

## Original Article

# FOXD1 predicts prognosis of colorectal cancer patients and promotes colorectal cancer progression via the ERK 1/2 pathway

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**Abstract:** Previous studies indicated a critical role of foxhead box D1 (FOXD1) in human cancers. However, its expression pattern in colorectal cancer (CRC) and the molecular mechanism of FOXD1 on cancer progression remain unknown. In this study, we found that FOXD1 was aberrantly overexpressed in human CRC tissues, and FOXD1 levels were correlated with tumor size, differentiation, TNM stage and lymph node metastasis and poor prognosis. Knockdown of FOXD1 attenuated CRC cell proliferation, migration and invasion. Overexpression of FOXD1 produced the opposite effects. These effects were mediated by activation of the ERK 1/2 signaling pathway, and inhibition of this pathway with a specific ERK 1/2 inhibitor (U0126) could impair the tumor-promoting effects induced by overexpression of FOXD1. Taken together, these findings indicate that FOXD1 promotes tumorigenesis and progression of CRC by activating ERK 1/2 signaling pathway and may represent a potential clinical target.

**Keywords:** FOXD1, colorectal cancer, proliferation, migration, invasion, ERK 1/2

## Introduction

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer-related death worldwide [1]. While we have witnessed great improvement in diagnosis and synthetic therapy, the average survival time of patients with advanced stage CRC remains pessimistic [2]. Thus, investigation the molecular mechanism that is responsible for the carcinogenesis and development of CRC is of great significance and could help to identify novel tumor biomarkers for early detection and therapeutic strategies.

Forkhead box D1 (FOXD1) is a newly discovered member of the FOX transcription factor family and is located on chromosome 5q12 [3]. Recently, a large body of evidence has indicated that FOXD1 plays a crucial role in carcinogenesis, tumor progression and metastasis in several types of human cancers [4, 5]. It was reported that FOXD1 is highly expressed and

correlates with poor survival in non-small cell lung cancer [6]. Further, inhibition of p27 by FOXD1 promotes breast cancer progression and chemotherapeutic drug resistance [7]. These findings suggest that FOXD1 functions as a tumor promoter in human cancers. However, little is known about the role of FOXD1 in the progression of CRC.

In the present study, we investigated the expression profiles of FOXD1 in CRC tissues and cell lines. We also demonstrated the roles and the underlying molecular mechanisms of FOXD1 in CRC. The goal of this study is to clarify the expression and functions of FOXD1 in CRC and offer a potential target for diagnosis and therapy for CRC.

## Materials and methods

### *Patients and immunohistochemical analysis*

Colorectal tumor and adjacent non-tumor tissues were obtained from 126 patients who

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underwent curative surgery at the First Affiliated Hospital of Zhejiang University and the First Affiliated Hospital of Jiaying University between December 2008 and January 2011. None of these patients received radiotherapy or chemotherapy before surgery. All tissue samples were fixed by formaldehyde and embedded by paraffin to produce tissue chips. Clinicopathological data were collected and the TNM-stage of patients was determined according to the UICC/TNM classification. All participants provided written informed consent before enrollment and the Ethic Committees of the First Affiliated Hospital of Zhejiang University and the First Affiliated Hospital of Jiaying University approved the study protocol.

Tissue chips were incubated with the anti-FOXD1 primary antibody (1:200; Abcam, USA) at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:1000, Santa Cruz, USA) for 30 min at room temperature. The expression status was independently examined by two pathologists according to the staining intensity and percentage of positive cells. The intensity of staining was classified by four grades: no staining (0), weak staining (1), moderate staining (2) and intense staining (3). The percentage of positive cells was also divided into four grades: 0% (0), 1-5% (1), 6-29% (2), 30-59% (3) and more than 60% (4). Finally, FOXD1 staining score = positive cell score + staining intensity score. For statistical analysis, each sample was grouped as either positive (score >3) or negative (score 0-3).

### *Cell culture*

CRC cell lines SW1116, SW480, Caco2, HCT1-16, Lovo and SW620 were obtained from American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### *Protein extraction and Western blot*

Cells were harvested and lysed using RIPA cell lysis buffer in the presence of protease inhibitor cocktail (Sigma, USA). A total of 50 µg protein lysates were resolved through 10% SDS-PAGE and then transferred to PVDF membranes. Proteins were visualized using an enhanced chemiluminescence detection sys-

tem in accordance with the manufacturer's protocol.

### *RNA isolation and quantitative real-time PCR (qRT-PCR)*

Total RNA was extracted from cell lines and tissues by Trizol Reagent (Invitrogen, USA). Subsequently, cDNA were synthesized by reverse transcription kit (Promega, USA) according to the manufacturer's instruction. Quantitative polymerase chain reaction (q-PCR) was conducted using SYBR Green PCR Master Mix (Applied Biosystems, UK). Relative mRNA expression was calculated by comparative Ct method and GAPDH was used as the control. All experiments were done in triplicate.

### *Establishment of stable cell lines*

pGLV-GFP-FOXD1 lentiviral vector and shRNA plasmids were purchased from Genepharma (Shanghai, China). Lentivirus particles were transfected into the CRC cells in the presence of polybrene and selected for 2 weeks using puromycin (5 µg/ml). Plasmids were transfected into cells with Lipofectamine 2000 (Invitrogen, USA) and stable transfected cells were selected by G418.

### *Cell proliferation assay*

Tumor cells (2×10<sup>3</sup>/well) were seeded into 96-well plates and cultured for 4 days. At the check point (0 h, 24 h, 48 h, 72 h and 96 h), 10 µL of CCK-8 solution was added into the wells and incubated at 37°C, 5% CO<sub>2</sub> for 3 h. The absorbance at 450 nm was assessed by a spectrophotometer.

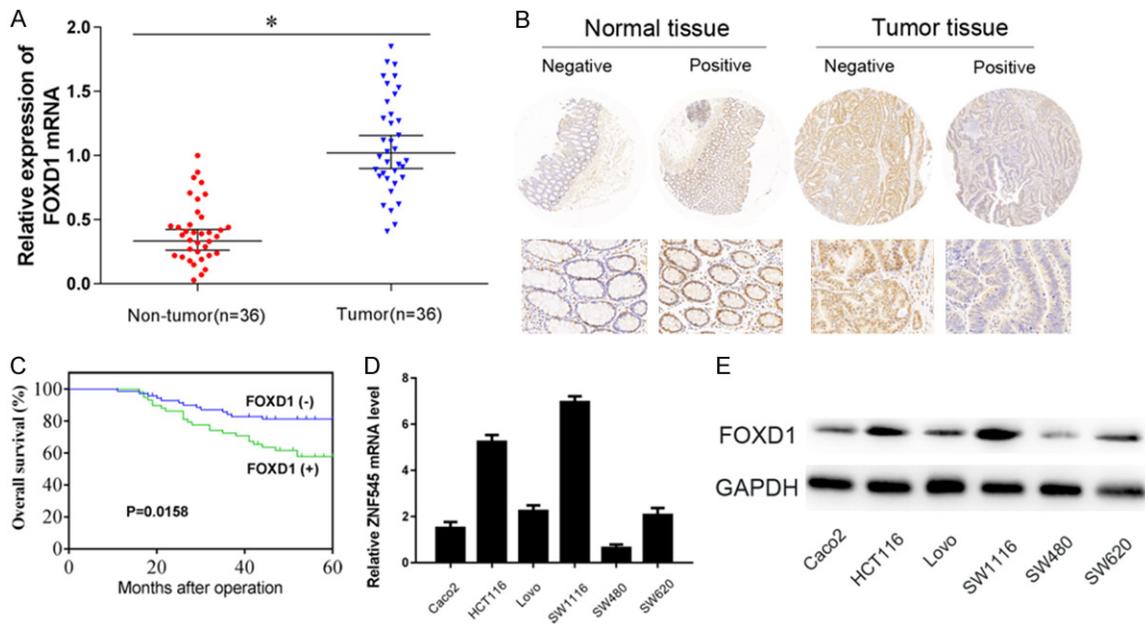
### *Plate colony formation assays*

Tumor cells (1×10<sup>3</sup>/well) were seeded into 6-well plates. The plates were gently shaken and incubated at 37°C, 5% CO<sub>2</sub> until visible cloning appeared. Then the supernatant was discarded, and the colonies were fixed with 4% formaldehyde for 30 min and later stained with crystal violet dye for 5 min.

### *Cell migration and invasion assay*

The ability of cell migration and invasion were detected by transwell chambers (Corning Costar, USA). Cells were culture in serum-free

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**Figure 1.** Expression of FOXD1 in the CRC tissues and cell lines. A. FOXD1 mRNA expression in 36 CRC patient tissues compared with its adjacent non-tumor tissues. B. Representative images of FOXD1 expression in 126 paired CRC samples examined by IHC. C. Kaplan-Meier analysis of survival in patients with CRC. Green: patients with negative FOXD1 expression; blue: patients with positive FOXD1 expression. D, E. FOXD1 mRNA expression in CRC cell lines was examined by qRT-PCR and Western blot. \* $P < 0.05$ .

RPMI-1640 for 12 h. For the migration assay,  $1 \times 10^5$  cells suspended in 200  $\mu$ L serum-free RPMI-1640 was seeded into the upper chamber, and 600  $\mu$ L RPMI-1640 with 10% FBS was added to the lower chamber as a chemoattractant. For the invasion assay, the insert membranes were coated with diluted matrigel (BD Bioscience, USA). Then, the chambers were incubated for 24 h at 37°C, and then cells that moved through the membranes were fixed by 10% formalin for 30 min and stained with 0.5% crystal violet for 15 min. Protein extraction and Western blot.

### Statistical analysis

All tests were performed by SPSS 19.0 software and shown as mean  $\pm$  SD. Student's t test was used to examine the statistical differences between the two groups. The relationship between the expression level of FOXD1 in CRC tissues and clinicopathological features were analyzed by Pearson  $\chi^2$  test or Fish's exact test. Survival time was evaluated with Kaplan-Meier method, and differences were compared by log-rank test. A two-tailed value of  $P < 0.05$  was considered statistically significant.

## Results

### Expression of FOXD1 and its clinicopathological significance in CRC patients

In order to investigate the potential roles of FOXD1 in CRC development, we first analyzed FOXD1 mRNA expression in CRC specimens. Surprisingly, we found that FOXD1 transcript levels were significantly decreased in CRC tissues compared to paired non-tumor tissues ( $P < 0.001$ ) (**Figure 1A**). In addition, immunohistochemical (IHC) analysis was performed to detect the expression of FOXD1 protein in 126 CRC tissues and paired non-tumor tissues (**Figure 1B**). Among these cases, 102 (80.95%) of the tumor tissues showed a positive FOXD1 signal, while FOXD1 positive expression was detected only in 56 (44.44%) of the adjacent non-tumor tissues ( $P < 0.001$ , **Table 1**).

To further investigate the clinicopathologic significance of FOXD1 in CRC, we compared several clinical and pathologic features with FOXD1 protein expression. As shown in **Table 1**, FOXD1 expression was significant associated with tumor size ( $P = 0.025$ ), differentiation ( $P = 0.036$ ), TNM stage ( $P = 0.023$ ) and lymph node metas-

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**Table 1.** Relationship between FOXD1 expression level and clinicopathologic features in 126 CRC cases

Variable	Case (n=126)	FOXD1 expression		P-value
		Negative	Positive	
<b>Tissues</b>				
Carcinoma	126	24	102	<0.001
Normal tissues	126	70	56	
<b>Age (years)</b>				
<65	47	29	18	0.284
≥65	79	41	38	
<b>Gender</b>				
Male	84	46	38	0.800
Female	42	24	18	
<b>Tumor size</b>				
≤5 cm	45	26	19	0.025
>5 cm	81	30	51	
<b>Differentiation</b>				
Well to moderate	48	27	21	0.036
Poor	78	29	49	
<b>Invasion depth</b>				
T1, T2	94	48	46	0.010
T3, T4	32	8	24	
<b>Lymphatic metastasis</b>				
Negative	51	29	22	0.021
Positive	75	27	48	
<b>Distant metastasis</b>				
Negative	102	55	47	0.447
Positive	24	15	9	
<b>TNM stage</b>				
I, II	60	33	27	0.023
III, IV	66	23	43	

tasis (P=0.021). Kaplan-Meier survival curves revealed that patients with high FOXD1 expression had shorter 5-year overall survival (OS) than those with low FOXD1 expression (**Figure 1C**). These results suggest that high expression of FOXD1 in CRC correlates with tumor progression and poor prognosis.

Then we examined FOXD1 expression level in seven CRC cell lines. As shown in **Figure 1D** and **1E**, FOXD1 was highly expressed in SW1116, while lowly expressed in SW480 and Caco2.

### *FOXD1 promotes CRC cell proliferation and clonogenicity*

To explore roles of FOXD1 in CRC cells, we generated Caco2 and SW480 cell lines ectopically

overexpressing FOXD1 and employed shRNA to knock down FOXD1 level in SW1116. The CCK-8 assay showed that the proliferation was markedly increased in the Caco2/FOXD1 and SW480/FOXD1 cells compared to corresponding control cells, whereas the proliferation was dramatically impaired in SW1116/sh-FOXD1 cells compared to SW1116/sh-NC cells (**Figure 2A-C**). Additionally, plate clone formation assay revealed that up-regulating FOXD1 expression in Caco2 and SW480 cells increased the number colony than that of control cells. Opposite results were observed with SW1116 transited with shRNA (**Figure 2D-F**).

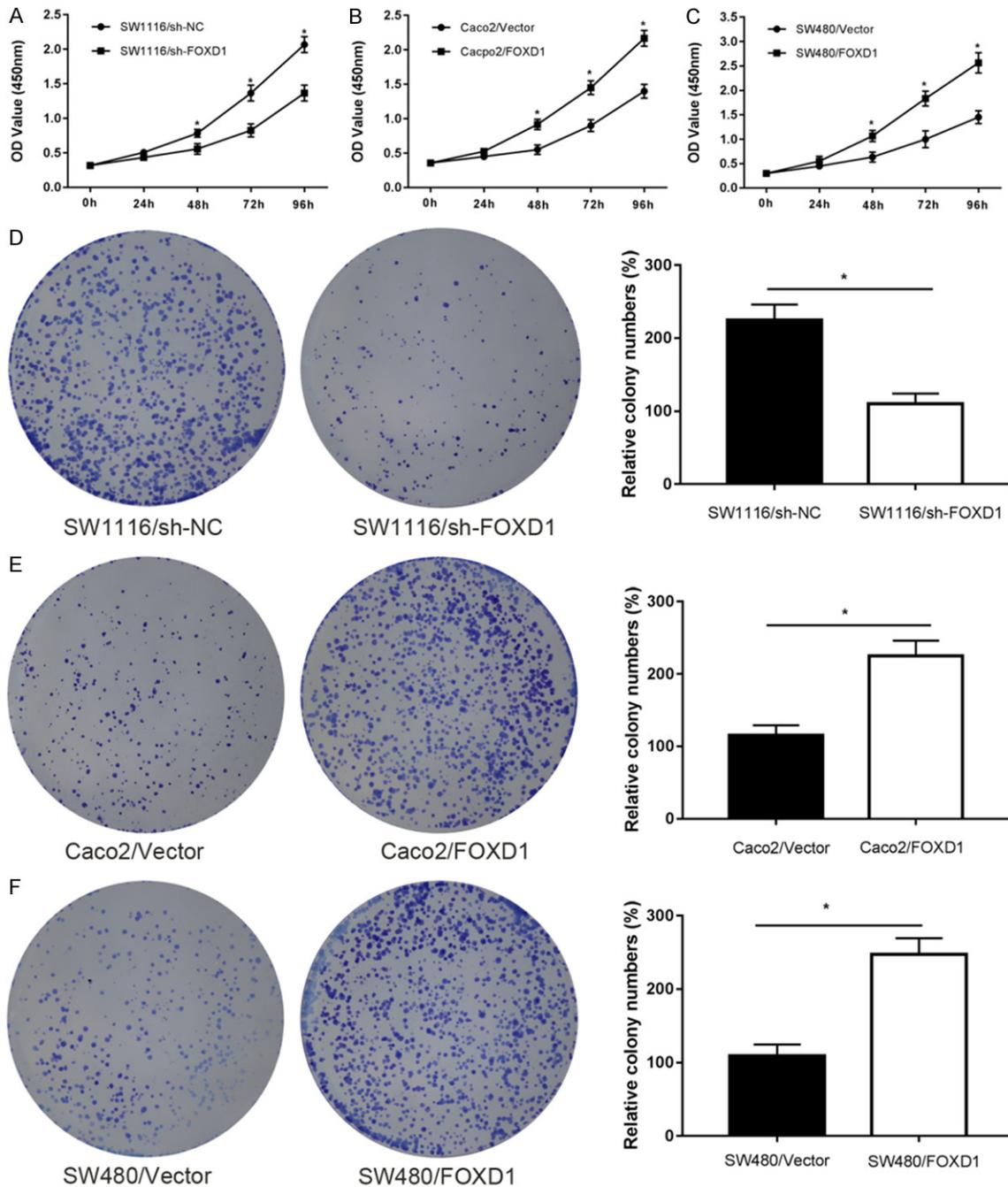
### *FOXD1 suppresses migration and invasion of CRC cells*

Migration and invasion are hallmarks of cancer metastasis. To investigate the functional role of FOXD1 in cell motility, we performed transwell assays. As shown in **Figure 3**, overexpression of FOXD1 increased the number of migration and invasion cells both in Caco2 and SW480 cells. Consistent with these observations, knockdown of FOXD1 decreased the number of migration and invasion cells in SW1116. Thus, FOXD1 promotes migration and invasion of CRC cells in vitro.

### *FOXD1 regulates cell proliferation, migration and invasion via the ERK 1/2 pathway*

In recent years, the ERK 1/2 signaling pathway has gained more attention due to its importance in the cell growth and migration. Therefore, we investigated whether FOXD1 could regulate the activation of the ERK 1/2 signaling pathway in CRC cells. As shown in **Figure 4A**, knockdown of FOXD1 significantly inhibited phosphorylation of ERK 1/2 in SW1116 cells. In contrast, FOXD1 overexpression elevated phosphorylation of ERK 1/2 both in Caco2 and SW480 cells. Thus, we hypothesized that FOXD1 may function as an upstream molecule in the ERK 1/2 pathway regulation. Next, we next investigated whether FOXD1 could regulate cell proliferation, migration and invasion via the ERK 1/2 signaling pathway by treating Caco2/FOXD1 and SW480/FOXD1 with an ERK 1/2 inhibitor (U0126) (**Figure 4B**). As shown in **Figure 4C** and **4D**, Caco2/FOXD1 and

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**Figure 2.** FOXD1 promotes CRC cell growth. A. Knockdown of FOXD1 inhibited cell proliferation of SW1116 by CCK-8 assay. B, C. Overexpression of FOXD1 enhanced cell proliferation of Caco2 and SW480 by CCK-8 assay. D. Knockdown of FOXD1 inhibited colony formation ability of SW1116. E, F. Overexpression of FOXD1 enhanced colony formation ability of Caco2 and SW480. \* $P < 0.05$ .

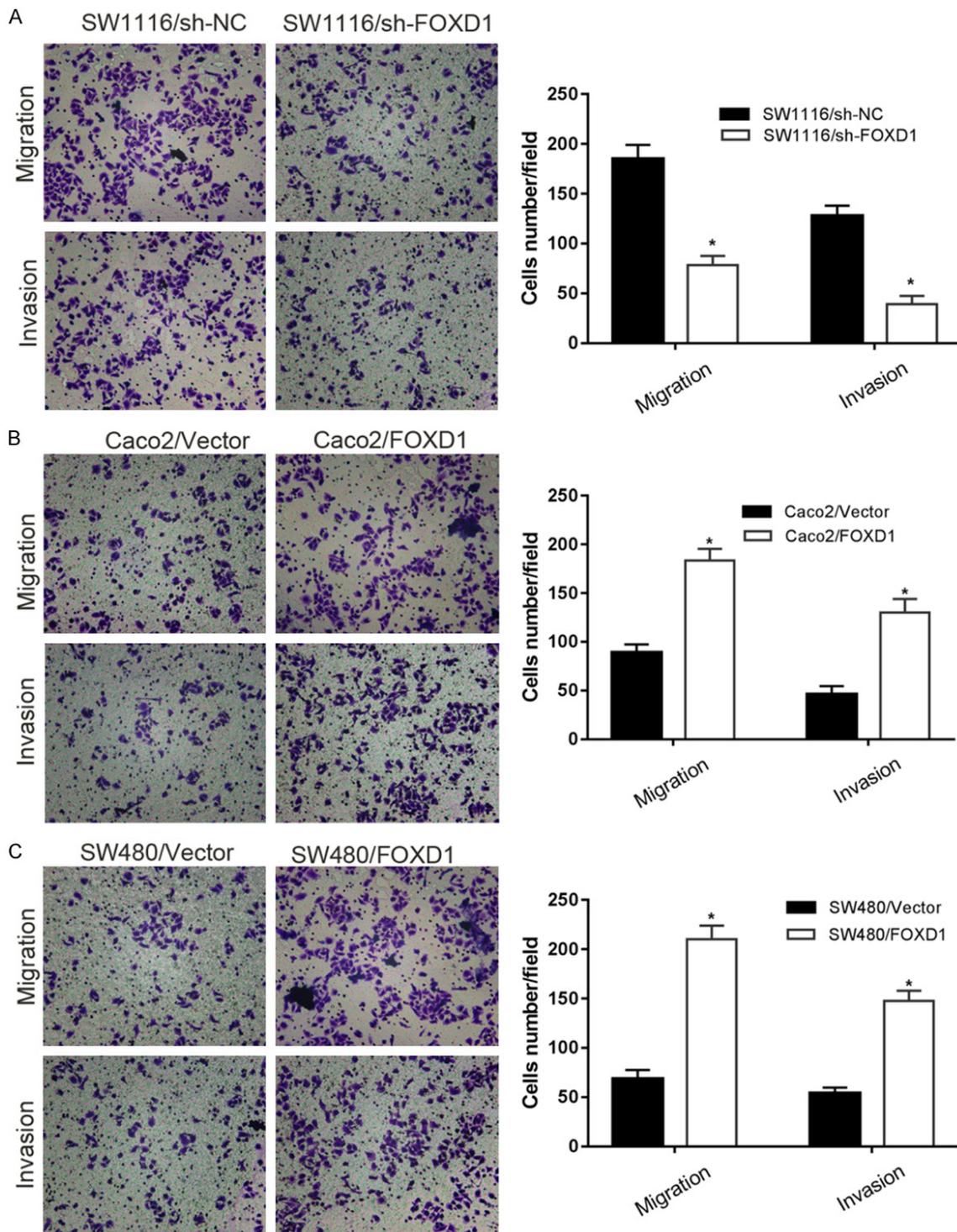
SW480/FOXD1 cells growth attenuated after treatment with U0126 compared with control cells. In addition, after treatment with U0126, Caco2/FOXD1 and SW480/FOXD1 cell migration and invasion were significantly inhibited (Figure 4E, 4F). Taken together, FOXD1 inhibits cell proliferation, migration and invasion via

blockade the activation of the ERK 1/2 signaling pathway.

### Discussion

The carcinogenesis and development of CRC are thought to mainly arise from epigenetic and

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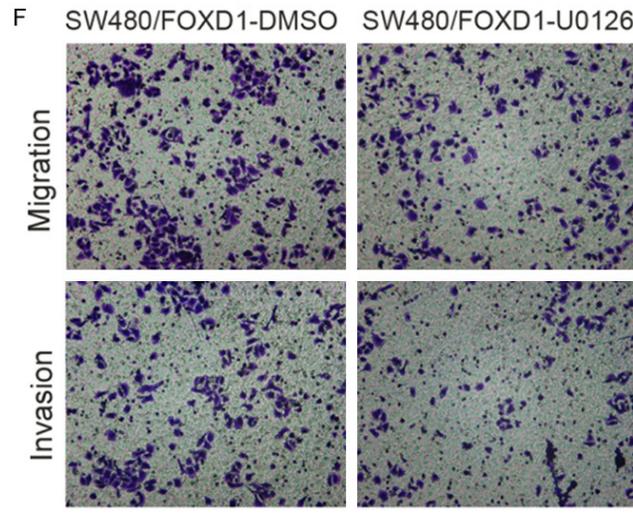
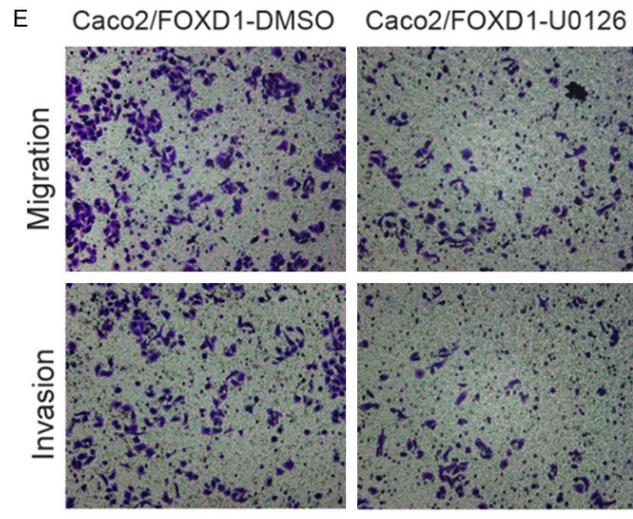
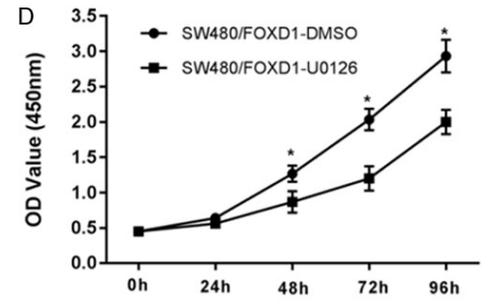
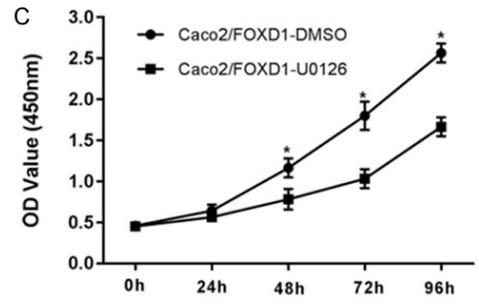
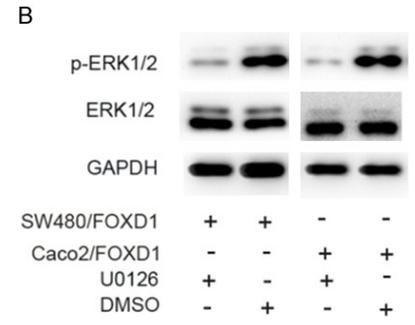
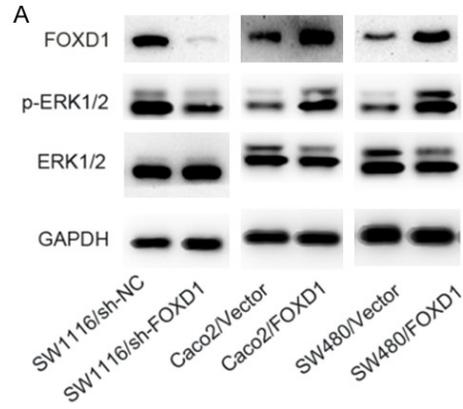


**Figure 3.** FOXD1 enhances migration and invasion of CRC cells. A. Knockdown of FOXD1 inhibited cell migration and invasion of SW1116. B, C. Overexpression of FOXD1 enhanced cell migration and invasion of Caco2 and SW480.

genetic alterations in some key genes that relate to cell proliferation, metastasis and genomic stability, such as p53, PTEN and Her2 [8, 9]. Growing evidences indicate that FOXD

family involved the tumorigenesis and metastasis of CRC [10, 11]. FOXD3 involved the carcinogenesis of colorectal cancer by regulation EGFP-Ras-Raf-MEK-ERK signal pathway [12].

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**Figure 4.** FOXD1 regulates cell proliferation, migration and invasion via activation of the ERK 1/2 pathway. A. Effect of FOXD1 on ERK 1/2 and phosphorylation were measured by Western blot. B. After treatment of U0126 at indicated times, ERK 1/2 and phosphorylation expression were analyzed. C, D. Caco2/FOXD1 and SW480/FOXD1 cell proliferation were examined after treatment with U0126. E, F. Caco2/FOXD1 and SW480/FOXD1 cell migration and invasion were examined after treatment with U0126.

However, the expression and prognostic value of FOXD1 in CRC has not been evaluated to date. In present study, we showed that the expression of FOXD1 was significantly overexpressed in CRC at both mRNA and protein levels in comparison with that in paired non-tumor tissues. High expression of FOXD1 is founded to be significantly correlated poor survival of CRC patients. In addition, a remarkable association between expression of FOXD1 with tumor size, differentiation, invasion depth, lymphatic metastasis and TNM stage was identified, suggesting that CRC cells with high level of FOXD1 have more invasive phenotype. These results implicate that high expression of FOXD1 might be served as a biomarker to identify patients with more aggressive CRC and poorer clinical outcomes.

The biological function of FOXD1 in human cancers has not been completely explored. Only one study reported that overexpression of FOXD1 could inhibit cell proliferation by inducing apoptosis in esophageal, nasopharyngeal and colorectal cancer cells. We established two stable FOXD1 up-regulated cell clones and a down-regulated cell clones to investigate the biological functions and possible mechanisms of FOXD1 in CRC cell lines. Our results revealed that overexpression of FOXD1 in CRC cell lines led to promotion of cell proliferation and clonogenicity. On the contrary, knockdown of FOXD1 induced converse results. We also identified that FOXD1 could regulate the motility and invasiveness of CRC cells. CRC cell migration and invasion were suppressed by FOXD1 knockdown and increased by overexpression FOXD1 in our transwell chamber assay. Proliferation, invasion and metastasis are the basic biological characteristics of cancer [13-16]. Therefore, FOXD1 function as a tumor promoter in CRC.

Previous studies indicate that activation of the ERK 1/2 pathway is needed for proliferation, migration and invasion of tumor and normal cells [17-19]. Here, we report that FOXD1 overexpression promoted activation of the ERK 1/2 pathway, whereas FOXD1 knockdown reversed these changes. In addition, inhibition of the

ERK 1/2 pathway by U0126, a specific ERK 1/2 inhibitor, could partially reverse tumor-inhibitory effects on cell proliferation, migration and invasion caused by FOXD1 overexpression, suggesting an involvement of the ERK 1/2 pathway in regulating FOXD1 function in CRC cells. Thus, FOXD1, as an upstream regulator of ERK 1/2, regulates CRC cell proliferation, migration and invasion by activating the ERK 1/2 pathway.

In summary, here we demonstrated that FOXD1 plays a crucial role in tumor growth, metastasis and prognosis via activation of the ERK 1/2 pathway. Our results provided a foundation for understanding the mechanism of FOXD1 overexpression in CRC progression and suggest that FOXD1 may be a promising therapeutic target for treating CRC.

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### Disclosure of conflict of interest

None.

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