FOXF1 Induces Epithelial-Mesenchymal Transition in Colorectal Cancer Metastasis by Transcriptionally Activating SNAI1[1,2]

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Abstract

Forkhead Box F1 (FOXF1) has been recently implicated in cancer progression and metastasis of lung cancer and breast cancer. However, the biological functions and underlying mechanisms of FOXF1 in the regulation of the progression of colorectal cancer (CRC) are largely unknown. We showed that FOXF1 was up-regulated in 93 paraffin-embedded archived human CRC tissue, and both high expression and nuclear location of FOXF1 were significantly associated with the aggressive characteristics and poorer survival of CRC patients. The GSEA analysis showed that the higher level of FOXF1 was positively associated with an enrichment of EMT gene signatures, and exogenous overexpression of FOXF1 induced EMT by transcriptionally activating SNAI1. Exogenous over-expression FOXF1 functionally promoted invasion and metastasis features of CRC cells, and inhibition of SNAI1 attenuates the invasive phenotype and metastatic potential of FOXF1-overexpressing CRC cells. Furthermore, the results of the tissue chip showed that the expression of FOXF1 was positively correlated with SNAI1 in CRC tissues chip. These results suggested that FOXF1 plays a critical role in CRC metastasis by inducing EMT via transcriptional activation of SNAI1, highlighting a potential new therapeutic strategy for CRC.

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Introduction

Colorectal cancer (CRC) is one of the most malignant tumors worldwide, and the morbidity and cancer-related mortality are among the upper third of all tumors [1]. Metastasis and recurrence are the leading causes of death in CRC, and more than half of patients with CRC have micro-metastases prior to radical surgery, which causes significantly poorer prognosis and higher mortality of CRC patients [2]. The carcinogenesis of CRC is a process with multiple steps, stages

and gene mutations, accompanied by oncogene activation, inactivation of tumor suppressor genes, apoptosis-regulating genes and DNA repair gene changes, as well as increased telomerase activity [3]. The molecular mechanisms underlying the tumorigenesis and metastasis of CRC remain completely unknown, although multiple genes mutations have been implicated in the development of CRC, such as mutational inactivation of APC [4] and TP53 [5] and activation of the β-catenin [6], KRAS and PIK3CA [7,8]. Other than somatic mutations or copy number variations (CNVs), recent studies have revealed that the different expressions of tumor related genes may play a major role in tumorigenesis and progression [9]. The function of most differentially expressed genes in CRC has not yet been fully understood, and developing targeted therapies focused on differentially expressed genes may provide more effective therapeutic strategies for improving CRC survival rate.

Forkhead box F1 (FOXF1) is a differentially expressed gene screened from public CRC GEO dataset (GSE41258) and belongs to the FOX-containing transcription factor family, a highly conserved transcription factor family that possesses a winged-helix DNA binding region (DBD) called the forkhead box. FOXF1 is a critical regulator of angiogenesis during embryonic development [10,11], and mainly expressed in fibroblasts, smooth muscle cells and endothelium [12]. As a transcription factor, FOXF1 binds to the promoter of mitogen-activated protein kinase MAP3K2 and WNK1 to promote prostate cancer [13]. FOXF1 can induce the EMT process by up-regulating lysine oxidase (LOX), down-regulating SMAD2/3, activating the MAPK pathway and promoting breast cancer cell invasion and metastasis in nude mice [14,15]. In lung cancer fibroblasts, FOXF1 promotes the expression of HGF, and the conditional supernatant of cultured lung cancer cell lines expressing FOXF1 can promote cell invasion and metastasis, and its expression in lung cancer fibroblasts is inversely proportional to the survival of the patients [16,17]. In contrast, FOXF1 inhibits hepatocellular carcinoma tumorigenesis and invasion and is negatively correlated with poor outcome [18]. The hypermethylation of the FOXF1 promoter was reported in breast cancer cell lines and demonstrated as a tumor suppressor [19]. These findings suggest that the role of FOXF1 in tumorigenesis is extremely complex and tissue-specific. In a previous study, we showed that FOXF1 expression was up-regulated in CRC; however, the clinicopathological significance of FOXF1 protein and the role of FOXF1 in the progression of CRC have not been well characterized.

Epithelial-mesenchymal transition (EMT) is a biological process by which polar epithelial cells are transformed into mesenchymal phenotypic cells by specifically inducing the down-regulation of epithelial markers (e.g., E-cadherin and β-catenin) and the up-regulation of mesenchymal markers (e.g., N-cadherin and Vimentin) [20,21]. The master regulators to control the gene expression changes of epithelial and mesenchymal phenotype include SNAIL, TWIST and ZEB [22]. The increasing studies have shown that EMT is one of the key steps in initiating tumor cell metastasis and is closely related to the ability of tumor invasion and metastasis [23]. Recent studies have shown that FOXF1 can induce EMT in breast and lung cancer cells to promote invasion and metastasis [15,16]. Similarly, a previous study and analysis of public databases revealed that FOXF1 can also induce EMT in CRC, but the underlying mechanism of FOXF1 inducing EMT remains unclear.

Here, we investigated the expression patterns and potential role of FOXF1 in the tumorigenesis and metastasis of CRC, and explored the potential mechanism of the FOXF1-mediated induction of EMT. The present study may help to provide a new theoretical basis for the individualized diagnosis and treatment of targeted CRC patients.

Methods and Materials

Patients and Tissue Specimens

A total number of 93 paraffin-embedded CRC samples were conducted in this study. All the clinical samples were collected from Nanfang Hospital, Southern Medical University between 2012 and 2014. Prior approval was obtained from Southern Medical University Institutional Broad (Guangzhou, China). Medical records regarding the samples were reviewed to summarize the clinical information in the Supplementary Materials and Methods.

Cell Culture

The human CRC cell lines SW480, RKO, HCT116 and SW837 were purchased from American Type Culture Collection. SW480, RKO, SW837 and HCT116 cells were cultured in RPMI 1640 medium (Gibco). All the medium was added with 10% FBS (Gibco). All the cells were cultured at 37 °C with 5% CO_2.

Real-Time Quantitative PCR, Western Blot and Immunohistochemistry

The Real-time Quantitative PCR (RT-PCR), Western blotting (WB) and immunohistochemistry (IHC) were conducted according to previously described methods [24]. Details are provided in the Supplementary Materials and Methods.

Plasmids

The FOXF1 or SNAI1 construct was generated by sub-cloning PCR-amplified full-length human FOXF1 or SNAI1 cDNA into plasmid plent-EF1α-Flag-puro. To knock down FOXF1 or SNAI1, 2 short hairpin RNA (shRNA) oligo nucleotides were respectively cloned into the pSuper-retro-puro (Oligo-Engine, Seattle, WA, USA)to generate pSuper-retro-FOXF1-shRNA (FOXF1 shRNA#1: 5′-CGAAAGGAGTTTGTCTTCT-3′; FOXF1 shRNA#2: 5′-GCATGATGAACGGCCACTT-3′; SNAI1 shRNA#1: 5′-ACTCAGATGTCAAGAAGTA-3′; SNAI1 shRNA#2: 5′-CCTTCGTCCTTCTCCTCTA-3′). Retroviral production and infection were performed as previously described [25].

Migration, 3-D Cell Culture, Wound Healing and Luciferase Assays

The migration, 3-D cell culture, wound healing and luciferase assays were performed according to previously described methods [24,26,27]. Further details are provided in the Supplementary Materials and Methods section.

Immunofluorescence

For immunofluorescence, cells were seeded on cover slips. Overexpression or shRNA knockdown treatment was performed after 24 h. After indicated treatment, the cells were cultured for 48 h and fixed with 4% formaldehyde for 10 min at room temperature (RT), washed thrice with wash buffer (0.02% Tween20/PBS). Then the cells were permeabilized with 0.5% Triton X-100/PBS for 5–10 min at RT. Washed the cells with wash buffer for three times (5 min for one time), then incubated the cells with 1.5% BSA/PBS solution (blocking solution) for 30 min at RT. Incubated E-cadherin antibody (1:200, Cell Signaling Technology, 3195) in blocking solution at 4°C

overnight. Rhodamine Phalloidin (1:1500, Cytoskeleton, PHDR1) which used for detecting F-catin was incubated in blocking solution at RT in the dark for 60 min. After washing, the cells were incubated with Alexa594-conjugated secondary antibodies for 1 h at RT protected in the dark (Life Technologies, A-21235, 1:500 in blocking buffer) followed by counterstaining with DAPI (Thermo Fisher). Samples were mounted with ProLong Gold antifade reagent (Life Technologies) and imaged on a confocal microscope (Carl Zeiss Jena, LSM 880 with Airyscan).

Chromatin Immunoprecipitation

ChIP assays were carried out using a kit (ACTIVE MOTIF, ChIP-IT Express, catalog # 53008). Briefly, cells (2×10^7) in a 10-cm culture dish were treated with 1% formaldehyde to cross-link chromatin-associated proteins to DNA. The cell lysates were subjected to ultrasound for 9–10 sets of 10-s pulses at 40% output to shear the DNA into fragments between 200 and 1000 bps. Equal cell lysates were respectively incubated with 1 μg of anti-Flag antibody (Sigma) and anti-IgG antibody (Millipore) as negative control. All the above chromatin supernatants were incubated with 20 μL magnetic protein G beads overnight at 4°C with rotation. Second day, the protein-DNA complexes were reversed and purified for pure DNA. The human SNAI1 promoter was amplified with RT-PCR.

Orthotopic Mouse Metastatic Model

A orthotopic implantation mouse model of CRC was examined as previously described [28]. Details are provided in the Supplementary Materials and Methods.

Statistical Analysis

All statistical analyses were performed using SPSS20.0 for Windows. Statistical tests included the Fisher exact test, log-rank test, χ^2 test, ANOVA and Student's t-test. Bivariate correlations between study variables were calculated by Spearman's rank correlation coefficients. Survival curves were plotted by the Kaplan–Meier method and were compared by the log-rank test. Data represent the mean ± SD. $P < .05$ was considered statistically significant.

Results

High Expression of FOXF1 was Associated With Aggressive Characteristics of CRC

Immunohistochemistry staining was used to detect the protein expression level and subcellular localization of FOXF1 in 93 cases of paraffin-embedded CRC tissues. The results showed that the FOXF1 expression was up-regulated in CRC cancer tissues compared to their matched adjacent normal tissues, especially higher in those with lymph node involvement or distant metastasis (Figure 1, A and B). Moreover, FOXF1 displayed as cytoplasmic (41/93, 44.1%) or cytoplasmic/nuclear (52/93, 55.9%) localization in CRC cells (Figure 1C), and high expression level of FOXF1 was positively correlated with cytoplasmic/nuclear localization of FOXF1 ($P < .001$, Supplementary Table S1). Up-regulation of FOXF1 expression was significantly associated more aggressive tumor phenotypes, such as lymph node involvement ($P = .018$) and distant metastasis ($P = .039$, Supplementary Table S2). Furthermore, the nuclear/cytoplasm localization of FOXF1 was highly associated with TNM stage ($P = .041$ for T classification, $P = .001$ for N classification and $P = .025$ for M

classification, Supplementary Table S2). Either up-regulation or nuclear localization of FOXF1 could reduce the overall survival of CRC patients (Figure 1D). Taken together, these observations showed that high expression and nuclear localization of FOXF1 was significantly correlated with invasive tumor phenotype and poorer prognostic of CRC patients.

### Exogenous Up-Regulation of FOXF1 Induced EMT in CRC Cells

Gene set enrichment analysis (GSEA) and GO analysis were used to analyze the FOXF1-regulated gene signatures. The results of the GSEA showed that higher level of FOXF1 was positively associated with an enrichment of EMT gene signatures (GSE17538, GSE41258, GSE13294, GSE42284, and GSE41568) (Figure 2A). These results indicated that FOXF1 might induce EMT through DNA binding during the progression of CRC.

To investigate whether FOXF1 affects EMT in CRC, we established stable FOXF1 over-expression lines in SW480 and HCT116 cells, and FOXF1 knockdown lines in SW837 and RKO cells (Supplementary Fig. S1A), and subsequently observed the morphological changes of the treated CRC cells, and detected the molecular indicators of EMT. The results showed that SW480 cells with FOXF1 ectopic over-expression exhibited weaker and patchier E-cadherin staining, and induced EMT-like morphological features, such as a spindle-shape and more formation of pseudo-foot. In contrast, the RKO cells with FOXF1 knockdown exhibited stronger E-cadherin staining and induced MET-like morphological features, such as an oval-shape and less formation of pseudo-foot (Figure 2B). Consistent with the switch in cellular appearance, FOXF1 overexpression led to a significant reduction in the expression of epithelial markers, such as E-cadherin and ZO-1, as well as an increase in the expression of mesenchymal markers, such as Snail, N-cadherin and Vimentin. The knockdown of FOXF1 increased the expression of E-cadherin and ZO-1 as well as decreased the expression of Snail, N-cadherin and Vimentin (Figure 2C). Taken together, these results suggested that FOXF1 is an enforcer of the mesenchymal phenotype and induces EMT in CRC.

We next explored the transcriptional influence of FOXF1 on the expression of well-established EMT-related genes at 48 h post-transduction of Flag-FOXF1 in SW480 and HCT116 cells (Figure 2D). As expected, overexpression of FOXF1 decreased the expression of epithelial marker CDH1 and increased the mesenchymal expression of markers CDH2, fibronectin and Vimentin. The expression of several master EMT-related transcription factors, such as SNAI1, SNAI2, TWIST1, TWIST2, Zeb1 and Zeb2 were detected, and that SNAI1 was most significantly increased by FOXF1 after transduction, suggesting SNAI1 as the potential direct target of FOXF1 in inducing EMT.

Up-Regulation of FOXF1 Induced EMT by Transcriptionally Activating SNAI1

The results showed that the overexpression or knockdown of FOXF1 could increase or decrease the expression of SNAI1 at both the mRNA and protein levels (Figure 3, A and B). As a transcriptional factor, FOXF1 binds to a target gene with the specific DNA sequence RTAAAYA to promote gene expression [29]. Furthermore, we analyzed the promoter sequence of SNAI1 gene, and detected the sequence of the binding site by FOXF1 (Figure 3C). We hypothesized that FOXF1 might induce the mesenchymal phenotype by directly binding to the SNAI1 promoter to activate its expression.
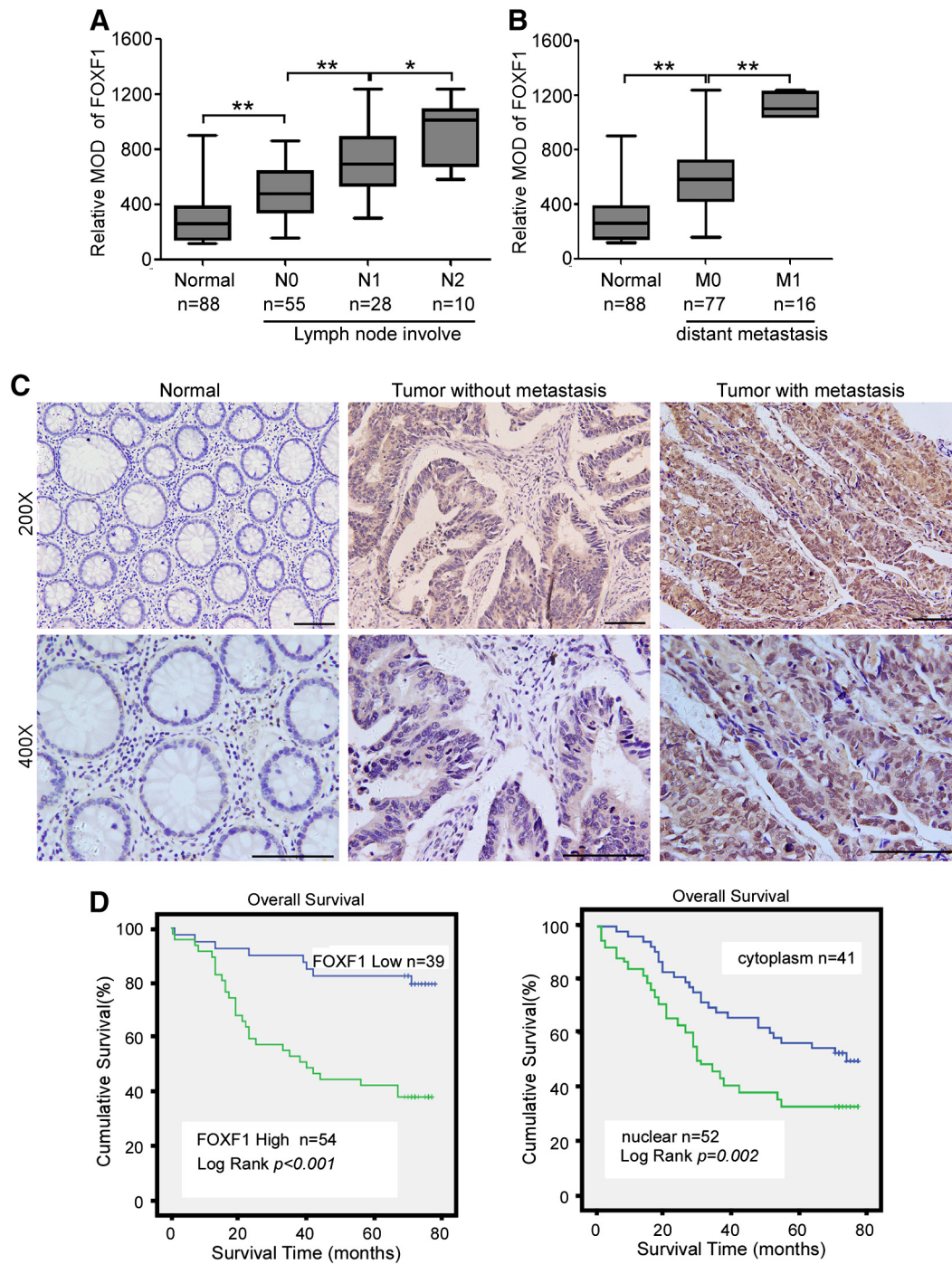
Figure 1. FOXF1 was up-regulated and correlated with poor prognosis in CRC. A and B, The average MOD statistics of correlation between FOXF1 IHC staining expression and N classification or M classification (normal intestines tissues compared to CRC tissues with different degrees of lymph node involve or distant metastasis), $**P < .01$. C, Representative images of IHC indicated the FOXF1 protein expression in normal intestine mucosa tissue, primary tumor collected from patients without metastasis and with metastasis. D, Overall survival of CRC patients with higher expression or nuclear localization of FOXF1.

Therefore, we performed chromatin immunoprecipitation (ChIP) assays of FLAG-FOXF1 in SW480 cells, followed by QPCR of the SNAI1 promoter and upstream regions, and the results revealed that FOXF1 protein could bind to SNAI1 promoter by the candidate site (Figure 3D). Moreover, the results of the dual luciferase reporter assay also revealed that FOXF1 activated the wild-type SNAI1 promoter but not the mutant promoter (Figure 3, E and F).

To further confirm whether the up-regulation of FOXF1 induced EMT by transcriptionally activating SNAI1, we knocked down SNAI1 in FOXF1 over-expressing cells and over-expressed SNAI1 in FOXF1 knockdown cells. The results showed that the ectopic expression of FOXF1 could reduce E-cadherin and increase ZEB1/2, MMP2, MMP9 and N-cadherin. The knockdown of SNAI1 restored the expression of epithelial markers and decreased the level of
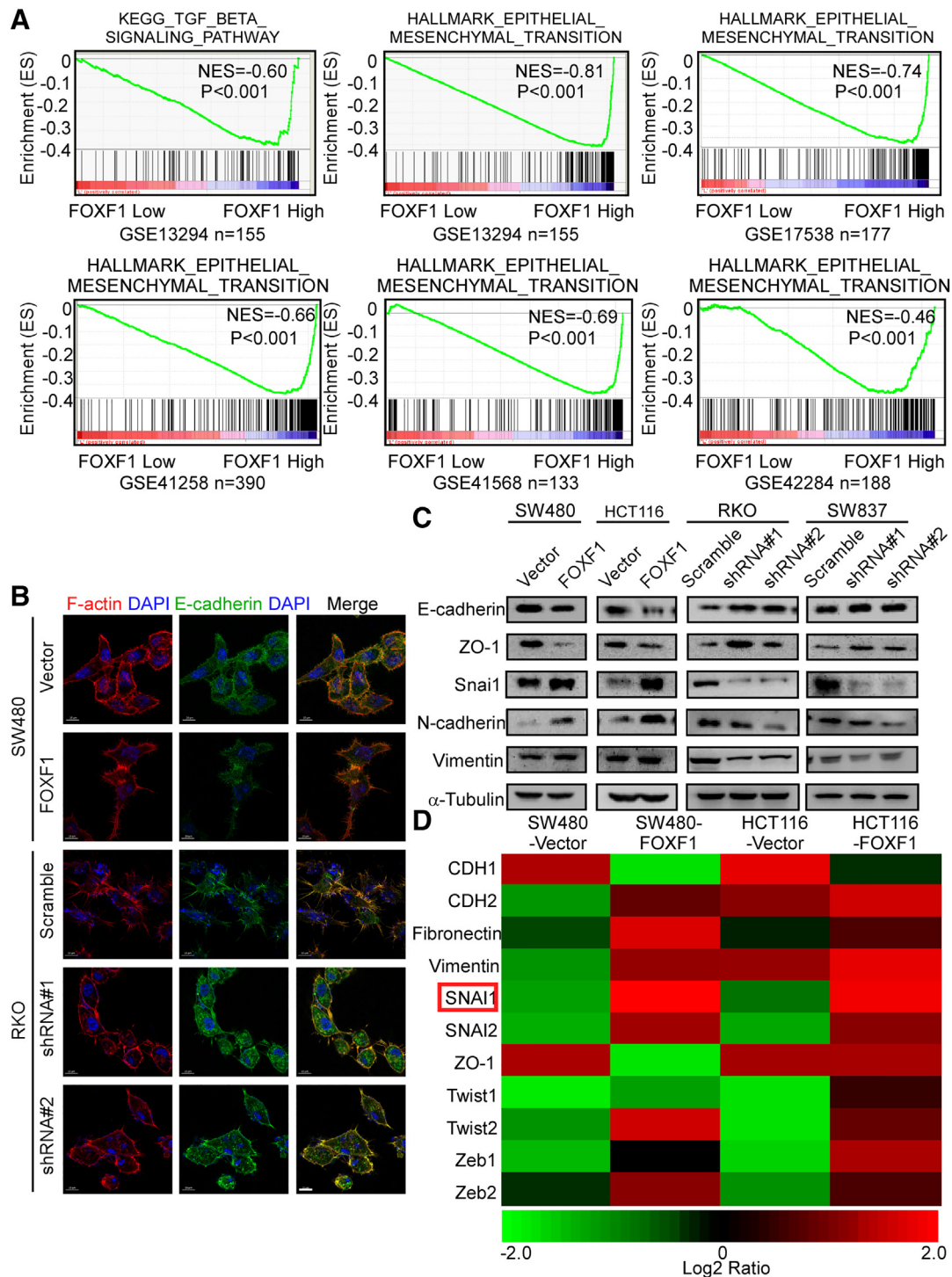
Figure 2. Up-regulation of FOXF1 induced epithelial-mesenchymal transition in CRC. A, GSEA showed positive association between high expression of FOXF1 and the enrichment of EMT-related molecules and pathways (KEGG_TGF_BETA_SIGNALING_PATHWAY and HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION) in published CRC patient gene expression profiles (NCBI/GEO/GSE13294, n = 155; GSE17538, n = 177; GSE41258, n = 390; GSE41568, n = 133; GSE42284, n = 188). B, Immunofluorescence staining of F-actin and E-cadherin in the indicated cells. C, Western blot analysis of EMT-relative gene expression in the indicated cells. α-Tubulin was used as a loading control. D, Heat map analysis indicated an apparent overlap master EMT-related gene expression by RT-PCR in the indicated cells.

mesenchymal markers and proteases, such as ZEB1/2, MMP2, MMP9 and N-cadherin (Figure 3G, left). Conversely, the over-expression of SNAI1 in FOXF1 knockdown cells reduced the expression of E-cadherin and attenuated the expression of ZEB1/2,

MMP2, MMP9 and N-cadherin (Figure 3G, right). Additionally, the silent expression of SNAI1 in SW480-FOXF1 cells exhibited stronger E-cadherin staining and a stronger MET-like morphological appearance, while weaker E-cadherin staining and an aggressive
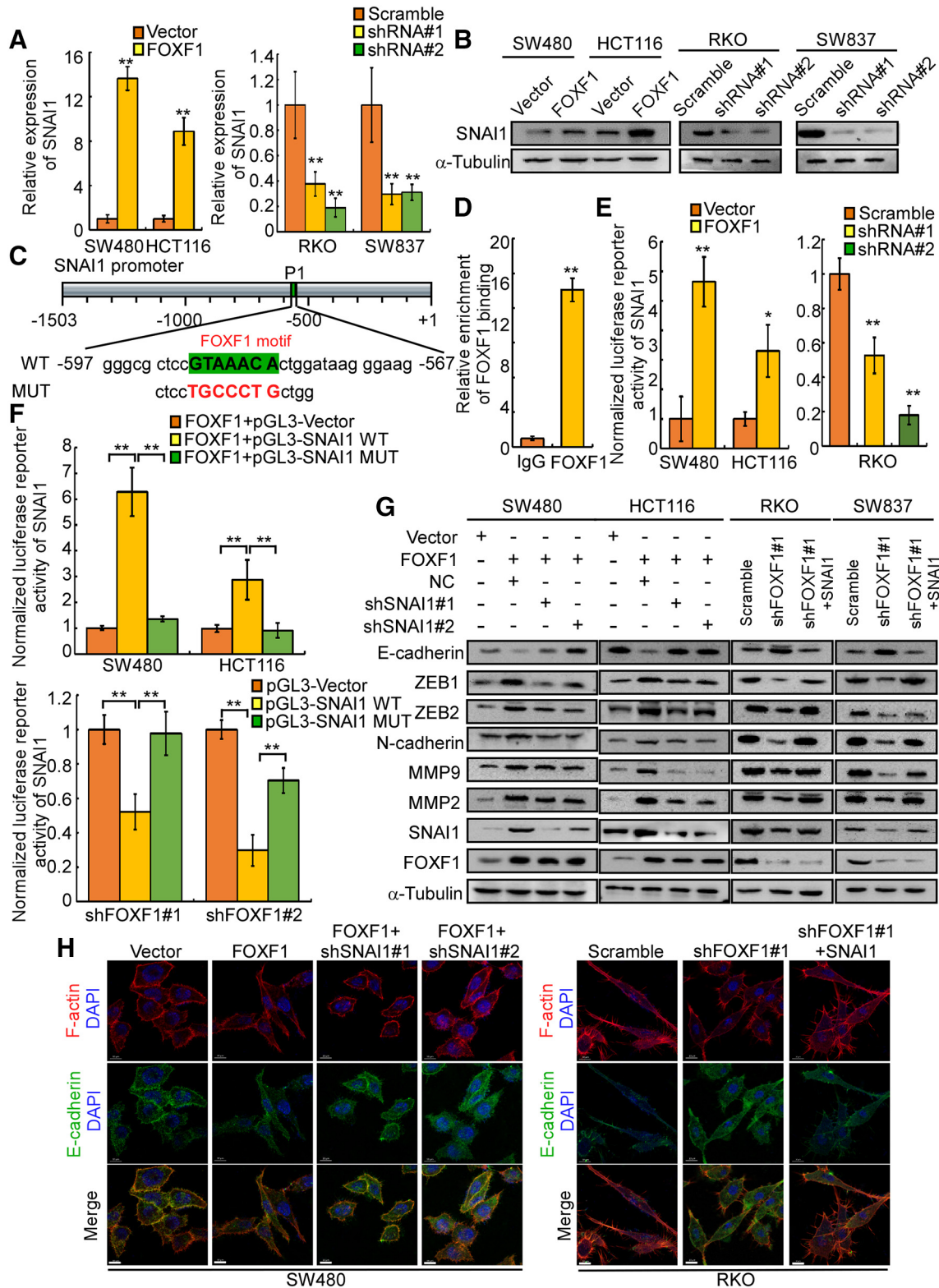
Figure 3. Up-regulation of FOXF1 induced epithelial-mesenchymal transition by transcriptional activation of SNAI1. A and B, Western blot or RT-PCR analysis of SNAI1 in the indicated cells. Expression of mRNA or protein was normalized to GAPDH or α-Tubulin. C, Schematic diagram of the SNAI1 promoter, the position with FOXF1 binding sites is indicated by green rectangles, and the mutated nucleotides of FOXF1 binding motif in SNAI1 promoter are highlighted in red. D, SW480 was transfected with plent-Flag-FOXF1 and ChIP was performed with an anti-Flag antibody to analyze FOXF1 binding to the SNAI1 promoter. RT-PCR experiments were detected with primers against the indicated area in SNAI1 promoter, and the indicated region showed significant enrichment with control of GAPDH. E, Luciferase assay analysis of wild type (WT) SNAI1 promoter-driven luciferase reporter in control or FOXF1-overexpression (left, SW480 and HCT116) or –knockdown (right, RKO) CRC cells. F, Relative expression of WT or mutant (MUT) SNAI1 promoter-driven luciferase reporters in FOXF1-overexpression (top, SW480 and HCT116) or -knockdown CRC cells (bottom, RKO). Error bars represent the mean of 3 independent experiments, $* P < .05$, $** P < .01$. G, Protein expression of E-cadherin, ZEB1, ZEB2, N-cadherin, MMP9, MMP2 and SNAI1 in the indicated cells. α-Tubulin was used as a loading control. H, Expression of F-actin and E-cadherin in the indicated cells by immunofluorescence staining.
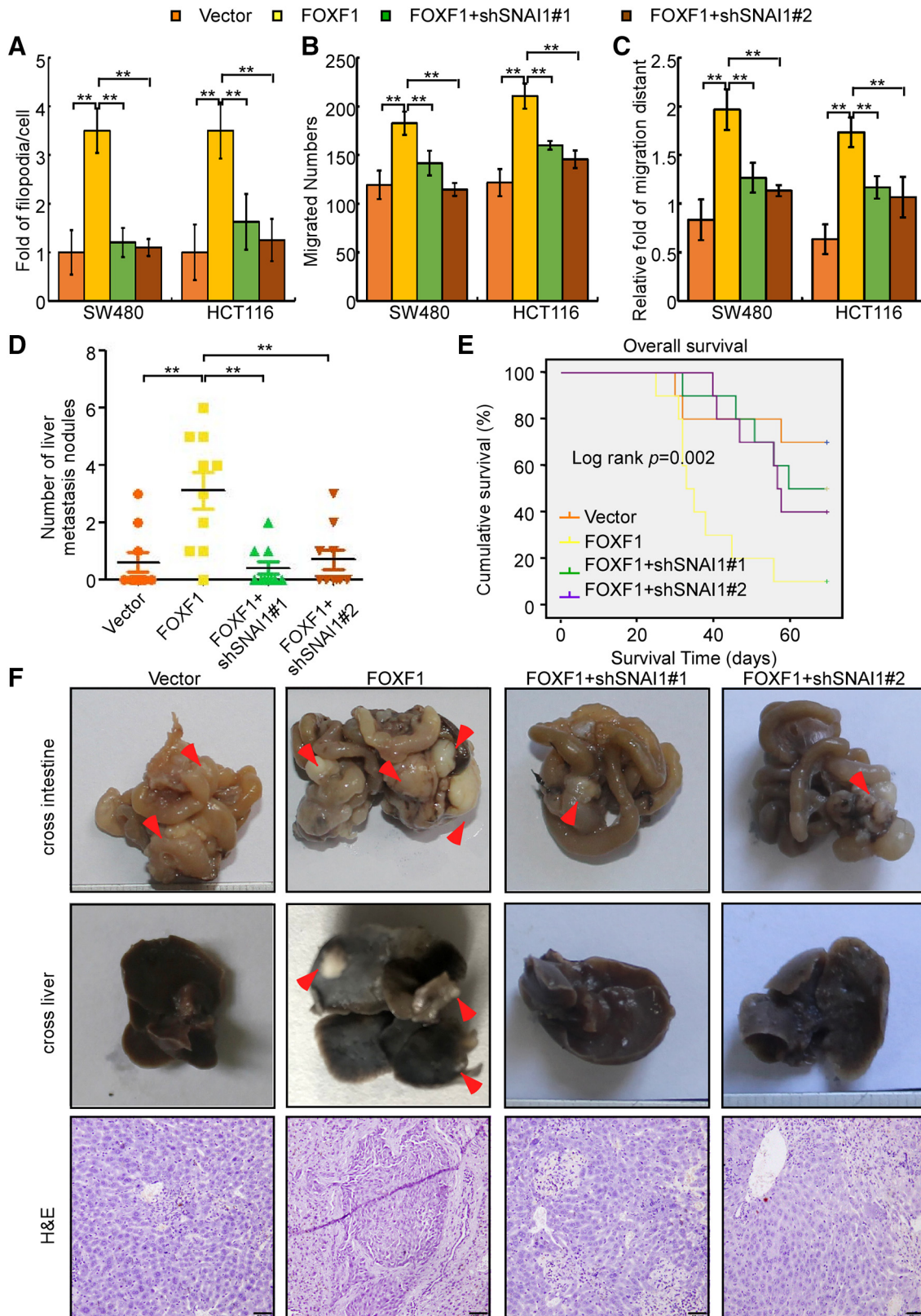
Figure 4. Up-regulation of FOXF1 increased the aggressive features and metastasis in CRC by activating SNAI1 in vitro and in vivo. A, Three-dimensional morphologies of the indicated CRC cells were analyzed under culture on Matrigel. B and C, The migrated and invasive abilities of CRC cells were analyzed by the Matrigel-coated Boyden chamber invasion assay (B) or wound-healing assay (C). The mice were orthotopically transplanted with the indicated cells in the cecum (n = 10 in each group). D, The number of liver metastases as observed in each group. E, Kaplan–Meier analysis of overall survival time for each group (log-rank test, $P < .05$) (right). F, Images of primary tumor in intestines and metastases in livers. Representative image of liver sections were stained with H&E. The primary tumor in the intestines and metastases in the liver were indicated by red arrows. ** $P < .01$.

EMT-like morphological appearance were observed in RKO-shFOXF1-SNAI1 cells (Figure 3H).

Up-Regulation of FOXF1 Promoted the Invasion and Metastasis of CRC Cells by Transcriptionally Activating SNAI1

Next, we detected the effects on invasion and metastasis of CRC cells by FOXF1 up-regulation through transcriptional activation of SNAI1. The results from the 3-D cell culture assay, transwell invasion assay and wound-healing assay revealed that over-expression of FOXF1 significantly promoted the migratory and invasive ability of CRC cells (Figure 4, A–C; Supplementary Fig. S3A and B, left); however, these affections were inhibited after knocking down FOXF1 in the indicated CRC cells (Figure 5, A–C; Supplementary Fig. S3A and B, right). Furthermore, the migratory and invasive abilities of FOXF1 over-expressing cells were partially blocked by silencing SNAI1 (Figure 4, A–C; Supplementary Fig. S2, A–C), while the ectopic expression of SNAI1 could reverse the effect of FOXF1 knockdown on cell migratory and invasive abilities (Figure 5, A–C; Supplementary Fig. S3, A–C).

Orthotopic implantation assay was used to examine the effects of FOXF1 up-regulation on metastasis in CRC by the transcriptional activation of SNAI1. As shown in Figure 4F, increased numbers of tumors in the intestines and the frequency of liver metastases were observed in SW480-FOXF1 cells (Figure 4D, Supplementary Fig. S2D), and the over-expression of FOXF1 also prolonged the overall survival time of nude mice (Figure 4E). However, knockdown of SNAI1 significantly decreased the number of enteral tumors and the frequency of liver metastases as well as reduced the overall survival time of nude mice (Figure 4, D–F); conversely, SNAI1 overexpression enhanced the metastatic ability and extended the overall survival time of nude mice in RKO cells with down-regulation of FOXF1 (Figure 5, D–F and Supplementary Fig. S3D).

Clinical Relevance of the Expression Between FOXF1 and SNAI1 in Human CRC Tissues

As mentioned above, the up-regulation of FOXF1 promoted the invasion and metastasis of CRC cells by transcriptionally activating SNAI1. To further verify this result, we examined the clinical relevance of the expression between FOXF1 and SNAI1 in human CRC tissues. RT-PCR and Western blot analyses indicated that FOXF1 was positively correlated with the level of SNAI1 mRNA and protein in 16 freshly collected CRC biopsies (Figure 6A, r = 0.935, $P < .001$; Figure 6B, r = 0.587, $P = .017$, Supplementary Fig. S4A). Moreover, the mRNA expression of FOXF1 was still positively correlated with SNAI1 in 40 CRC biopsies (Supplementary Fig. S4B, r = 0.669, $P < .001$). Furthermore, analysis of a tissue chip (including 80 CRC tissue and 5 normal colon tissue specimens) revealed a consistently positive correlation between the expression of FOXF1 and SNAI1 (Figure 6C, Tissue chip, r = 0.707, $P < .001$ and Supplementary Fig. S4C). In addition, analysis of the TCGA database still showed a consistent correlation between FOXF1 and SNAI1 (Supplementary Fig. S4D, r = 0.41, $P < .05$). Taken together, these data support the opinion that the up-regulation of FOXF1 directly transcriptionally activates SNAI1, subsequently induces EMT, and finally leads to the invasion and metastasis of CRC cells (Figure 6D).

Discussion

Metastasis is a multistep and complex process that requires local invasion, intravasation, survival in circulation, and subsequent extravasation and colonization. During cell progression, multiple driven genes and the abnormal activation of complex signal pathways are involved, representing a major obstacle to treating cancer. Not only somatic mutations, such as APC, KRAS or TP53 but also a large number of differentially expressed genes play an important role in tumorigenesis and progression in CRC [9].

FOXF1, a member of the forkhead box family, is a differentially expressed gene that has been screened by the public CRC GEO database. Several studies have shown that many members of the FOX family participate in the progression of human cancers. For example, FOXO proteins play critical roles in proliferation, apoptosis, metastasis, cell metabolism, aging and cancer biology in multiple type of malignancies [30]. The overexpression of FOXM1 has an important role in the progression of multiple types of malignant tumor, such as breast cancer [31], CRC [32] and gastric cancer [33]. Additionally, in a previous study, we revealed that FOXC2 could promote progression and metastasis by the activation of the HGF-MET and MAPK-AKT signaling pathway in CRC [34,35]. Recent research reveals that FOXF1 could control the transcription of KIT and ETV1 to promote the progression of gastrointestinal stromal tumor [36]. Altogether, the dysfunction of FOX gene protein could influence the cell fate, tumorigenesis and metastasis cancer [37]. However, the functional role and downstream targets of FOXF1 have been limited characterized. Our present study revealed that both high expression and nuclear/cytoplasm localization of FOXF1 were positively associated with aggressive phenotype and poorer outcomes of CRC, indicating that FOXF1 might contribute to the progression and metastasis of CRC.

FOXF1 is normally expressed in mesenchymal cells [38] and is important for mesoderm differentiation, vasculogenesis [39] and organogenesis [40]. FOXF1 promotes EMT in breast cancer [15] or lung cancer [16] to promote cell invasion and metastasis. Several FOX transcription factors (FOX TFs) induce EMT (e.g., FOXC1, FOXC2 and FOXQ1), whereas some FOX TFs play an opposite role in the progress of EMT (e.g., FOXM1) [37,41]. Our results show that the up-regulation of FOXF1 could promote invasion and metastasis by in vivo and vitro experiments in CRC, but the underlying molecular mechanisms remain unknown. Interestingly, we found that the high expression of FOXF1 was also associated with the induction of EMT, a fundamental process in cancer invasion and metastasis [42]. In invasive tumors, there are several EMT-induced abnormal activation of genes and signaling pathways, including the Snail / Slug family of transcription factors, the Twist transcription factor family, and the TGF-β signaling pathways [20]. This study revealed that FOXF1 induced EMT in CRC by decreasing the expression of epithelial markers, such as E-cadherin and ZO-1, as well as increasing the levels of mesenchymal markers, N-cadherin and Vimentin. The up-regulation of FOXF1 generated multiple mesenchymal characteristics in CRC cells and promoted invasion and metastasis both in vitro and in vivo. The results suggest that the up-regulation of FOXF1 facilitates the metastasis of CRC by inducing of EMT.

In the present study, the up-regulation of FOXF1 could induce EMT in CRC, but the specific molecular mechanisms by which FOXF1 induces EMT require further discussion. As a transcription factor, FOXF1 binds to the promoter of the target genes to regulate gene expression and the activity of multiple signaling pathways that
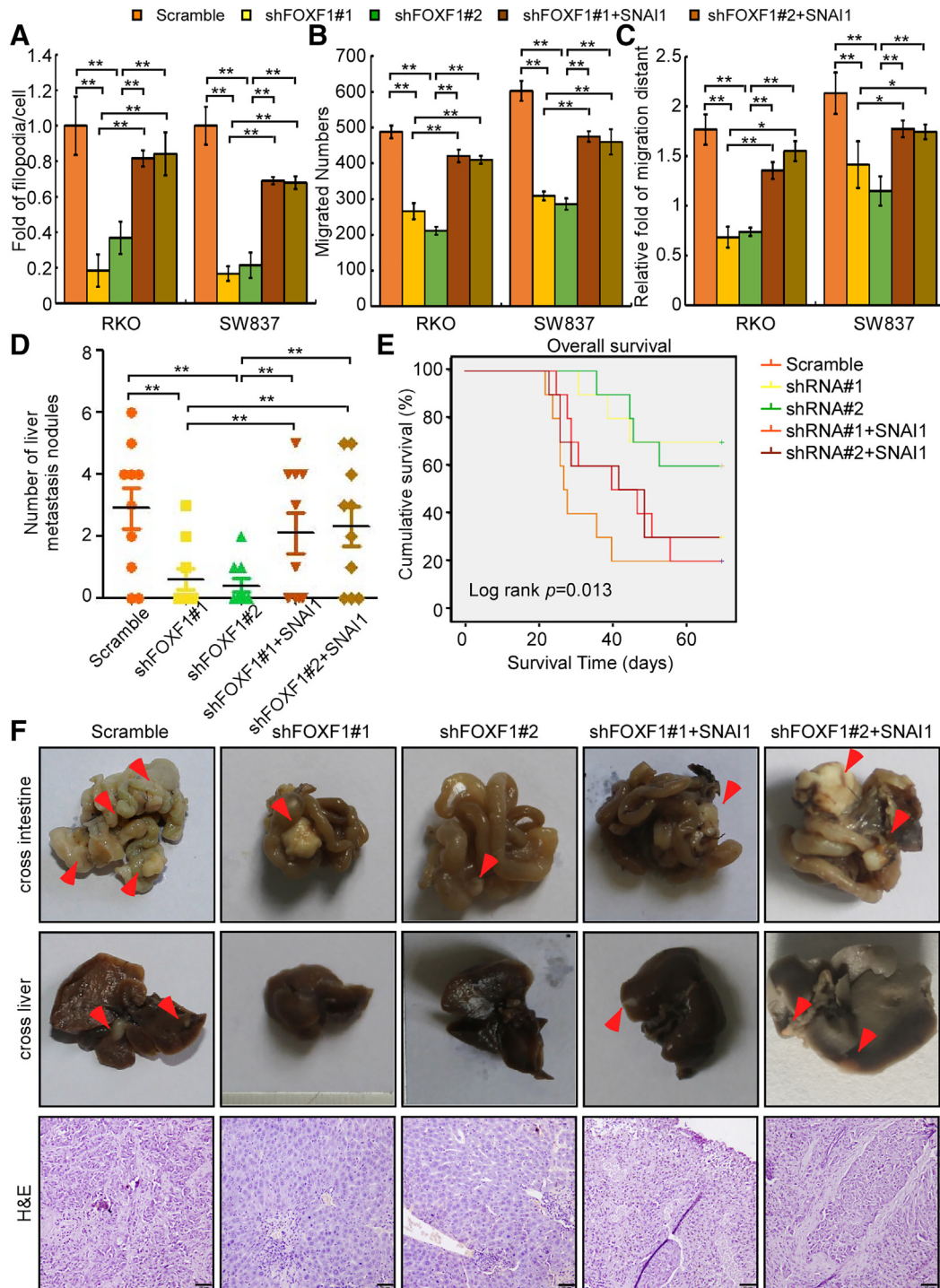
Figure 5. Down-regulation of FOXF1 inhibited the aggressive features and metastasis in CRC by suppressing SNAI1. A, Three-dimensional morphologies of the indicated CRC cells were examined. B, The migrated and invasive abilities of CRC cells were detected by the Matrigel-coated Boyden chamber invasion assay. C, The relative migration distance was determined by the wound-healing assay. D and E, In orthotropic transplantation assay, the number of liver metastases were determined by microscope in each group (D) and Kaplan–Meier analysis of mice outcome in each group (log-rank test, $P < .05$) (E). F, Representative images and histopathology of primary tumor and metastases in orthotropic transplantation assay of the intestines and livers. The liver metastases were under H&E staining. Arrows indicate the primary tumors in intestines and metastases in the livers. ** $P < .01$.

play an important role in physiological or pathological processes [10,11,13,43], suggesting that FOXF1 might directly regulate master EMT-related genes to induce EMT in CRC. The SNAI1 protein has been identified as a master transcription regulator of EMT [44]. As a highlighted inducer of EMT, SNAI1 recruits other factors (ex. PRC2)

to promote histone hypermethylation and deacetylation [45], and finally represses epithelial genes, such as E-cadherin [46,47]] to facilitate cellular detachment and enhance mesenchymal genes, such as N-cadherin and Zeb [48], and proteases, such as MMP2 and MMP9, to facilitate an invasive phenotype [49–51]. The present
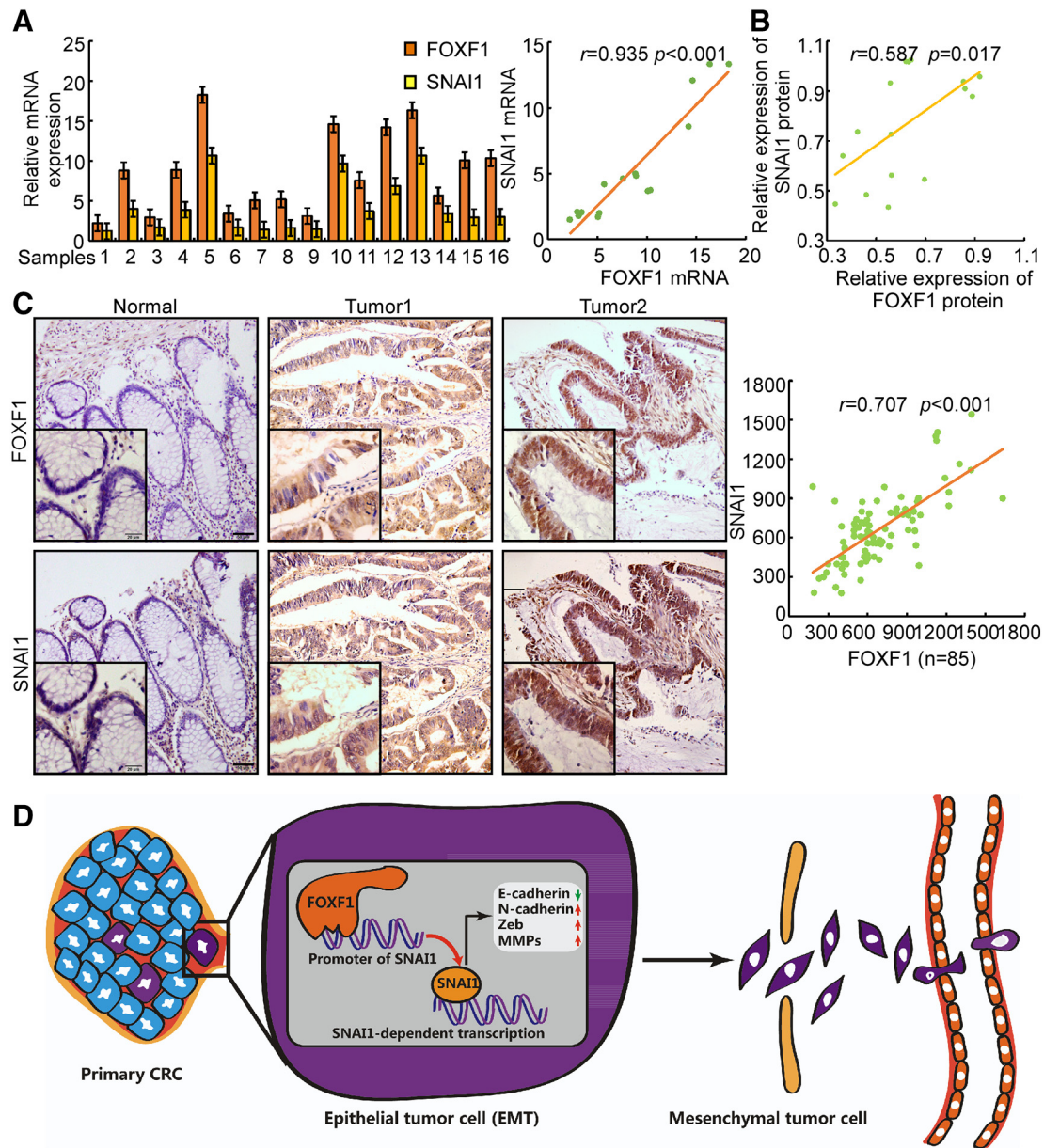
Figure 6. FOXF1 expression in CRC tissue was positively correlated with SNAI1. A, Expression of FOXF1 and SNAI1 mRNA in 16 fresh human CRC samples. Error bars represent the mean ± SD of 3 parallel experiments. The left graph represents the relationship by Spearman's correlation. B, Correlation analysis of the FOXF1 and SNAI1 protein expression in 16 fresh CRC samples by analysis of gray level with Quantity One software. C, The IHC staining of FOXF1 and SNAI1 in 80 CRC and 5 normal intestinal epithelium tissue arrays showed a positive correlation between FOXF1 and SNAI1. Four representative images are shown in left. Spearman's correlation analysis of average MOD of staining in 85 specimens was used to show the correlation between FOXF1 and SNAI1 in right. D, Model: Up-regulation of FOXF1 transcriptional activation SNAI1 and induces EMT, finally increasing the invasion and metastasis of CRC.

study revealed that FOXF1 could transcriptionally bind to the promoter of SNAI1, with the specific sequence RTAAAYA, and promote the transcriptional activation of SNAI1. By targeting on SNAI1, FOXF1 facilitates the EMT to promote the invasion and metastasis of CRC both in vitro and in vivo. Overall, we demonstrated a new mechanism of inducing EMT by targeting the master regulator SNAI1 during the metastasis of CRC.

In conclusion, the present study shows that the expression of FOXF1 is up-regulated in CRC tissues, and its high expression or nuclear/cytoplasm localization is associated with the advanced progression and poorer diagnosis of CRC. Furthermore, FOXF1 promotes invasion and metastasis by inducing EMT in CRC

progression through transcriptionally activating SNAI1. These data highlight the FOXF1-SNAI1 axis as a potential therapeutic target in CRC progression.

Conflict of Interests
The authors declare that they have no competing interests.

Author Contributions
YYP, DYQ and JHL designed the experiments; WSY, YSS and ZSW analyzed the data, wrote the manuscript, and directed the research. XZY, ZYL, BJX, and QJF performed experiments. ZD, ZLJ and HCM conducted vector construction. HZX and YJY directed animal

study and primary cell culture. LTT, LL and LWT provided research materials and methods.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.08.004.

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