

E2F1/SP3/STAT6 axis is required for IL-4-induced epithelial-mesenchymal transition of colorectal cancer cells

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Abstract. Colorectal cancer (CRC) is a type of cancer with a mortality rate among the highest worldwide owing to its high rate of metastasis. Therefore, inflammation-associated metastasis in the development of CRC is currently a topic of considerable interest. In the present study, the pro-inflammatory cytokine interleukin-4 (IL-4) was identified to promote the epithelial-mesenchymal transition (EMT) of CRC cells. However, the enhancing effect of IL-4 was more evident in HCT116 cells compared with in RKO cells. Accordingly, an increased expression level of STAT6 was observed in HCT116 cells compared with RKO cells. Further investigations identified that E2F1 was required for maintaining the level of signal transducer and activator of transcription 6 (STAT6) in HCT116 cells. Mechanistically, E2F1 induced specificity protein 3 (SP3) directly by binding to the promoter of the STAT6 gene and activating its transcription in CRC cells. As a result, phosphorylation-activated STAT6 increased the expression of several EMT drivers, including zinc finger E-box-binding homeobox (Zeb)1 and Zeb2, which serve a critical function

in IL-4-induced EMT. Rescue experiments further confirmed that IL-4-induced EMT relied on an intact E2F1/SP3/STAT6 axis in CRC cells. Finally, analysis of clinical CRC specimens revealed a positive correlation between E2F1, SP3 and STAT6. The ectopically expressed E2F1/SP3/STAT6 axis indicated a poor prognosis in patients with CRC. In conclusion, the E2F1/SP3/STAT6 pathway was identified to be essential for IL-4 signaling-induced EMT and aggressiveness of CRC cells.

Introduction

Colorectal cancer (CRC) is one of the most common causes of malignancy-associated mortality, and tumor metastasis is the predominant component that leads to disease recurrence and mortality in patients with CRC (1,2). Previous studies have demonstrated that chronic intestinal inflammation such as inflammatory bowel disease is associated with the risk of CRC, and the inflammatory microenvironment around the tumor serves a central function in the development and progression of CRC (3-5). Various pro-inflammatory cytokines released by infiltrating immune cells and other cells in the microenvironment have been identified to induce CRC metastasis (6). In particular, among these cytokines, including interleukin (IL)-6, IL-8, IL-13 and IL-17 are involved in the pathogenesis of CRC metastasis (7-10). In addition, previous studies have focused on the overactivation of IL-4 signaling in association with CRC cell invasiveness and metastasis. In CRC cells expressing the IL-4 receptor (IL-4R), IL-4 stimulation downregulates the expression of the cell adhesion molecule epithelial (E)-cadherin, suggesting that the cytokine may be involved in the epithelial-mesenchymal transition (EMT) of CRC (11). Additionally, IL-4R α appears to promote CRC growth, and blockade of the IL-4R α -IL-4 interaction decreases proliferation and increases apoptosis (12).

IL-4 initiates the signaling pathway by binding to IL-4R α , triggering a cascade, including the activation of Janus kinase (JAK)1 and JAK3, which phosphorylates and dimerizes signal transducer and activator of transcription 6 (STAT6).

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Finally, STAT6 homodimers translocate to the nucleus and bind to the promoters of IL-4-responsive genes to induce various biological functions (13). Therefore, STAT6 mediates signal transduction and determines the cellular response to IL-4 stimulation. However, abnormal STAT6 activation induced by IL-4 promotes pro-metastatic processes including migration and invasion, proliferation, and survival in cancer cells. For example, in colorectal cancer stem cells (CR-CSCs), the IL-4/STAT6 signaling pathway helps cells to escape cell death by increasing the nuclear survivin pool (14). In addition, Liu *et al* (15) identified that the IL-4/STAT6 signaling pathway accelerates CRC cell proliferation through enhancing the expression of nicotinamide-adenine dinucleotide phosphate oxidase 1.

Although E2F1 is recognized as a strong apoptosis-driver following chemotherapy-induced DNA damage in all types of human cancer, evidence suggests that E2F1 is associated with cancer progression. Increased abundance of E2F1 in various cancer cells has been identified to trigger invasion and metastasis by activating cytokine receptor signaling pathways. In malignant melanoma, E2F1-dependent progression is mediated via upregulation of epidermal growth factor receptor (EGFR) and activation of the mitogen-activated protein kinase/extracellular-signal-regulated kinase and phosphoinositide 3-kinase/protein kinase B signaling cascades (16). Other evidence indicates that E2F1 transactivates integrin-linked kinase, which drives the IL-6/nuclear factor- κ B (NF- κ B) signaling loop in triple-negative breast cancer (17). In our previous study, different expression levels of E2F1 were detected in several CRC cell lines, and knockdown of E2F1 was identified to impair the aggressive phenotypes of CRC cells with highly expressed E2F1 (18). Therefore, whether E2F1 contributes to CRC development promoted by inflammatory cytokines requires further investigation.

In the present study, a previously unknown function of E2F1 as an enhancer in the IL-4/STAT6 signaling pathway was identified. The results supported the hypothesis that E2F1 is able to induce STAT6 expression by upregulating specificity protein 3 (SP3), which was identified as a transcription activator of the STAT6 gene in CRC cells. An increase in total STAT6 protein led to a strong response to IL-4 stimulation, as indicated by a high level of STAT6 phosphorylation. Finally, as targets of the activated STAT6, zinc finger E-box-binding homeobox (Zeb)1 and Zeb2 boosted EMT and aggressiveness of CRC cells. Thus, the existence of an aberrant E2F1/SP3/STAT6 axis may amplify the IL-4 signaling which facilitates CRC metastasis.

Materials and methods

Cell lines and culture. Human CRC cell lines SW480, HCT116, RKO, HT-29 and DLD1 were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured and stored according to the supplier's protocol. In detail, all the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% CO₂. Cell passage was performed every 2 days. Short hairpin RNA targeting E2F1 (shE2F1, 5'-CUGCAGAGCAGAUGGUUAU-3'; GenePharma,

Shanghai, China)-stably transfected HCT116 cells were derived from the parental cells using G418 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) selection. Human recombinant IL-4 (R&D Systems, Minneapolis, MN, USA) at 20 ng/ml was used to treat CRC cells. Cell morphology was visualized by phase-contrast microscopy (magnification, x400).

Reagents and antibodies. Recombinant human IL-4 was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Antibodies against E-cadherin (cat. no. sc-7870; 1:1,000), vimentin (cat. no. sc-6260; 1:1,000), E2F1 (cat. no. sc-193; 1:1,000) and GAPDH (cat. no. sc-365062; 1:5,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against zonula occludens-1 (ZO-1; cat. no. ab59720; 1:500), fibronectin (cat. no. ab2413; 1:1,000) and SP3 (cat. no. ab129099; 1:1,000) were purchased from Abcam (Cambridge, UK). Antibodies against phosphorylated (p-)JAK1 (cat. no. 3331; 1:500), JAK1 (cat. no. 3332; 1:1,000), p-STAT6 (cat. no. 9361; 1:500) and STAT6 (cat. no. 9362; 1:1,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from the CRC cell lines (HCT116, RKO, HT-29 or DLD1) with RNAiso Plus (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. RNA quality and concentration was evaluated using a NanoDrop ND-1000 spectrophotometer. A total of 0.5 μ g RNA was reverse-transcribed using a cDNA Reverse Transcription kit (Takara Bio, Inc.), according to the manufacturer's protocol. The resulting cDNA was analyzed in triplicate using qPCR (95°C for 5 min, followed by 40 cycles of 95°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min) on a Step One Plus Real-Time PCR system (Thermo Fisher Scientific, Inc.) with SYBR-Green Master Mix (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. Target gene expression was normalized to GAPDH levels in respective samples as an internal control and calculated using the 2^{- $\Delta\Delta C_q$} method (19). The sequences of the qPCR primers are listed in Table I.

Western blotting. Cells were harvested and lysed using radio-immunoprecipitation lysis buffer [50 mM Tris/HCl, pH 7.4, 100 mM 2-mercaptoethanol, 2% (w/v) SDS and 10% glycerol]. The protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The proteins (40 μ g/lane) were separated by SDS-PAGE (10 gels) and transferred onto nitrocellulose membranes (Whatman, Maidstone, UK). The membrane was blocked with 5% non-fat milk powder (diluted in Tris-buffered saline with Tween-20) for 2 h at room temperature and then incubated with diluted primary antibodies followed by IRDye® 800CW- or IRDye 680RD-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA): IRDye 680RD goat anti-rabbit immunoglobulin G (IgG) [heavy and light chains (H + L)] (cat. no. 926-68071), IRDye 800CW goat anti-rabbit IgG (H + L) (cat. no. 926-32211), IRDye 680RD goat anti-mouse IgG (H + L) (cat. no. 926-68070) and IRDye 800CW goat anti-mouse IgG (H + L) (cat. no. 926-32210). All the secondary antibodies were diluted at 1:20,000, and detection

Table I. Sequences of qPCR primer pairs.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
STAT6	AGGTGTACCCACCACACTCT	GGTCACATCTGAGCAGAGCA
Slug	CAGTATGTGCCTTGGGGGAG	AGGCACTTGGAAAGGGGTATTG
Zeb1	AGAGCGCTAGCTGCCAATAA	GGGCGGTGTAGAATCAGAGT
Zeb2	GGCCTACACCTACCCAACCTG	ACAGGAGTCGGAGTCTGTCA
Snail1	CCTGTCTGCGTGGGTTTTTG	ACCTGGGGGTGGATTATTGC
Twist1	GCATTCTCAAGAGGTCGTGC	GGTTTTGCAGGCCAGTTTGAT
GAPDH	ATGGGGAAGGTGAAGGTCGGAGT	TGACAAGCTTCCC GTTCTCAGCC

STAT6, signal transducer and activator of transcription 6; Zeb, zinc finger E-box-binding homeobox.

was performed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The obtained signals were converted into grayscale images (Application Software, version 2.1.12; LI-COR Biosciences).

Luciferase assay. In total, 1×10^5 HCT116 cells were plated onto 24-well plates. The next day, cells were co-transfected with firefly luciferase reporter constructs and pRL-SV40 *Renilla* luciferase reporter plasmids (Promega Corporation, Madison, WI, USA). The pRL-SV40 plasmid was used to normalize the transfection efficiency. The luciferase activities were measured using a dual-luciferase reporter assay system (Promega Corporation) and a luminometer (LB 9507; Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany). All experiments were performed in triplicate.

Chromatin immunoprecipitation (ChIP). Chromatin was cross-linked with 1% formaldehyde and ChIP assays were performed using a SimpleChIP® Enzymatic Chromatin IP kit (Cell Signaling Technology, Inc.; cat. no. 9003), according to the manufacturer's protocol. The immunoprecipitated DNA fragments were quantified using RT-qPCR using the following primer pairs for the STAT6 promoter: -1,300 bp, 5'-TGTTTAGCAGCATTAGC AGG-3' (forward) and 5'-CAGCTCCTCCTCCTCCATCC-3' (reverse); -400 bp, 5'-TCAGTCCAAGGGACTCCTAG-3' (forward) and 5'-GCTCGATGCCAGCCAACCTCC-3' (reverse); +500 bp, 5'-GGTTTCAGCTAGTGTGGTG-3' (forward) and 5'-GAGGGGCACTCCTTCACCTC-3' (reverse).

Wound healing assay. Cells were seeded in 6-well plates. Following transfection for 24 h, the monolayer was gently and slowly scratched with a pipette tip across the center of the well. Following scratching, the well was gently washed several times with PBS to remove the detached cells. The wells were replenished with fresh medium without serum, and the cells were allowed to grow for an additional 48 h, and the images of the stained monolayer were captured at x40 magnification under a light microscope (Eclipse Ti, Nikon, Kyoto, Japan). The wound was evaluated using ImageJ (version 1.4.3.67; National Institutes of Health, Bethesda, MD, USA).

Cell invasion assays. Cell invasion was assessed in Boyden chambers with Matrigel (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. First, an

8-mm-porosity polycarbonate membrane was covered with 200 μ l serum-free medium containing 1×10^5 cells/well. The plates were then incubated with 10% FBS for 48 h at 37°C in a humidified atmosphere containing 5% CO₂. The invading cells on the lower surface of the filter were fixed, stained and counted under a light microscope (Eclipse Ti) at x100 magnification.

Xenografts. Animal experiments were performed with the approval of the Ethics Committee of Sanmen People's Hospital (Sanmen, China) and the Animal Care and Use Committee of Zhejiang University (Hangzhou, China). A total of 28 female BALB/c nude mice (5-week-old; weight, 18 g; housed at 25°C with filtered air by efficiency particulate air filters with 40-60% humidity on a 10-h light/14-h dark cycle, with; free access to food and water) were purchased from the Laboratory Animal Center of Zhejiang University and randomly divided into HCT116/shCtrl, IL-4-HCT116/shCtrl, HCT116/shE2F1 and IL-4-HCT116/shE2F1 groups (n=7 per group), where shCtrl is control short hairpin RNA (5'-UUC UCCGAACGUGUCACGU-3'; GenePharma, Shanghai, China), and equal amounts of indicated cells (5×10^6) were injected subcutaneously into each mouse. IL-4 was administered by intraperitoneal injection at a dose of 10 μ g/kg three times weekly for 2 weeks. Treatment began on day 7, when the tumor sizes were measurable. The tumors were examined every 2 days, the length and width measurements were obtained with calipers, and the tumor volumes were calculated. On day 21, the animals were euthanized using decapitation under Midazolam anesthesia (5 mg/kg), and the tumors were excised and weighed. The IL-4/STAT6 signaling in the tumor tissues was analyzed by immunohistochemistry.

Immunohistochemistry. Experiments using human tissues were performed with the approval of the Ethics Committee of Sanmen People's Hospital. In total, 218 human CRC samples were collected at Sanmen People's Hospital, and written informed consent was obtained from all patients. Immunohistochemistry was performed and analyzed as described in our previous study (20).

Statistical analysis. Statistical analysis was performed with SPSS software (version 22.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 5.0; GraphPad Software, Inc.,

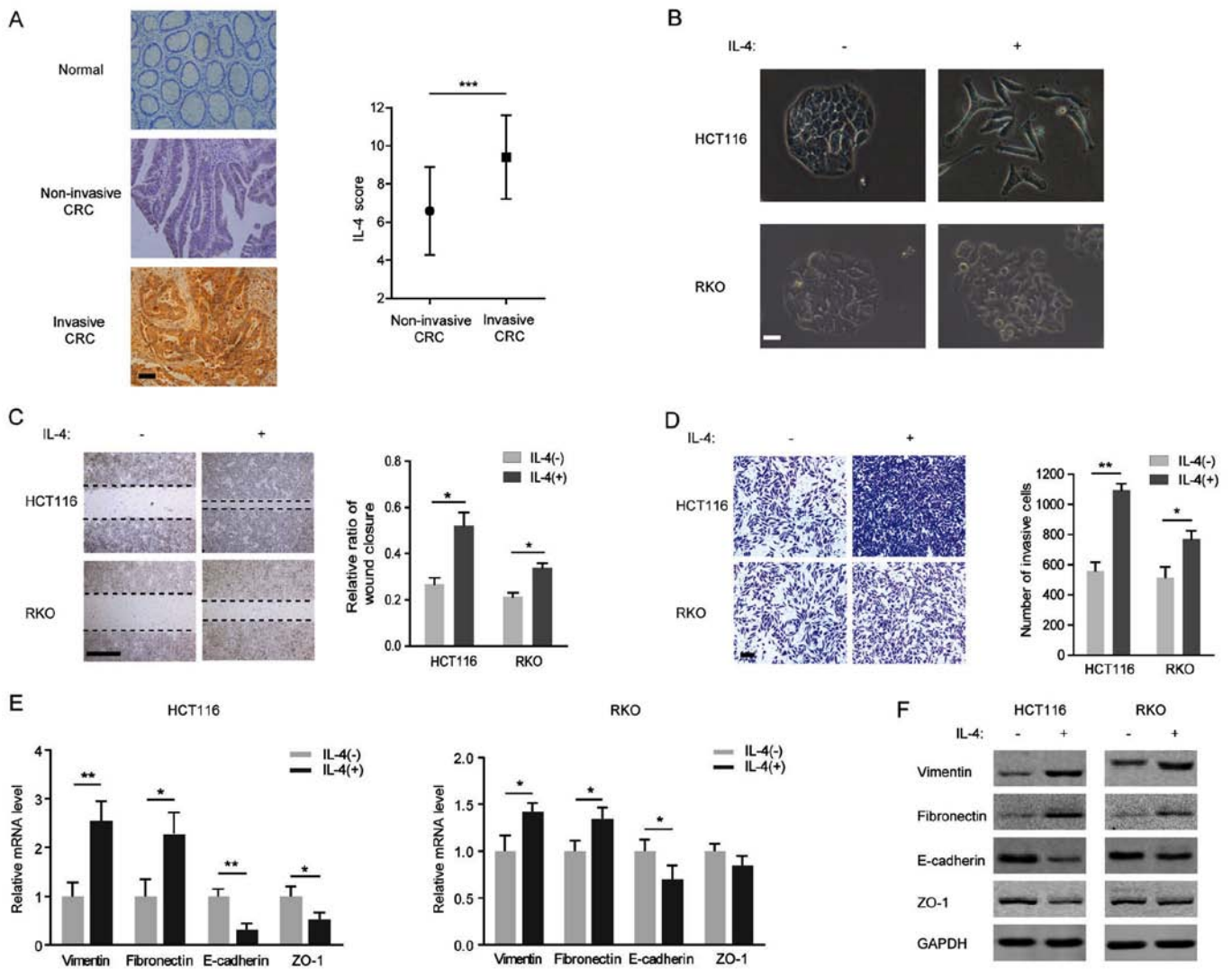


Figure 1. IL-4-induced epithelial-mesenchymal transition program is more evident in HCT116 cells compared with in RKO cells. (A) Left: Representative images of IL-4 immunostaining in human colorectal cancer tissue and para-tumor tissue. Scale bar, 200 μ m. Right: Immunostaining scores of IL-4 in invasive or non-invasive CRC tissues. (B) Morphology of HCT116 and RKO cells treated with or without IL-4 (20 ng/ml) for 72 h visualized using phase-contrast microscopy. Scale bar, 500 μ m. (C) Left: Representative images from wound healing assays with HCT116 and RKO cells treated with 20 ng/ml IL-4. Scale bar, 100 μ m. Right: Percentage wound closure 48 h after addition of IL-4. (D) Left: Representative images of HCT116 and RKO cells penetrating the Matrigel in invasion assays following treatment with 20 ng/ml IL-4. Scale bar, 100 μ m. Right: numbers of invasive cells treated with IL-4 for 48 h. (E) Relative mRNA levels of vimentin, fibronectin, E-cadherin and ZO-1 in HCT116 (left) and RKO (right) cells treated with 20 ng/ml IL-4 for 48 h. (F) Western blots of vimentin, fibronectin, E-cadherin and ZO-1 with specific antibodies in HCT116 (left) and RKO (right) cells treated with 20 ng/ml IL-4 for 48 h. * $P < 0.05$; ** $P < 0.01$. IL, interleukin; CRC, colorectal cancer; E-cadherin, epithelial cadherin; ZO-1, zonula occludens-1.

La Jolla, CA, USA). Analysis of differences was performed using a two-tailed Student's *t*-test and analysis of variance (one-way with Tukey's post hoc test; two-way with Sidak's post hoc test). The results are presented as the mean \pm standard deviation of three separate experiments. χ^2 test or Fisher's exact probability test was used to compare the clinicopathological features of the patients with protein expression. A Spearman's rank correlation test was used for analyzing the correlation. Kaplan-Meier plots and log-rank tests were used for survival analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

IL-4-induced EMT is more evident in the CRC cell line HCT116 compared with the RKO. Although expression of IL-4 and

IL-4R is involved in the process of local metastases in colorectal cancer (21), the underlying molecular mechanisms have not been clearly elucidated. In the present study, IL-4 immunoreactivity was observed in clinical CRC tissues, but not in adjacent normal tissues, and a higher IL-4 level was detected in invasive CRC tissues compared with in non-invasive ones (Fig. 1A). Additionally, IL-4 was identified to induce an EMT-like morphological change in CRC cells (Fig. 1B). The potential function of IL-4 in EMT induction in CRC cells was investigated. Following exogenous IL-4 stimulation, the migration of HCT116 and RKO cells was identified to be increasing, and the CRC cells exposed to IL-4 also acquired the capacity for migration and invasion (Fig. 1C and D). Furthermore, IL-4 treatment decreased the expression of the membranous epithelial marker E-cadherin and increased the expression of the cytoplasmic mesenchymal marker vimentin at the mRNA and protein levels in HCT116

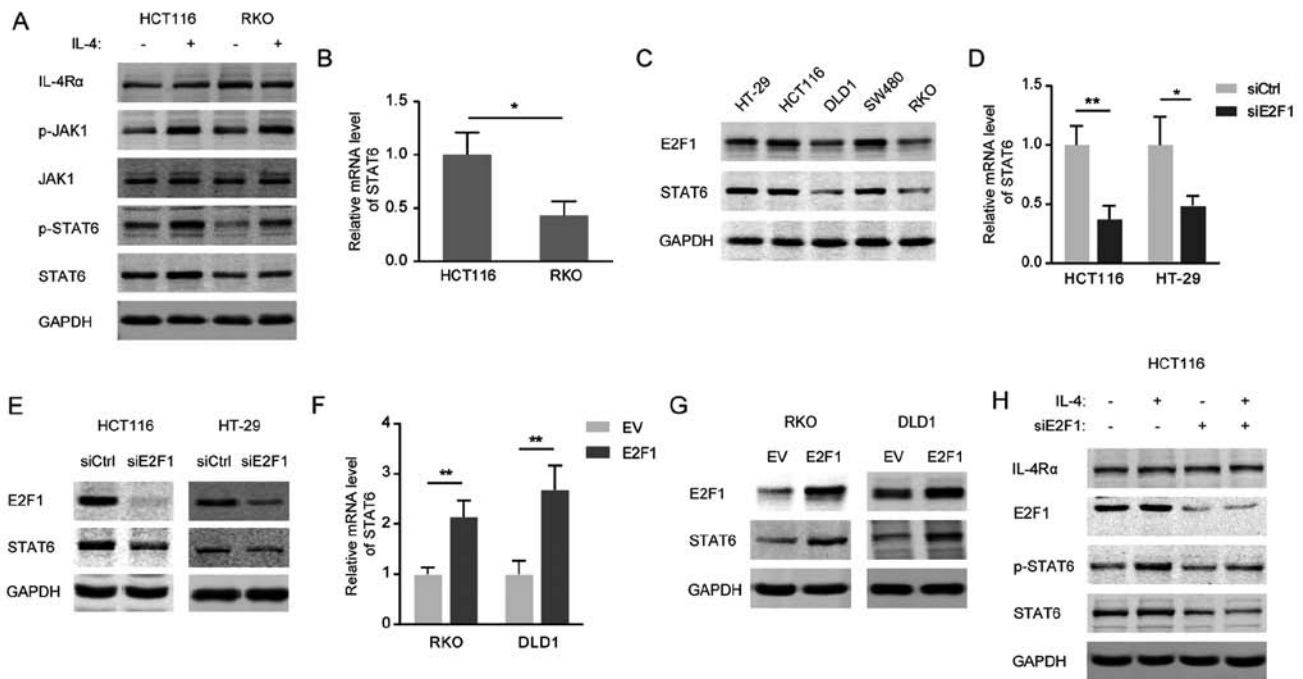


Figure 2. Constitutive expression of STAT6 is dependent on E2F1 in CRC cells. (A) Western blots of the indicated proteins with specific antibodies in HCT116 (left) and RKO (right) cells exposed to 20 ng/ml IL-4 for 12 h. (B) Relative mRNA levels of STAT6 in HCT116 and RKO cells (normalized to GAPDH). (C) Expression of E2F1 and STAT6 proteins in 5 CRC cell lines was analyzed by western blotting. (D) mRNA and (E) protein levels of STAT6 in HCT116 and HT-29 cells with knockdown of E2F1. (F) mRNA and (G) protein levels of STAT6 in RKO and DLD1 cells with E2F1 overexpression. (H) Western blots of the indicated proteins with specific antibodies in HCT116 cells with E2F1 knockdown and treatment with 20 ng/ml IL-4. * $P < 0.05$; ** $P < 0.01$. STAT6, signal transducer and activator of transcription 6; CRC, colorectal cancer; IL, interleukin; IL-4R α , IL-4 receptor α ; p-, phosphorylated; JAK1, Janus kinase 1; si, small interfering RNA; Ctrl, control; EV, empty vector.

and RKO cells (Fig. 1E and F). In addition, repression of ZO-1 accompanied by the induction of fibronectin was observed in IL-4-treated HCT116 and RKO cells (Fig. 1E and F). However, it is notable that IL-4 exposure led to an increased EMT change in HCT116 cells compared with in RKO cells.

Constitutive expression of STAT6 is dependent on E2F1 in CRC cells. To understand the different responses to IL-4 in HCT116 and RKO cells, the IL-4/STAT6 signaling pathway was investigated in the two CRC cell lines. As presented in Fig. 2A, exogenous IL-4 resulted in a marked increase in p-STAT6 in HCT116, but not in RKO, cells due to a high level of STAT6 protein in HCT116 cells. Consistently, the mRNA level of STAT6 was significantly increased in HCT116 cells compared with in RKO cells (Fig. 2B). Furthermore, the expression levels of STAT6 were paralleled with those of E2F1 in different CRC cell lines (Fig. 2C). Small interfering (siRNA)-mediated E2F1 knockdown markedly decreased STAT6 expression at the mRNA (Fig. 2D) and protein (Fig. 2E) levels in HCT116 and HT-29 cells, and STAT6 was identified to be markedly upregulated following overexpression of E2F1 in RKO and DLD1 cells (Fig. 2F and G). As expected, the induced activation of IL-4/STAT6 signaling was blunted in the E2F1-silenced HCT116 cells, which exhibited attenuated STAT6 expression (Fig. 2H). From these results, it may be concluded that ectopically expressed E2F1 is required for the abnormal activation of IL-4/STAT6 signaling in CRC cells.

E2F1-induced SP3 transactivates STAT6 in CRC cells. Considering E2F1 is a well-known transcriptional activator,

serial deletion constructs of the STAT6 gene promoter were examined using luciferase reporter assays to identify the transcriptional regulatory region responsive to E2F1 in HCT116 cells. It was identified that the STAT6 promoter without the region between -500 and -350 lost the ability to respond to E2F1 silencing (Fig. 3A). However, ChIP-PCR analysis revealed no enrichment of E2F1 protein into the STAT6 promoter (Fig. 3B). The promoter sequence (-500/-350) was analyzed and two potential GT-box elements predicted to be bound by SP3 were identified (Fig. 3C). Mutations in either the GT#1 or GT#2 sites decreased the reporter activity of the STAT6 gene promoter in HCT116 cells, and knockdown of SP3 mimicked the effect of these mutations (Fig. 3D and E). In turn, SP3 overexpression induced the reporter activity of the wild-type STAT6 promoter, but failed to induce the GT#1/2-mutated STAT6 promoter (Fig. 3F). Consistently, the mRNA level of STAT6 was decreased following knockdown of SP3 in HCT116 cells (Fig. 3G). ChIP-PCR analysis also confirmed that SP3 directly bound to the STAT6 promoter at ~-400 bp, upstream of the transcription start site (Fig. 3H). Furthermore, E2F1 knockdown diminished the expression of SP3 and overexpression of SP3 rescued the attenuated STAT6 expression that resulted from E2F1 deprivation, indicating that SP3 mediates the E2F1-dependent increased level of expression of STAT6 in CRC cells (Fig. 3I).

Highly expressed E2F1 promotes the IL-4-induced aggressiveness by upregulating STAT6. On the basis of the aforementioned results, the function of E2F1 in the IL-4/STAT6 signaling-induced EMT process was next investigated. In IL-4-stimulated

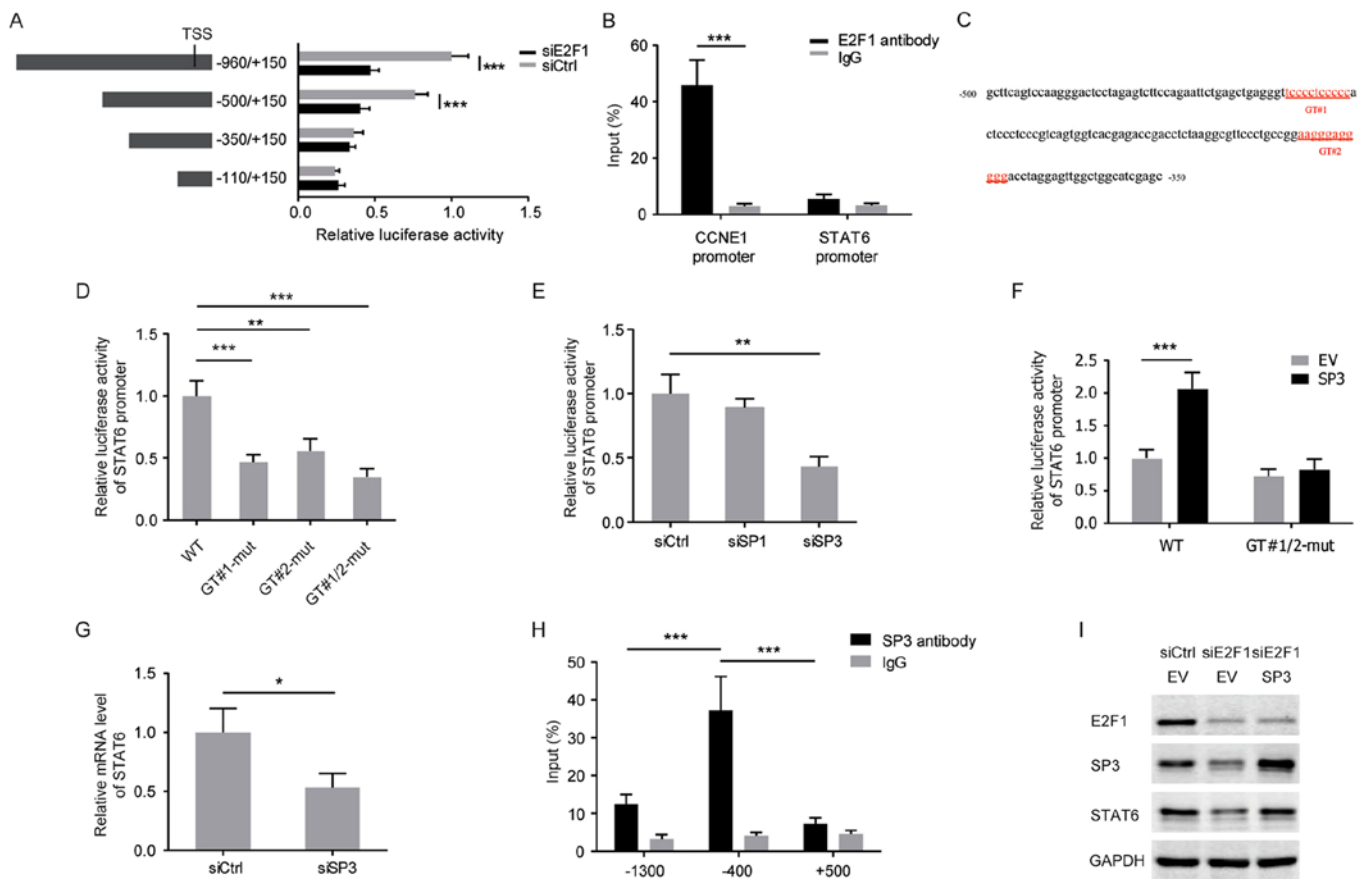


Figure 3. E2F1-induced SP3 transactivates STAT6 in CRC cells. (A) Relative luciferase activities were measured with a series of truncated constructs of the STAT6 promoter in HCT116 cells with E2F1 knockdown (internal control, pRL-SV40). (B) HCT116 cells were analyzed by ChIP assays using anti-E2F1 antibody and IgG. qPCR was performed with the immunoprecipitated DNA or soluble chromatin using the specific primer pairs for STAT6 promoter (~400 bp). CCNE1 served as a positive control. (C) The STAT6 promoter region (-500/-350) with two potential GT-box elements. (D) Relative luciferase activities in HCT116 cells transfected with the wild-type or GT-box-mutated STAT6 promoter reporter constructs. (E) Relative luciferase activity of STAT6 promoter in HCT116 cells with knockdown of SP1 or SP3. (F) Relative luciferase activity of the wild-type or GT-box-mutated STAT6 promoter in HCT116 cells with overexpression of SP3. (G) mRNA level of STAT6 in HCT116 cells with SP3 knockdown. (H) HCT116 cells were analyzed by ChIP assays using anti-SP3 antibody and IgG. qPCR was performed with the immunoprecipitated DNA or soluble chromatin using the specific primer pairs for STAT6 promoter regions (~1300, -400 and +500 bp). (I) Western blots of the indicated proteins with specific antibodies in HCT116 cells with E2F1 knockdown and SP3 overexpression. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. SP3, specificity protein 3; STAT6, signal transducer and activator of transcription 6; CRC, colorectal cancer; ChIP, chromatin immunoprecipitation; IgG, immunoglobulin G; qPCR, quantitative polymerase chain reaction; TSS, transcription start site; si, small interfering RNA; Ctrl, control; CCNE1, cyclin E1; WT, wild-type; mut, mutant; EV, empty vector.

HCT116 cells, a decrease in a mesenchymal marker (vimentin) and an increase in epithelial markers (E-cadherin and ZO-1) were observed following knockdown of E2F1, whereas overexpression of STAT6 partially recovered the mesenchymal phenotype (Fig. 4A). In addition, the acquisition of migratory and invasive capabilities in HCT116 cells exposed to IL-4 was attenuated by E2F1 silencing, but restored when STAT6 was overexpressed (Fig. 4B). Similarly, overexpression of E2F1 enhanced the promoting effect of IL-4 on the mesenchymal phenotype in RKO cells; however, knockdown of STAT6 impaired the E2F1-dependent EMT properties (Fig. 4C). As expected, E2F1 overexpression strengthened the IL-4-induced migration and invasion of RKO cells, but failed to disrupt STAT6 (Fig. 4D). These results demonstrate that the regulation of STAT6 by E2F1 is essential for IL-4-induced malignancy in CRC cells.

As a target gene of STAT6, Zeb1 is important in IL-4 triggered-EMT. Cao *et al* (22) identified that the IL-13/STAT6/Zeb1 pathway serves a critical function in promoting EMT and

aggressiveness of CRC cells. The alterations in EMT-associated transcription factors including Snail1, Slug, Zeb1, Zeb2 and Twist1 in response to IL-4/STAT6 signaling were investigated. As presented in Fig. 5A and B, the mRNA and protein levels of Zeb1 and Zeb2 were markedly increased in HCT116 cells following IL-4 stimulation. However, in HCT116 cells with STAT6 knockdown, IL-4 was not able to induce Zeb1 and Zeb2 (Fig. 5C and D). Furthermore, it was identified that knockdown of STAT6 prevented IL-4 from inducing Zeb1 and EMT markers, whereas ectopic expression of Zeb1 recovered the cell's EMT properties (Fig. 5E). Therefore, consistent with a previous study (22), Zeb1 functions as a critical downstream effector of IL-4/STAT6 signaling during EMT process.

E2F1 is required for IL-4-induced CRC tumorigenesis in vivo. To further determine the involvement of E2F1 in IL-4-induced tumorigenesis, HCT116 cells with stable knockdown of E2F1 were constructed and subcutaneously injected into nude mice. Tumor formation was evaluated during the 21 days after injection. It was identified that IL-4 significantly promoted the

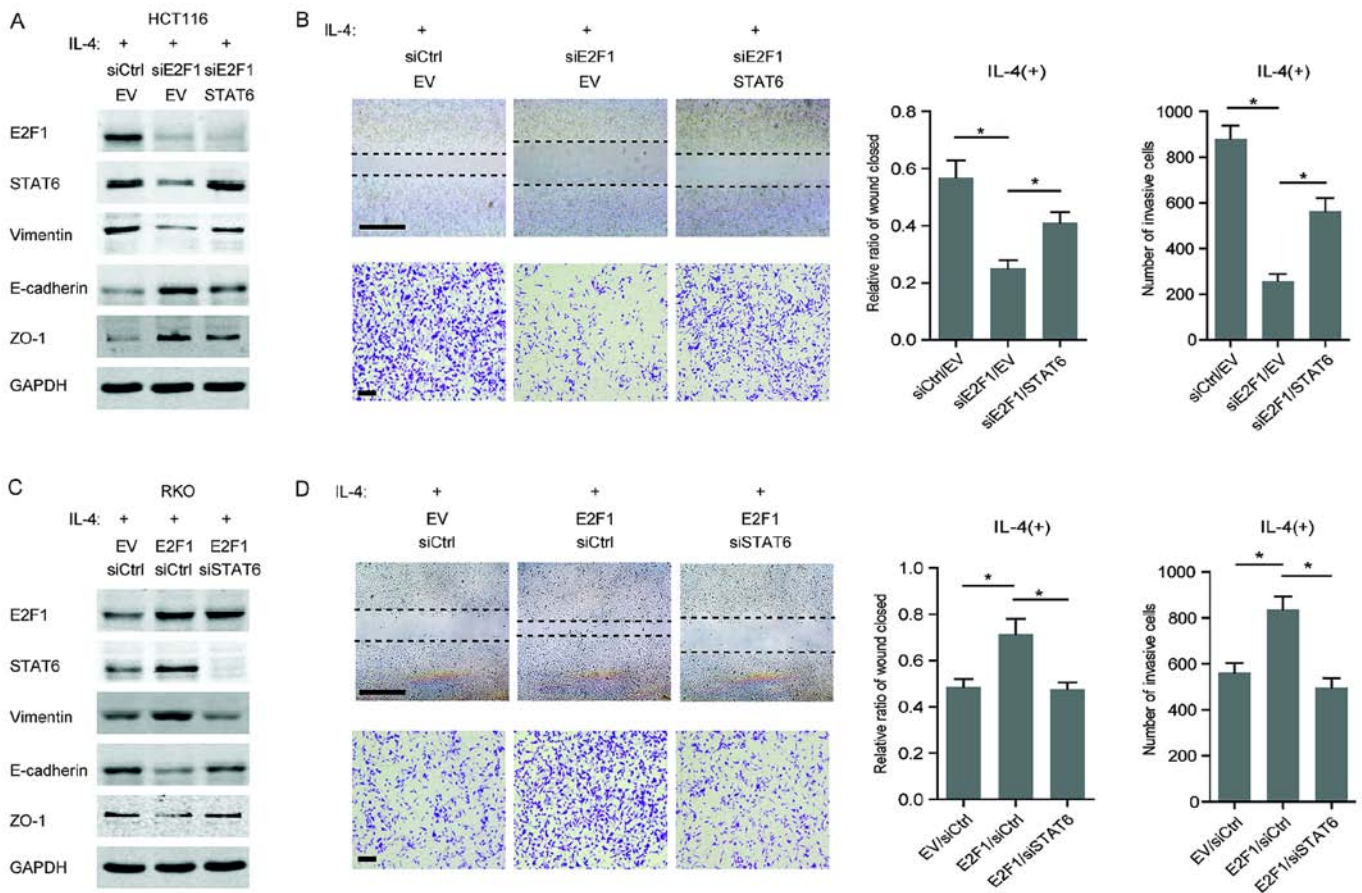


Figure 4. Highly expressed E2F1 promotes IL-4-induced aggressiveness by upregulating STAT6. (A) Western blots of the indicated proteins with specific antibodies in HCT116 cells co-transfected with E2F1 siRNA/STAT6 overexpression plasmid and exposed to 20 ng/ml IL-4 for 48 h. (B) Left: Representative images from wound healing and invasion assays with HCT116 cells co-transfected with E2F1 siRNA/STAT6 overexpression plasmid and exposed to 20 ng/ml IL-4 for 48 h. Scale bar, 100 μ m. Right: Percentage wound closure and numbers of invasive cells 48 h after IL-4 addition. (C) Western blots of the indicated proteins with specific antibodies in the RKO cells co-transfected with STAT6 siRNA/E2F1 overexpression plasmid and exposed to 20 ng/ml IL-4 for 48 h. (D) Left: representative images from wound healing and invasion assays with the RKO cells co-transfected with STAT6 siRNA/E2F1 overexpression plasmid and exposed to 20 ng/ml IL-4 for 48 h. Scale bar, 100 μ m. Right: Percentage wound closure and numbers of invasive cells 48 h after IL-4 addition. * $P < 0.05$. IL, interleukin; STAT6, signal transducer and activator of transcription 6; si, small interfering RNA; Ctrl, control; EV, empty vector; E-cadherin, epithelial cadherin; ZO-1, zonula occludens-1.

tumorigenesis of HCT116 cells, but not HCT116 cells with stable knockdown of E2F1 (Fig. 6A and B). Consistent with these results, immunostaining for the xenograft indicated that silencing of E2F1 inhibited IL-4-induced accumulation of p-STAT6 through decreasing the total STAT6 level (Fig. 6C). Thus, *in vivo* experiments suggest that E2F1 may exert a crucial function in IL-4-induced CRC tumorigenesis by regulating STAT6 signaling.

Abnormal activation of E2F1/SP3/STAT6 axis is associated with cancer aggressiveness in CRC samples. Immunohistochemical staining was performed to evaluate the protein levels of E2F1, SP3, STAT6 and p-STAT6 in human CRC specimens. As presented in Fig. 7A, different immunoreactivity intensities of E2F1, SP3, STAT6 and p-STAT6 were observed in CRC specimens. Further analysis revealed that all three factors were significantly correlated with the TNM stage and distant metastasis, but not with other clinical characteristics (Table II). In addition, immunostaining using consecutive sections revealed a positively correlation of the expression levels of STAT6 with the levels of E2F1 and SP3 (Fig. 7B and C). The potential associations between immunostaining and overall survival were retrospec-

tively evaluated in 218 patients with CRC. A Kaplan-Meier analysis identified that increased expression of E2F1, SP3 or STAT6 significantly indicated poor survival (Fig. 7D-F). These results suggested that the E2F1/SP3/STAT6 axis is involved in CRC progression and metastasis.

Discussion

It is well-accepted that cytokines including ILs produced by tumor cells or, more often, by the B- or T-lymphocytes recruited to the tumor microenvironment promote the proliferation of tumor cells, perturb their differentiation and support the metastasis of cancer cells (23). Several lines of evidence suggest that IL-4 may suppress cancer-directed immunosurveillance and enhance tumor metastasis. For example, IL-4 has the ability to exert an autocrine proliferation stimulation effect in pancreatic cancer cells and furthermore serves paracrine functions on the surrounding infiltrating immune cells, repressing immunoresponses (24). In addition, it has been observed that secretion of IL-4, for instance, by malignant cells from the bladder, lung and colon, confers resistance to chemotherapy-induced cell death (25,26). Particularly for

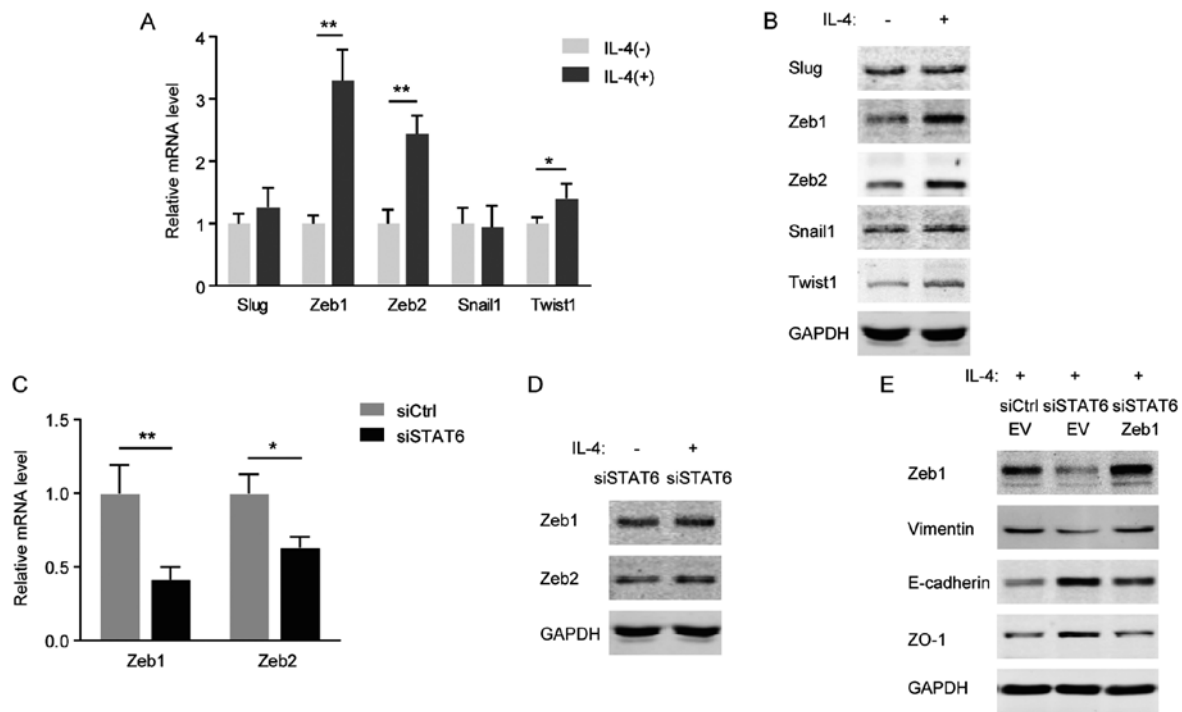


Figure 5. As a target gene of STAT6, Zeb1 is important in EMT triggered by IL-4. (A) mRNA levels of EMT transcription factors in HCT116 cells treated with 20 ng/ml IL-4 for 24 h. (B) Western blots of the indicated proteins with specific antibodies in HCT116 cells treated with 20 ng/ml IL-4 for 24 h. (C) mRNA levels of Zeb1/2 in HCT116 cells with STAT6-knockdown. (D) Western blots of the indicated proteins with specific antibodies in HCT116 cells transfected with STAT6 siRNA and exposed to 20 ng/ml IL-4 for 24 h. (E) Western blots of the indicated proteins with specific antibodies in HCT116 cells co-transfected with STAT6 siRNA/Zeb1 overexpression plasmid and exposed to 20 ng/ml IL-4 for 48 h. * $P < 0.05$; ** $P < 0.01$. STAT6, signal transducer and activator of transcription 6; EMT, epithelial-mesenchymal transition; IL, interleukin; Zeb, zinc finger E-box-binding homeobox; si, small interfering; Ctrl, control; E-cadherin, epithelial cadherin; ZO-1, zonula occludens-1; EV, empty vector.

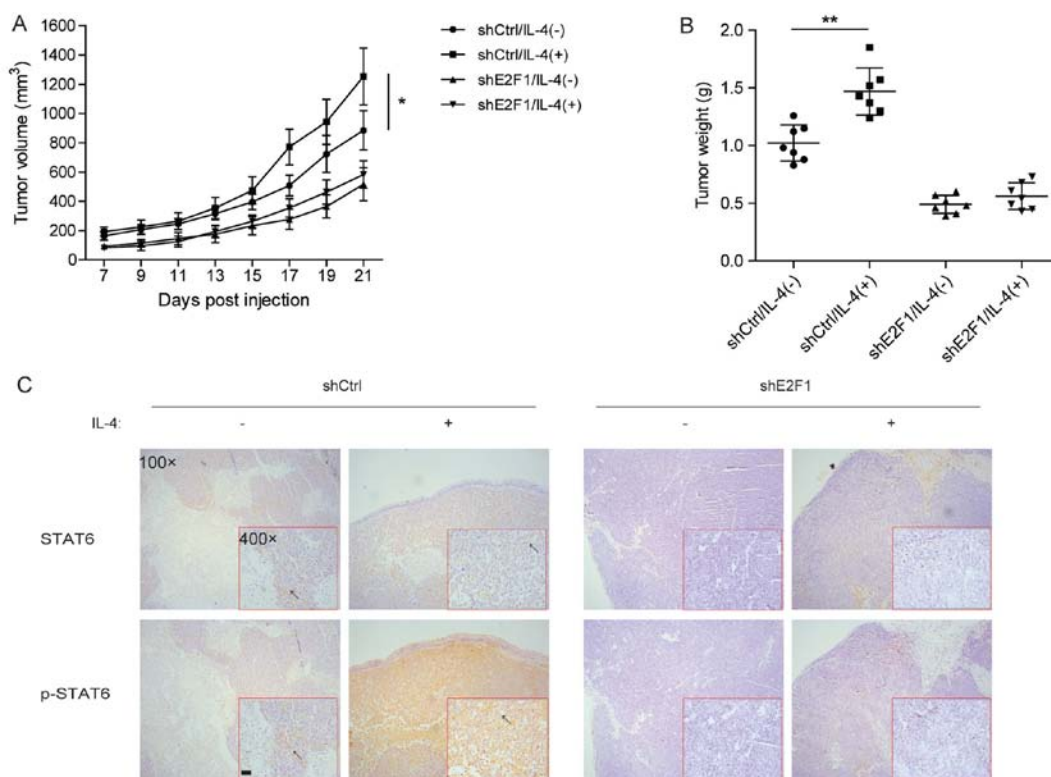


Figure 6. E2F1 is required for IL-4-induced CRC tumorigenesis *in vivo*. (A) Growth curves of the tumors formed by the indicated HCT116 cells. Results are presented as the mean \pm SD ($n = 7$ mice per group). (B) Weight differences in tumors formed by the indicated HCT116 cells. Results are presented as the mean \pm SD ($n = 7$ mice per group). (C) Immunohistochemical staining of STAT6 and p-STAT6 proteins in the indicated xenograft tissues. Scale bar, 500 μ m. The arrow indicates positive cells. * $P < 0.05$. IL, interleukin; CRC, colorectal cancer; SD, standard deviation; sh, short hairpin RNA; Ctrl, control; STAT6, signal transducer and activator of transcription 6; p-, phosphorylated.

Table II. Correlation of the expression of SP3, E2F1 and STAT6 with clinicopathological features in CRC.

Characteristic	Total	SP3 expression			E2F1 expression			STAT6 expression		
		Low	High	P-value	Low	High	P-value	Low	High	P-value
N	218	103	115		111	107		113	105	
Tumor location				0.5832			0.7549			0.6933
Colon	90	41	49		44	46		46	44	
Rectum	128	62	66		67	61		67	61	
Sex				0.9837			0.3220			0.1288
Male	116	56	60		54	62		54	62	
Female	102	47	55		57	45		59	43	
Age, years				0.8908			0.7756			0.6932
≤65	88	43	45		44	44		46	42	
>65	130	60	70		67	63		67	63	
Differentiation status				0.9279			0.1521			0.6771
Well	46	24	22		22	24		25	21	
Moderate	147	68	79		82	65		78	69	
Poor	25	11	14		7	18		10	15	
Tumor size, cm				0.2307			0.3672			0.3444
<5	105	46	59		55	50		50	55	
≥5	113	57	56		56	57		63	50	
Lymph node metastasis				0.0194 ^a			0.0957			0.0079 ^a
N0	125	67	58		70	55		71	54	
N1	69	28	41		33	36		35	34	
N2	24	8	16		8	16		7	17	
TNM				0.0055 ^a			0.0126 ^a			0.0439 ^a
I	35	20	15		19	16		17	18	
II	83	39	44		43	40		50	33	
III	83	41	42		45	38		41	42	
IV	17	3	14		4	13		5	12	
Distant metastasis				0.0005 ^a			0.0010 ^a			0.0089 ^a
M0	201	102	99		107	94		114	87	
M1	17	3	14		4	13		5	12	

^aP<0.05. TNM, tumor-node-metastasis.

CR-CSC cells, resistance to oxaliplatin is dependent on auto-crine production of IL-4 (27). In the present study, the acquired EMT-like characteristics of several CRC cell lines stimulated by IL-4 were identified. However, it was observed that different CRC cell lines (HCT116 and RKO) with different expression levels of STAT6 exhibited entirely different responses to IL-4. As a key signaling transducer in the IL-4 pathway, STAT6 may determine the IL-4-induced aggressiveness of CRC cells. Consistent with this, it was identified previously that a lack of STAT6 attenuates inflammation and prevents early steps of colitis-associated CRC (28). Furthermore, HT-29 cells with an active STAT6 phenotype exhibit more aggressive metastasis compared with Caco-2 cells with defective STAT6 (29). Additionally, knockdown of STAT6 inhibits proliferation and induces apoptosis in HT-29 cells (30). Therefore, the STAT6 level may determine the CRC development facilitated by inflammatory cytokines.

Our previous study focused on E2F1, a critical transcription factor for CRC development, which promoted the migration and invasion of CRC cells (18). In the present study, it was identified that the levels of STAT6 protein were paralleled with E2F1 in several CRC cell lines. Further *in vitro* experiments confirmed an E2F1-dependent transcription of STAT6. However, no evident enrichment of E2F1 into the E2F1-response region located at the STAT6 promoter was identified in HCT116 with increased expression of STAT6, indicating an indirect regulation for STAT6 by E2F1. In addition, SP3, a member of the SP transcription factor family, was verified to mediate the transcription of the STAT6 gene. SP transcription factors including SP3 have been identified to be overexpressed in tumors, whereas SP levels are relatively low in non-tumor tissues (31). Functional studies have identified that SP transcription factors have a function in cancer cell proliferation, survival, angiogenesis, migration and invasion by inducing oncogenes including

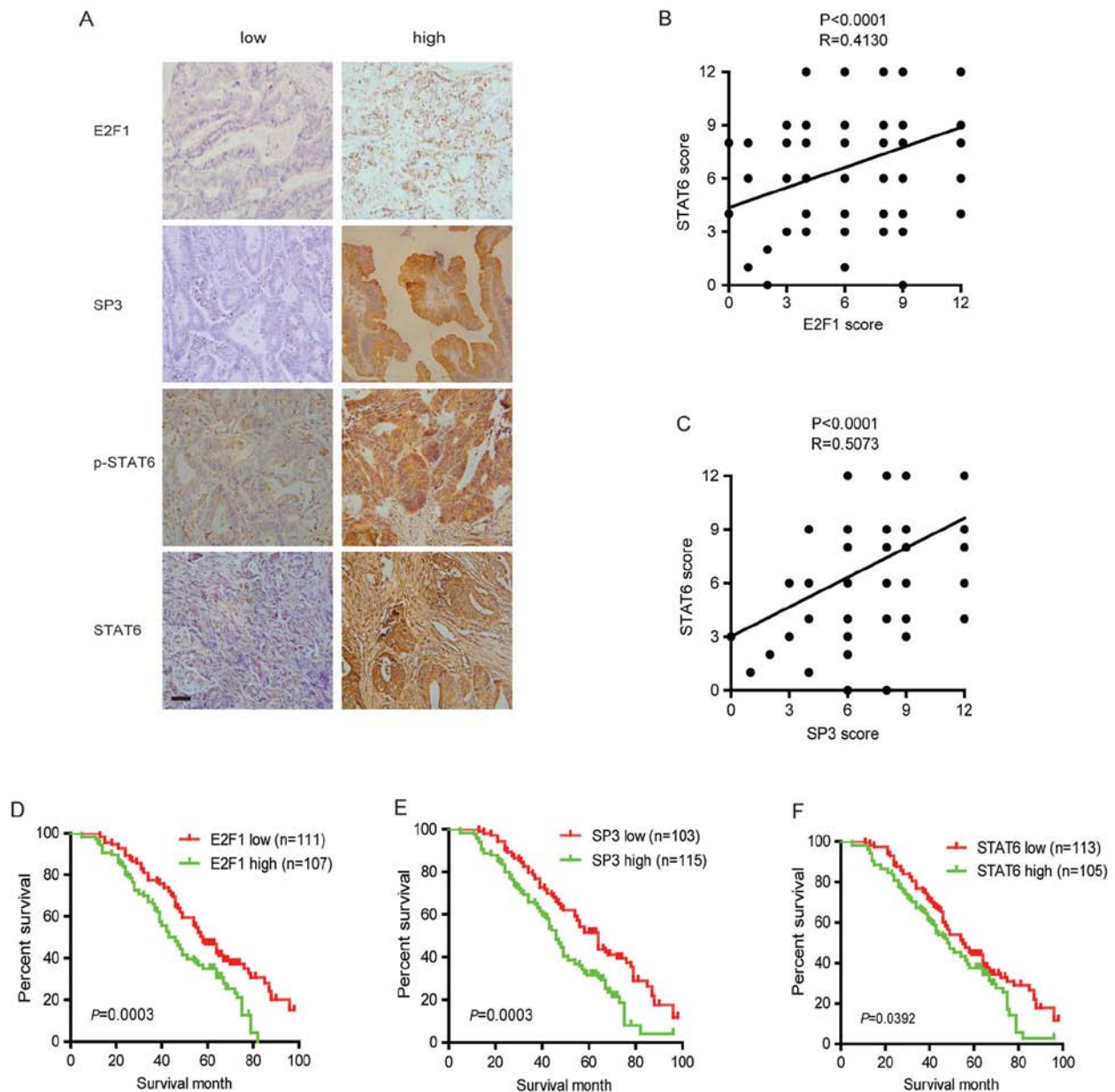


Figure 7. Abnormal activation of E2F1/SP3/STAT6 axis is associated with cancer malignancy in CRC samples. (A) Representative images of E2F1, SP3, p-STAT6 and STAT6 immunostaining in human CRC tissues. Scale bar, 200 μ m. (B) Linear regression between immunostaining intensity of E2F1 and STAT6. (C) Linear regression between immunostaining intensity of SP3 and STAT6. Overall survival of patients with low and high expression of (D) E2F1, (E) SP3 or (F) STAT6. SP3, specificity protein 3; STAT6, signal transducer and activator of transcription 6; CRC, colorectal cancer; p-, phosphorylated.

survivin, B-cell lymphoma 2, p65 (NF- κ B), c-MET, EGFR and other receptor tyrosine kinases (32). The overexpressed SP members in CRC cells, SP1 and SP3, are required for migration and invasion (33). Even though SP1 and SP3 share >90% DNA sequence homology in the DNA-binding domain and bind to the same DNA element with similar affinity (34), it was identified in the present study that SP3 rather than SP1 contributed to the transcription activation of the STAT6 gene in CRC cells. More importantly, increased expression of SP3 appeared to depend on E2F1, suggesting the E2F1/SP3/STAT6 axis as a potential regulatory pathway for STAT6 signaling in CRC. However, the reasons for SP3, but not SP1, being the major regulatory activator of the transcription of the STAT6 gene remain to be determined, and the details of how E2F1 regulates SP3 remain unknown.

On the surface of many solid tumors, IL-4R consists of the IL-4R α and IL-13R α 1 subunits. Thus, IL-4R may be bound and activated by IL-13, although IL-4 binds with higher affinity (35). Previous studies demonstrate that IL-4 and IL-13 share signaling events by initiating a signaling cascade that activates the JAK/STAT pathway (particularly STAT6) in human CRC cells (36). Recent study by Cao *et al* (22) identified that IL-13/STAT6 signaling induces the aggressive properties of HT29 and SW480 cells through transactivating Zeb1, which is a well-known EMT core regulator and triggers malignant transformation. Therefore, in the present study, the expression patterns of EMT-associated transcription factors, including Snail1, Slug, Zeb1, Zeb2 and Twist1, were also investigated. However, the results of the present study were not completely consistent with those of

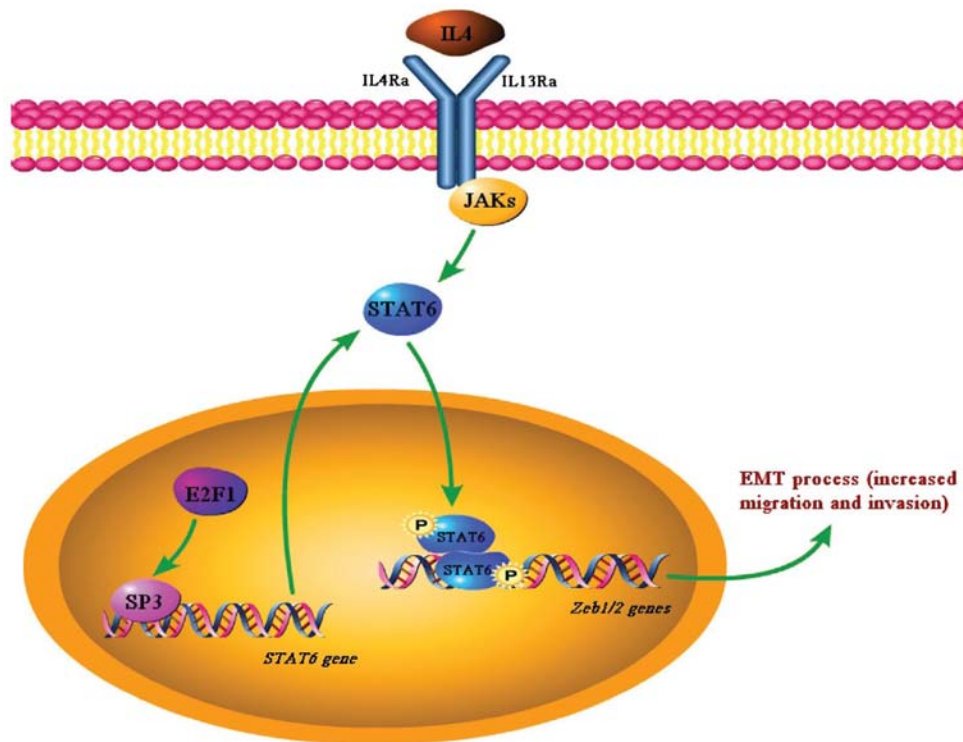


Figure 8. Schema indicating the crucial function of the E2F1/SP3/STAT6 axis in IL-4-induced EMT. In IL-4-responsive CRC cells such as HCT116, highly expressed E2F1 induces the expression of SP3, which transactivates STAT6. At the same time, IL-4 signaling leads to STAT6 phosphorylation, which then promotes the EMT of CRC cells by activating the transcription of Zeb1/2. SP3, specificity protein 3; STAT6, signal transducer and activator of transcription 6; IL, interleukin; EMT, epithelial-mesenchymal transition; CRC, colorectal cancer; Zeb, zinc finger E-box-binding homeobox; IL4Ra, IL-4 receptor α ; IL13Ra, IL-13 receptor α ; JAK, Janus kinase.

Cao *et al* (22), as demonstrated by the critical function of Zeb2 in the EMT progress driven by IL-4/STAT6 signaling. Despite STAT6 functioning as the common transducer in IL-4 and IL-13 signaling, activated STAT6 induces different expression profiles of downstream EMT-drivers following IL-4 and IL-13 stimulation.

In summary, we propose a model in which the high expression of STAT6 arose from abnormal activation of the E2F1/SP3/STAT6 axis and is phosphorylated by IL-4 and then promotes EMT progression by upregulating Zeb1/2, therefore enhancing the migration and invasion of CRC cells (Fig. 8). The discovery of such a regulatory pathway for IL-4 signaling suggests the potential of the E2F1/SP3/STAT6 axis to serve as a biomarker and represents a promising target for therapeutic intervention against CRC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HL and XC conceived and designed the study. JC, CG, HM, ZL, ZF, QC, ML, XJ, YH, WW, XZ, XC and HL performed the experiments and wrote the manuscript. JC, CG, HM, ZL and ZF analyzed and interpreted data. JC, HL, XC, ZF and WW contributed reagents and materials. All authors helped to draft the manuscript and read and approved the final manuscript.

Ethics approval and consent to participate

Experiments using human tissues were performed with the approval of the Ethics Committee of Sanmen People's Hospital (Sanmen, China). Animal experiments were performed with the approval of the Ethics Committee of Sanmen People's Hospital and the Animal Care and Use Committee of Zhejiang University (Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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