*p< 0.05.

Figure 4
FOXD1 increased the transcription of SNAI1 through the direct binding to its promoter. A. The relative expression changes of SNAI1 were measured in FOXD1-up-regulated TE1 cells and FOXD1-knockdown KYSE150 cells. B. The DNA-binding motifs of FOXD1 were analyzed by HumanTFDB. C. The predicted FOXD1-binding sites were shown in the proximal promoter region of SNAI1. D. The luciferase activity of the SNAI1 promoter containing or lacking FOXD1-binding elements was explored by a dual-luciferase reporter assay. E. CHIP assays were used to measure the binding of FOXD1 to the promoter region of SNAI1 with containing putative binding elements in the designated cells using anti-FOXD1 antibodies or IgG control. *p< 0.05.
Figure 5

FOXD1 upregulated the proliferation, migration, and invasion of ESCC cells and promoted the EMT process through up-regulating SNAI1 in TE1 cells. A-B. The transfection efficiency of FOXD1(A) and SNAI1(B) in the four experimental groups was determined by qRT-PCR assays. C-D. MTS (C) and colony formation (D) assays were used to measure the proliferation capacity by cotransfection with si-SNAI1 in FOXD1-overexpressing cells. E-F. Transwell assays were used to confirm the invasion of cells after
cotransfection with si-SNAI1 in FOXD1-overexpressing cells. G. A positive correlation between FOXD1 and SNAI1 in ESCC specimens. H. Kaplan-Meier method was used to explore the effect of the expression of FOXD1 and SNAI1 on the prognosis of patients. *p< 0.05.

**Supplementary Files**

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- [SupplementaryMaterials.zip](#)