

SPIB Acts as a Tumor Suppressor by Activating NFkB and JNK Signaling Pathways Through MAP4K1 in Colorectal Cancer Cells

Xunping Zhao

The First Affiliated Hospital of Chongqing Medical University

Lin Li

The First Affiliated Hospital of Chongqing Medical University

Shiyun Yuan

The First Affiliated Hospital of Chongqing Medical University

Qia Zhang

Yongchuan Hospital of Chongqing Medical University

Xianyao Jiang

The First Affiliated Hospital of Chongqing Medical University

Tao Luo (✉ luotao@hospital.cqmu.edu.cn)


The First Affiliated Hospital of Chongqing Medical University <https://orcid.org/0000-0001-8357-6918>

Primary research

Keywords: SPIB, colorectal cancer, MAP4K1, tumor suppressor

Posted Date: December 31st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-136420/v1>

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Abstract

Background

Colorectal cancer (CRC) is one of the major cancers in the world. Spi-B Transcription Factor (SPIB) is one member of the E-twenty-six (ETS) transcription factor family. Previous studies have shown that the expression of SPIB is down-regulated in human colorectal cancer tissues. However, its biological function in colorectal cancer cells is not reported. The purpose of our study is to explore the biological function and related mechanism of SPIB in colorectal cancer cells, to provide reference for the molecular detection and targeted drug therapy of colorectal cancer.

Methods

The biological function of SPIB in colorectal cancer cells were studied by colony formation assay, CCK-8 cell proliferation assay, transwell assay, tube formation assay, flow cytometry analysis. Growth inhibition assay was used to measure the impact of SPIB on oxaliplatin and 5-fluorouracil (5-FU). Double luciferase reporter assay and western blot were used to detect mechanism of SPIB in colorectal cancer cells.

Results

SPIB mRNA was down-regulated in CRC cell lines and CRC tissues. SPIB can inhibit the proliferation, migration and invasion of CRC cells; can inhibit angiogenesis; and induce the cell cycle of CRC cells arrest in G2/M phase and promote the apoptosis of CRC cells. In the growth inhibition assay we found that compared with the control group, the 50% inhibitory concentration(IC50) values of oxaliplatin and 5-FU in the SPIB overexpression group were significantly reduced. Western blot results showed that the overexpression of SPIB up-regulated cleaved-PARP(c-PARP), nuclear factor kB p65 (NFkB p65), phospho-NFkB p65(p-NFkB P65), JNK1, and C-Jun proteins expression level compared with the control group. Double luciferase report experiment showed that SPIB can activate the promoter of MAP4K1 and enhance the expression of MAP4K1. After silencing MAP4K1, the protein expressions of c-PARP, NFkB P65, p-NFkB P65, JNK1, and C-Jun were down-regulated.

Conclusions

In this study, we found that SPIB is a tumor suppressor in colorectal cancer cells, SPIB sensitizes colorectal cancer cells to oxaliplatin and 5-FU. we also found that MAP4K1 is a target gene of SPIB, SPIB exerts its anti-colorectal cancer effect by activating NFkB and JNK signaling pathways through MAP4K1. The above findings may provide reference for new molecular markers and therapeutic targets for CRC.

Background

CRC is one of the most common cancers in the world, with the third highest incidence and the second highest mortality among malignant tumors [1]. More and more evidence show that CRC is not a single gene disease but a disease caused by a series of genetic changes. Molecular marker detection and molecular targeted drug therapy are the focus of current research.

The ETS transcription factor family is one of the largest transcription factor families. This family has 28 genes, and its members are related to the development of different tissues and cancer. All ETS family proteins have a highly conserved DNA-binding domain, namely ETS domain, which has a flanking helix and can bind to the DNA-binding site of GGA (A/T) sequence [2]. The ETS family has a variety of functions, including regulation of cell differentiation, cell cycle, cell migration, cell proliferation, apoptosis, angiogenesis and so on[3]. Many members of the ETS family have been shown to be associated with cancer.

SPIB transcription factor protein belongs to the ETS transcription factor family. Human SPIB is located on chromosome 19q13.3-q13.4. It is mainly expressed in mature B cells, plasma cell-like cells and T cell precursor cells. Real-time polymerase chain reaction and immunohistochemical detection were performed on 28 CRC tissues and paired non-tumor tissues by some researchers, the results showed that SPIB was mainly distributed in the nucleus and down-regulated in CRC tissues [4]. However, there is no report of the biological function and related mechanism of SPIB in CRC cells. The purpose of this study is to explore the biological function and mechanism of SPIB in human CRC cells, in order to provide reference for molecular detection and targeted drug therapy of Colorectal cancer.

Materials And Methods

Cells lines and tumour samples

Human CRC cell lines (HCT116, CaCO2, RKO, SW480, LoVo, HT-29) and Human umbilical vein endothelial cells (HUVECs) were selected. CaCO2, RKO, LoVo and SW480 cells were purchased from the Chinese Academy of Sciences. HCT116 and HT-29 were presented by Professor Q.tao of the Chinese University of Hong Kong. HUVECs were purchased from American Type Culture Collection. The cells were cultured in RPMI-1640 medium (Gibco-BRL, Karlsruhe, Germany) containing 10% fetal bovine serum (ExCell Bio, Shanghai, China), cultured in 5%CO2, 37 °C humidified atmosphere [5]. The CRC tissues and paired non-tumor tissues of 25 patients in the tumor tissue specimen database of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) were collected. All the tissues have been identified and kept at -80 °C. The research scheme was approved under the guidance of the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Nucleic acid and protein extraction

Total RNA was extracted from CRC tissues and cell lines with TRIzol® reagent (Life Technologies, Carlsbad, CA, USA). The extracted RNA was measured by NanoDrop 2000 spectrophotometer (Thermo Scientific, Rockford, IL, USA) and stored at -80 °C. Total protein was extracted from HCT116, RKO and HT-29 cells. The cells were lysed by protein extraction reagent (Thermo Scientific) in the experimental group and the control group, then the lysate was collected into EP tubes and homogenized by ultrasonic cell grinder (Scientz, Ningbo). After centrifugation at 4°C, the supernatant was transferred to new EP tubes. The protein concentration was measured by BCA protein kit (Thermo Scientific) and then stored in -80 °C refrigerator [6].

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

The expression of SPIB in CRC cells and tissues was detected by RT-PCR and qRT-PCR. 1 µg RNA was reverse transcribed into 20 µg cDNA using the reverse transcription system (Promega, Madison, WI, USA). For RT-PCR, the SPIB gene was amplified by GoTaq DNA polymerase (Promega) with 32 cycles, the internal control was β-actin. SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Hong Kong, China) was used in the qRT-PCR, ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) was used for amplification, and β-actin as control. The primer sequences are shown in Table 1.

Construction of SPIB overexpressed stable cell lines

The pCMV6-SPIB plasmid (OriGene Technologies, Inc. USA) and pCMV6-Entry plasmid were transfected into RKO and HT-29 cells with the transfection reagent Lipofectamine® 2000 (Invitrogen, Thermo Fisher Scientific, Inc.) respectively. After being cultured in serum-free RPMI-1640 medium for 4-6h, the fresh medium containing 10% FBS was used to replace the medium. After 48h, the G418 (Invitrogen/Gibco) was added to the medium containing 10% FBS for about 20 days to obtain the stable cell lines of SPIB overexpressed. RKO and HT-29 cells were screened at concentrations of 800ug/ml and 1200ug/ml. RT-PCR, qRT-PCR and western blot were used to verify the results.

SPIB gene was silenced in CRC cells

HCT116 cells were plated in a 6-well plate, the next day the confluence degree of 70% could be reached. siRNA-SPIB (Ribobio, China) was transfected into HCT116 cells with the transfection reagent Lipofectamine® 2000, siRNA-NC was used as control. After being cultured in serum-free RPMI-1640 medium for 4-6h, the fresh medium containing 10% FBS was used to replace the medium. Cultured for 48 hour, the cells were collected for later experiments.

Colony formation assay

Cells of experimental group and control group were cultured in a 6-well plate (200,400,800 cells / well) for 10-14 days. Fixed with 4% paraformaldehyde for 30 minutes, then stained with Gentian Violet (ICM pharma, Singapore) for 15 minutes.

CCK-8 Cell proliferation assay

Cells of experimental group and control group were planted in a 96-well plate at the density of 3000-6000/well, cultured in 100ul/well complete medium containing 10% FBS. At 0h, 24h, 48h and 72h, 100µl RPMI-1640 containing 10% FBS with 10µl CCK-8 (CCK-8, Beyotime, Shanghai, China) solution was used to replace the culture medium of each well and incubated at 37 °C for 2 hours. The

absorbance was measured with a microplate reader (Multiskan MK3; Thermo Fisher Scientific, former Fermentas, Schwerte, Germany) at 450nm, all the experiments were repeated three times.

Transwell assays

Without or with Matrigel barrier (BD Biosciences) in the top of transwell chamber (8µm pore size; Corning, New York, New York, USA) to detect the migration or invasion ability of CRC cells [7]. Cells were digested and centrifuged. After serum-free medium suspension, cells (RKO cells 4×10^4 , HT-29 cells 12×10^4) were made into 200 µl cell suspension added to the upper transwell chamber, and 700µl RPMI-1640 containing 30% FBS was added to the lower chamber. After incubating at 37°C and 5%CO₂ for 48-56 h, the cells in the lower part of the chamber were fixed in 4% paraformaldehyde for 30 minutes, and stained in Gentian Violet for 15 min. The cells in upper part of the chamber were wiped off with cotton buds. These cells in 5 random fields were counted under 400x magnifying microscope (CTR4000; Leica, Wetzlar, Germany). All the experiments were repeated three times.

Tube formation assay

Matrigel barrier diluted (Matrigel: RPMI-1640 =1:1) and placed into a 96-well plate according to 50ul/well. When the Matrigel barrier was solidified, the HUVECs cells were placed into the 96-well plate at a density of 60-70% / well. The control group was added with the original culture medium of RKO cells transfected with pCMV6-Entry and the experimental group was added with the original culture medium of RKO cells transfected with pCMV6-SPIB, 100ul/well. After 8 hours of culture, images were collected using a fluorescence microscope. All the experiments were repeated three times.

Flow cytometry analysis of cell cycle and apoptosis

In the determination of cell cycle, stably overexpressing cells were collected, after digesting, centrifuge with 1000rpm for 5 minutes, then with 4ml PBS re-suspension, centrifugation for 5 minutes again, fixed it with ice-cold 70% ethanol for 5 days at -20°C. The fixed cells were stained with 50mg/l propidium iodide (PI) to avoid light for 37°C, 30 min. In the determination of apoptosis, the original culture medium was collected. After digesting, centrifugating, resuspending and centrifugating Annexin V-FITC (BD Pharmingen, San Jose, CA, USA) and PI were used to stain in the dark for 20 minutes, then analyzed by flow cytometry (BD Bioscience, Franklin Lakes, New Jersey, USA). The results were analyzed by Cell Quest software (BD Biosciences, San Jose, California, USA). All the experiments were repeated three times.

Western blot assay

The 40ug protein was used for western blot as previously mentioned [8]. The main antibodies to be used were as follows: GAPDH (1:1000) (2118, Cell Signaling Technology), Bax (1:1000) (5023, Cell Signaling Technology), SPIB (1:1000) (15768-1-AP, Proteintech), PARP/cleaved-PARP (1:750) (WL01932, Wanleibio), MAP4K1 (1:1000) (23950-1-AP, Proteintech), NFκB p65 (1:500) (WL01980, Wanleibio), P-NFκB p65 (1:1000) (WL02169, Wanleibio), JNK1 (1:1000) (TA500043, Origene), C-Jun (1:1000) (TA500226, Origene). The enhanced chemiluminescence detection system (PierceChemical Co, Rockford, IL) was used for Proteins imaging.

Growth inhibition assay

Cells were inoculated into 96-well plates, 8000/well. 6h later, the cells were exposed to oxaliplatin (EAIE000ZA, QILU) with increased concentration gradient (0, 0.5, 1, 2, 4, 8ug/ml) or 5-FU (0, 0.01, 0.1, 1, 10 ug/ml). After 48 hours of incubation, 100µl RPMI-1640 containing 10% FBS with 10µl CCK-8 solution was used to replace the culture medium of each well, incubated at 37 °C for 2 hours. The absorbance was measured with a microplate reader at 450nm, and the IC₅₀ value was calculated by Prism statistical software (GraphPad Software, San Diego, CA). All the experiments were repeated 3 times.

Identification of SPIB associated target genes

In order to further analyze the molecular mechanism of SPIB, we downloaded RNA-sequencing data from TCGA database, and the samples were divided into SPIB high expression group (237 CRC patients) and SPIB low expression group (236 CRC patients) by median expression value of SPIB gene. The top 50 p-value < 0.05 signaling pathways were obtained through gene set enrichment analysis. The signal pathways with great correlation with CRC were found by literature review. Through the analysis of the signal pathways, the target gene that may be related to SPIB was found.

Double luciferase reporter assay

In order to explore the interaction of transcription factor SPIB and the MAP4K1 promoter region, 293T cells were inoculated into a 24-well plate, the cell density increased to 60%-70% per well on the second day. Four groups were set up and transfected with pHG-PromDetect-NC+pCMV6-Entry, pHG-PromDetect-NC+pCMV6-SPIB, pHG-PromDetect-MAP4K1p+pCMV6-Entry and pHG-PromDetect-m4K1p+pCMV6-SPIB respectively. Double luciferase reporter plasmid was purchased from HonorGene (China). After 48 hours, luciferase activity was detected by double luciferase reporter kit (PROMEGA) and expressed as relative activity. All the experiments were repeated three times.

Statistical analysis

For all analyses, a double-tailed t-test from the GraphPad software was used to assess the difference between the cell control group and the experimental group. P-values < 0.05 was considered to be statistically significant.

Results

SPIB mRNA is down-regulated in CRC cell lines and CRC tissues

The online database UALCAN (<http://ualcan.path.uab.edu/analysis.html>) showed that the expression of SPIB mRNA in Rectum adenocarcinoma (READ) and Colon adenocarcinoma (COAD) tissues was lower than that in normal colorectal tissues (Fig. 1a). We used RT-PCR to detect the expression of SPIB mRNA in colorectal cancer cell lines (HCT116, HT-29, LoVo, SW480, CaCO2 and RKO), the colorectal non-tumor tissues were used as control. The result showed that the expression of SPIB mRNA was significantly down-regulated in 6 CRC cell lines (Fig. 1b). We used qRT-PCR to detect the expression of SPIB mRNA in 25 CRC tissues and paired non-tumor tissues. The results showed that the expression level of SPIB mRNA in 88% (22/25) CRC tissues was significantly lower than that in the paired non-tumor tissues (Fig. 1c) ($P < 0.05$). The clinical information of CRC patients is shown in Table 2.

mRNA and protein levels were verified after the SPIB gene was overexpressed and silenced in CRC cells

As the expression of SPIB mRNA in HT-29 and RKO cells was significantly reduced, the overexpressed plasmid pCMV6-SPIB was transfected to construct SPIB overexpressed stable strain, and transfection of plasmid pCMV6 - Entry as control. Since the expression of SPIB mRNA in HCT116 cells was higher than that in the other 5 CRC cell lines, SPIB in HCT116 cells was silenced by transfection siRNA-SPIB, and siRNA-NC was used as the control. Total mRNA and protein of the experimental group and the control group were extracted respectively. RT-PCR, qRT-PCR and western blot were used for detection. The results showed that the mRNA and protein levels of SPIB in HT-29 and RKO cells were significantly higher than those in the control group, and the mRNA and protein levels of SPIB in HCT116 cells were significantly lower than those in the control group (Fig. 2a, 2b and 2c). These results suggested that SPIB overexpressed stable cell lines and SPIB silenced cell lines were successfully constructed.

SPIB inhibits the proliferation of CRC cells

Colony formation assay showed that compared with the control group, the number of clones of HT-29 and RKO cells after SPIB overexpression was significantly reduced ($P < 0.001$) (Fig. 2d and 2e). The results of CCK-8 cell proliferation assay showed that compared with the control group, HT-29 and RKO cell lines with stable overexpression of SPIB decreased significantly in cell proliferation at 24h, 48h and 72h ($P < 0.01$) (Fig. 2f and 2g). The proliferation of HCT116 cells of SPIB silenced was significantly increased at 24h, 48h and 72h ($P < 0.05$) (Fig. 2h). These results suggested that SPIB could inhibit the proliferation of CRC cells.

SPIB inhibits migration and invasion of CRC cells

Transwell assay was used to detect the effect of SPIB on the migration and invasion ability of CRC cells. For migration, the number of cells passing through the chamber membrane is shown below: HT-29 cells in the SPIB overexpression group (22 ± 2.646) were significantly less than those in the control group (94.67 ± 19.40), RKO cells in the SPIB overexpression group (13 ± 5.92) were significantly less than those in the control group (147 ± 9.849), and HCT116 cells in the SPIB silence group (146.7 ± 6.11) were significantly more than those in the control group (70.67 ± 2.517) (Fig. 3a–3b and 3c). With statistically significant differences ($P < 0.01$). These results suggested that SPIB can reduce the migration ability of CRC cells. For invasion, the number of cells passing through the chamber membrane is shown below: HT-29 cells in the experimental group (7.333 ± 2.517) were significantly less than those in the control group (43.33 ± 1.528), RKO cells in the experimental group (18 ± 4) were significantly less than those in the control group (194 ± 19.08), and HCT116 cells in the experimental group (168.3 ± 3.512) were significantly more than those in the control group (64.3 ± 3.512) (Fig. 3d–3e and 3f). With statistically significant differences ($P < 0.01$). These results suggested that SPIB can reduce the invasion ability of CRC cells.

SPIB inhibits angiogenesis

In the tube formation assay, we found that the number of tubules formed in the SPIB overexpression group was significantly lower than that of the control group (Fig.3g). It suggested that SPIB can inhibit angiogenesis.

SPIB can induce the cell cycle of CRC cells arrest in G2/M phase and promote the apoptosis of CRC cells

Flow cytometry was used to detect the effect of SPIB on the cell cycle and apoptosis of CRC cells. When the cell cycle was detected, we found that in RKO(Fig.4a~4b) and HT-29(Fig.4c~4d) cells, the number of cells in the G2/M phase was significantly higher in the SPIB overexpression group than in the control group ($P < 0.05$). The results suggested that SPIB could induce the cell cycle of CRC cells arrest in G2/M phase. In the detection of cell apoptosis, the results showed that the apoptosis percentage of HT-29 and RKO cells in SPIB overexpression group was significantly higher than that in the control group ($P < 0.001$)(Fig. 4e and 4f).The results suggested that SPIB could promote the apoptosis of CRC cells.

SPIB up-regulates c-PARP protein expression and activates NFkB and JNK signaling pathways

Western blot was used to detect the protein expression level associated with apoptosis, we found that in HT-29 and RKO cells, overexpression of SPIB up-regulated NFkB P65, p-NFkB P65, c-PARP, JNK1, and C-Jun proteins expression level compared with the control group (Fig. 4g).

SPIB sensitizes colorectal cancer cells to oxaliplatin and 5-FU

The effects of SPIB on oxaliplatin and 5-FU against CRC were investigated by growth inhibition assay. The IC₅₀ value of oxaliplatin was as follows: IC₅₀ pCMV6-Entry =1.83, IC₅₀ pCMV6-SPIB =0.5964. (Fig.5a). The IC₅₀ value of the control group was 3.068 times higher than that of the SPIB overexpression group. The IC₅₀ value of 5-FU was as follows: IC₅₀ pCMV6-Entry =1.214, IC₅₀ pCMV6-SPIB =0.1033. (Fig.5b). the IC₅₀ value of the control group was 11.75 times higher than that of the SPIB overexpression group. These results suggest that SPIB can up-regulate the sensitivity of colorectal cancer cells to oxaliplatin and 5-FU.

SPIB can activate the promoter of MAP4K1 and enhance the expression of MAP4K1

In order to further study the mechanism of SPIB in CRC, we obtained SPIB-related signaling pathways in CRC through gene set enrichment analysis, including MAPK, JAK-STAT and APOPTOSIS signaling pathways (Fig.6a).In the MAPK pathway, we found that the expression level of SPIB in CRC tissues affected the expression level of MAP4K1 (Fig.6b).In GEPIA database (<http://gepia.cancerpku.cn/detail.php>), the expression of SPIB was positively correlated with MAP4K1 (Fig. 6c).By using qRT-PCR and western blot to detect the correlation between the mRNA and protein levels of SPIB and MAP4K1, we found that the expression of MAP4K1 up-regulated after SPIB was overexpressed and decreased after SPIB was silenced ($P < 0.05$)(Fig. 6d and 6e). In order to further explore the interaction between SPIB and the promoter region of MAP4K1, we conducted double luciferase reporter assay, the results showed that SPIB overexpression could enhance the luciferase activity of MAP4K1 compared with the control group ($P < 0.01$)(Fig. 6f). The results suggested that SPIB can activate the promoter of MAP4K1 and enhance the expression of MAP4K1.

SPIB up-regulates c-PARP protein expression and activates NFkB and JNK signaling pathways by upregulation of MAP4K1

In order to explore whether MAP4K1 is the target gene of SPIB, RKO cells with SPIB overexpression were transfected with siRNA-MAP4K1 and siRNA-NC (Ribobio, China) respectively, confirmed by qRT-PCR($P < 0.001$)(Fig. 7a) and western blot(Fig. 7b).The results suggested that the MAP4K1 gene was successfully silenced in the RKO cells that stably overexpressing of SPIB. Subsequently, western blot was used to detect the protein expression level of the above associated with apoptosis after silencing MAP4K1, and the results showed that the protein expressions of c-PARP, NFkB P65, p-NFkB P65, JNK1, and C-Jun were down-regulated (Fig.7c). The above results indicated that MAP4K1 is the target gene of SPIB, and SPIB activates NFkB and JNK signaling pathways by upregulation of MAP4K1 (Fig.7d).

Discussion

SPIB is one member of the ETS transcription factor family, which is abnormally activated at all stages of oncogenesis [3].Previous studies have found that SPIB plays an important role in the occurrence and development of tumors. In acute lymphoblastic leukemia of children B lymphocyte line, the high expression of SPIB can inhibit cell proliferation and induce cell apoptosis [9].SPIB is an important transcriptional regulator of B-cell differentiation and a new tumor suppressor in the B-cell lineage [10].However, SPIB also plays a pro-

cancer role in some tumors. For example, SPIB can promote the invasion of lung cancer cells [11]. SPIB is anti-apoptotic through PI3K-AKT pathway in diffuse large B-cell lymphoma [12]. The expression of SPIB may be related to the poor prognosis of liver cancer and may be used as a clinical prognostic indicator of liver cancer [13]. Previous Study has shown that the expression level of SPIB in CRC tissues is lower than that in paired non-tumor tissues [4]. In our study, we also found that the expression of SPIB in CRC tissues and cell lines were silenced or down-regulated, suggesting that SPIB may play a role of cancer inhibition in CRC. However, the biological function and mechanism of SPIB in CRC have not been reported so far, so we conducted further research on it.

The effect of SPIB on the growth and metastasis of CRC cells was studied by CCK-8 cell proliferation assay, clone formation assay, flow cytometry, transwell assay and other in vitro assays. This study found that SPIB overexpression inhibited the proliferation, migration and invasion of CRC cells, promoted the apoptosis of CRC cells and induced cell cycle arrest in G2/M phase. It has been reported that ETS family members have the ability to regulate angiogenesis [14]. In our study, tube formation assay has shown that SPIB can inhibit angiogenesis. All the above results indicate that SPIB may be a tumor suppressor gene in CRC. To further confirm this conclusion, SPIB was silenced in CRC cells, and the results showed that the ability of CRC cells to proliferate, migrate and invade was significantly enhanced after SPIB silencing, which further confirmed the anticancer effect of SPIB in CRC cells.

Oxaliplatin is the third generation of platinum anticancer drugs, is the most commonly used chemotherapy drugs of I, II stage of colorectal cancer patients after surgical resection [15]. Through the growth inhibition assay, we found that the IC₅₀ value of the control group was 3.068 times higher than that of the SPIB overexpression group, indicating that the high expression of SPIB could increase the sensitivity of CRC cells to oxaliplatin. Since the activation of JNK can promote oxaliplatin induced apoptosis [16], we believe that the high expression of SPIB induces the sensitivity of CRC cells to oxaliplatin by activating the JNK pathway. 5-FU is a commonly used chemotherapy drug in tumor therapy [17], which mainly targets at the S phase of cell cycle and other phases to play an anti-tumor role [18]. In this study, the growth inhibition assay found that the IC₅₀ value of the control group was 11.75 times higher than that of the SPIB overexpression group, suggesting that SPIB could increase the sensitivity of CRC cells to 5-FU. We believed that the high expression of SPIB could induce the cycle arrest of CRC cells in the G2/M phase, leading to increase sensitivity to 5-FU. Since clinical chemotherapy failure is often caused by the resistance of tumor cells to chemotherapy drugs, our study provides a new probability for the treatment of CRC patients.

In order to explore how SPIB acts on colorectal cancer, we conducted corresponding studies.

1. PARP is considered to be an important protein involved in DNA repair [19]. Proteolysis of PARP is considered to be an early molecular marker of programmed cell death, PARP cleavage during apoptosis promotes cell disintegration [20]. c-PARP is the product of PARP cleavage. In our study, c-PARP expression was up-regulated after SPIB overexpression. Therefore, we believe that the overexpression of SPIB induced apoptosis of colorectal cancer cells.
2. NFκB is a family of transcription factors that play an important role in tumor proliferation, migration and apoptosis [21]. Activated NFκB has been reported to promote the apoptosis of ovarian cancer cells [22]. NFκB p65 is the core of NFκB signaling pathway, and the level of p-NFκB P65 can reflect the activation degree of NFκB signaling pathway [23]. When exposed to specific stimuli, NFκB signaling pathways are activated and free p-NFκB P65 is introduced into the nucleus and combined with corresponding DNA sequences to induce transcription of target genes [23], thus affecting tumor growth and metastasis. In this study, the expressions of NFκBp65 and p-NFκBP65 were up-regulated after SPIB overexpression. Therefore, we believe that SPIB may also induce the apoptosis of CRC cells by activating NFκB signaling pathway.
3. JNK is a key protein in the downstream of the three-level activation system of MAPK protein kinase. JNK plays an important role in biological processes such as cell proliferation, differentiation and apoptosis [24]. The transcription factor C-Jun is a downstream target of JNK and can regulate the expression of pro-apoptotic or anti-apoptotic genes [25]. Studies have shown that the activation of the JNK/C-Jun pathway can lead to the apoptosis of CRC cells [26]. Our data showed that the overexpression of SPIB upregulated both JNK1 and C-Jun proteins. Therefore, we believe that SPIB may induce the apoptosis of CRC cells by activating the JNK/C-Jun pathway.

In order to further study the mechanism of SPIB in CRC, we found that MAP4K1 may be the target of SPIB through gene set enrichment analysis. MAP4K1, known as human hematopoietic progenitor cell kinase 1, is a member of the MAP4K family of kinases [27]. It has been reported that MAP4K family kinases can activate the upstream molecules of MAP3Ks and MAP2Ks, thus activating the MAPK/JNK signaling pathway [28–31], and MAP4K1 proteins can be activated during apoptosis [32]. By western blot, qRT-PCR and double luciferase reporter assay, we found that SPIB can enhance the expression of MAP4K1. We also found that the protein expressions of c-PARP, NFκB P65, p-NFκB P65, JNK1 and C-Jun were down-regulated in the SPIB overexpressed stable strain after MAP4K1 silence. Therefore, we

believe that MAP4K1 is a target gene of SPIB, and SPIB may induce the apoptosis of CRC cells by activating NFkB and JNK signaling pathways through MAP4K1.

However, the number of clinical specimens used in this study was insufficient to determine the possible relationship between SPIB and the pathological features and survival prognosis of colorectal cancer. In future, we will collect more clinically matched samples to further study the relationship between SPIB and clinicopathology and prognosis, so as to provide reference for molecular detection and targeted drug therapy of Colorectal cancer.

Conclusion

In summary, this study found that SPIB acts as a tumor suppressor by activating NFkB and JNK signaling pathways through MAP4K1, and increases the sensitivity of CRC cells to oxaliplatin and 5-FU. SPIB may provide reference for new molecular markers and therapeutic targets for CRC.

Abbreviations

CRC Colorectal cancer

ETS E-twenty six

5-FU 5-fluorouracil

c-PARP cleaved-PARP

NFkB p65 Nuclear factor kB p65

p-NFkB P65 Phospho-NFkB p65

RT-PCR Reverse Transcription Polymerase Chain Reaction

qRT-PCR Quantitative Real-time Polymerase Chain Reaction

READ Rectum adenocarcinoma

COAD Colon adenocarcinoma

IC₅₀ 50% inhibitory concentration

Declarations

Ethics approval and consent to participate

This work was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, the Approval Number is 2020 Research Ethics 2020-340.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded by Tao Luo's personal funds (202257GRJFY).

Authors' contributions

XPZ designed the technical route of this study; XPZ, LL, SY, XYJ were responsible for data acquisition and data analysis; XPZ was involved in data statistics; XPZ, QZ were responsible for paper writing and revision; TL was responsible for the project design and the supervision and management of the whole experiment process. All the authors approved the final manuscript.

Acknowledgements

We would like to thank Professor Tingxiu Xiang for technical assistance, and help with experimental design. We also would like to thank all those who contributed to this study.

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Tables

Table 1

The primers used in this study

PCR	Primer	Sequences (5'-3')	Product size(bp)	Annealing temperature (°C)	Cycles
RT-PCR	SPIB-F	GGAAGACTTACCGTTGGACAG	115bp	55	32
	SPIB-R	CAGCTTCTTGCGAGTCCCT			
	MAP4K1-F	AACGGAAAGAGACCAGAGCA	205bp	55	32
	MAP4K1-R	ATGGGCTGGTACCACTGAAG			

Table 2

The clinical information of colorectal cancer tissues

NO.	Age	Gender (1M2F)	Size (cm)	Grade (1234x)	Lymph metastasis	Distant Metastasis	Localization	T- stage	N- status	Stage	Pathological type
1	63	2	2.5,3	1-2	12	0	Down	2	2b	ⅢB	Ad
2	82	1	-	2	0	0	Right	3	0	Ⅰ	Ad
3	63	2	-	2	0	0	Down	2	0	Ⅰ	Ad
4	72	2	-	1	11	0	Left	4	2	Ⅰ	Ad
5	66	1	3	1-2	0	0	Right	2	0	Ⅰ	Ad
6	60	1	4Ⅹ7	-	1	0	Right	4a	1a	ⅢB	Ad
7	63	1	8	-	1	0	Down	4b	1a	ⅢC	Ad
8	64	1	1,2.5	2	0	0	Down	4b	0	ⅢC	Ad
9	52	2	-	2	2	0	Down	4	1	ⅢB	Ad
10	93	2	-	2	1	0	Down	4	1	ⅢB	Ad
11	55	1	-	2	0	0	Down	2	0	Ⅰ	Ad
12	47	1	3	2	0	0	Left	4a	0	ⅢB	Ad
13	46	2	-	1-2	9	0	Down	4b	2b	ⅢC	Ad
14	52	2	-	2	0	0	Down	4a	0	ⅢB	Ad
15	62	2	-	2	0	0	Right	4a	0	ⅢB	Ad
16	75	1	-	2	0	0	Right	4a	0	ⅢB	Ad
17	52	2	-	2	0	0	Down	4	0	ⅢB	Ad
18	77	2	-	2	1	0	Left	4a	1	ⅢB	Ad
19	68	1	-	1-2	0	0	Left	1	0	Ⅰ	Ad
20	46	1	-	2	0	0	Left	4a	0	Ⅰ	Ad
21	74	2	5,5	2	0	0	Left	4	0	ⅢB	Ad
22	72	1	-	2	1	0	Down	4a	1a	ⅢA	Ad
23	75	2	-	2	0	0	Right	4a	0	ⅢB	Ad
24	64	2	-	1	0	0	Right	4a	0	ⅢB	Ad
25	76	2	-	2	0	0	Left	4a	0	ⅢB	Ad
Abbreviations: Ad, adenocarcinoma.											

Figures

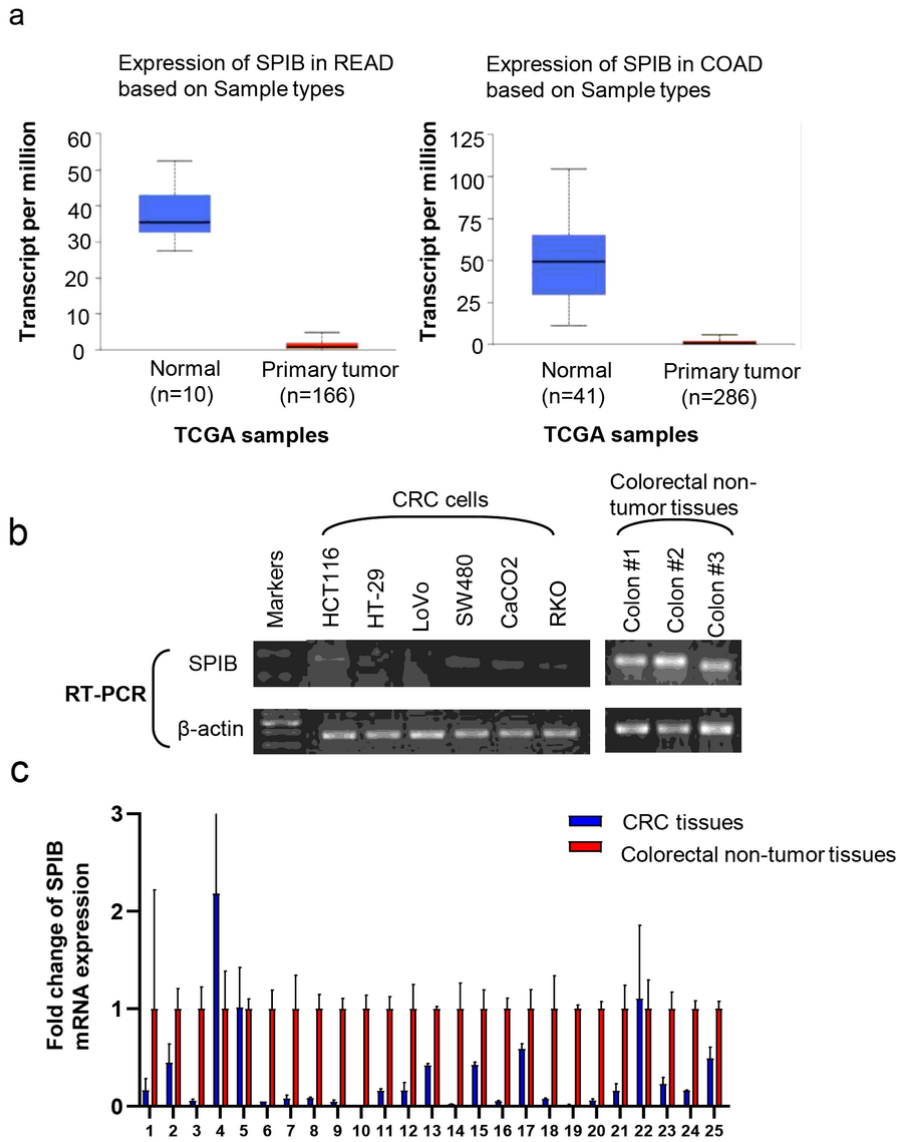


Figure 1

The expression of SPIB mRNA in CRC cell lines and tissues. (a) The expression of SPIB mRNA in Rectum Adenocarcinoma (READ) and Colon Adenocarcinoma (COAD) on UALCAN website. (<http://ualcan.path.uab.edu/analysis.html>). (b) The expression of SPIB mRNA in CRC cell lines and colorectal non-tumor tissues. (c) QRT-PCR was used to detect the expression of SPIB mRNA in 25 pairs of CRC and colorectal non-tumor tissues. Abbreviations: CRC, colorectal cancer.

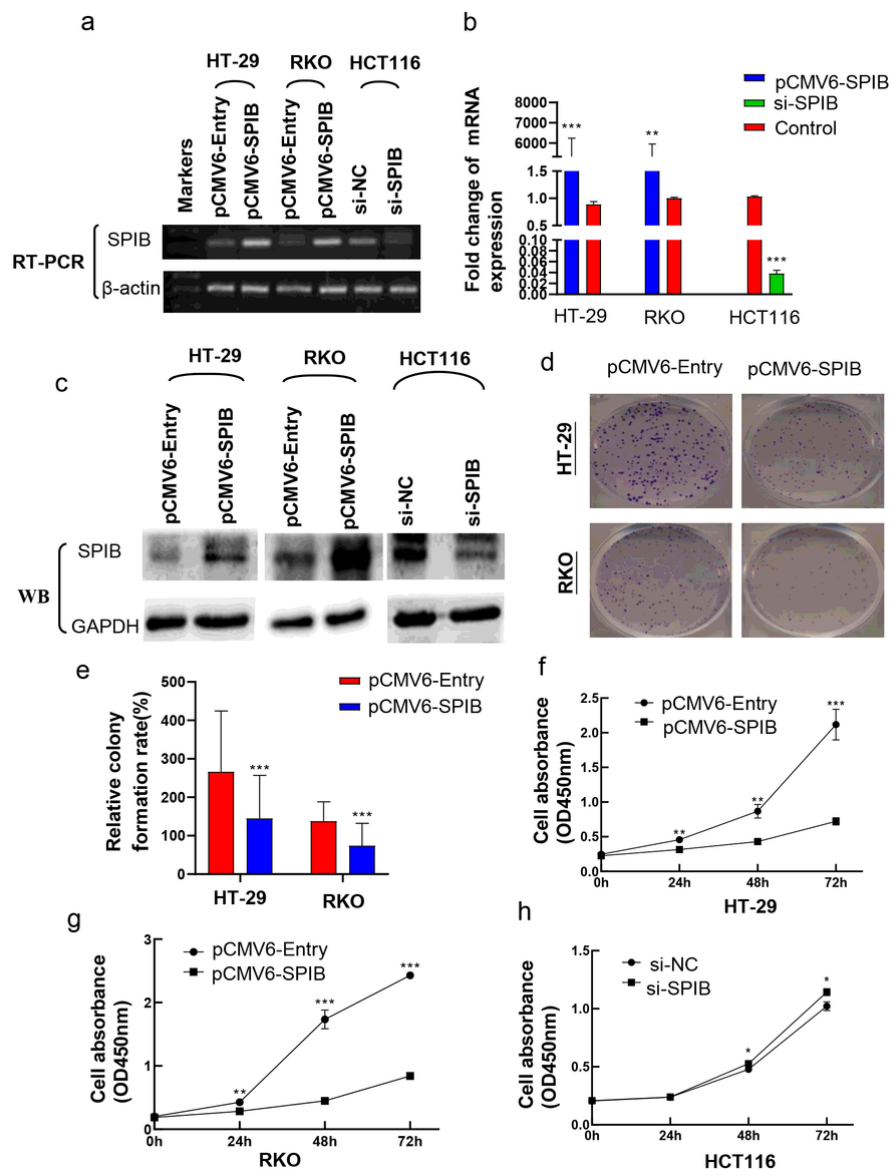


Figure 2

SPIB inhibits the proliferation of CRC cells. The mRNA and protein levels of SPIB in overexpressed and knockdown cell lines were verified by (a) RT-PCR (b) qRT-PCR (***) $p<0.001$, ** $p<0.01$) and (c) western blot. (d) The effect of SPIB on colony formation of CRC cells and (e) the quantitative analysis of colony formation (***) $p<0.001$. (f-g-h) The effect of SPIB on the proliferation of CRC cell lines was detected by CCK-8 (***) $p<0.001$, ** $p<0.01$, * $p<0.05$).

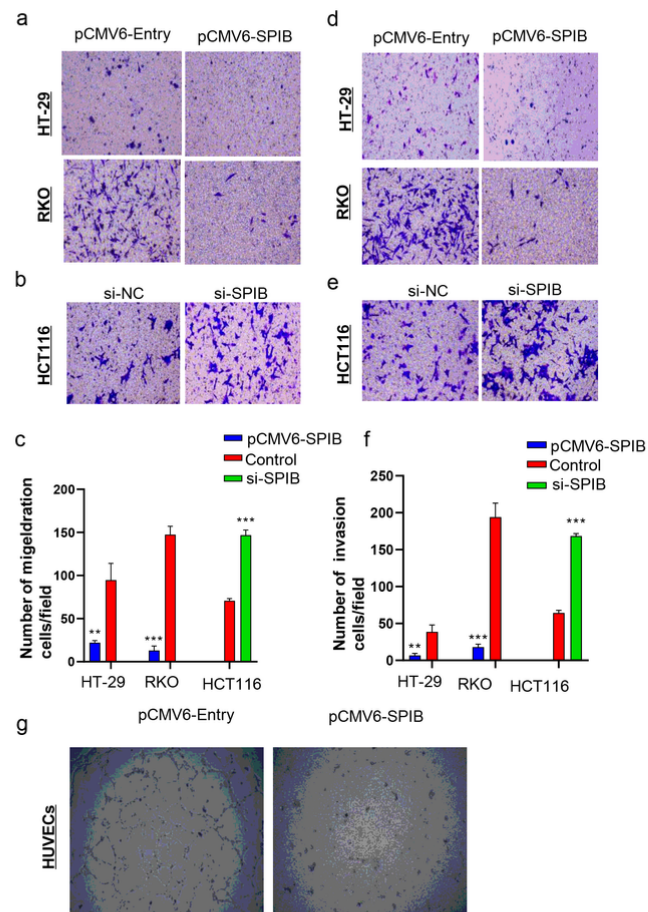


Figure 3

SPIB inhibits CRC cells migration, invasion and inhibits vascularization. Transwell assay was used to detect the (a-b) migration and (d-e) invasion capabilities of CRC cells, the number of migration (c) and invasion (f) cells after overexpression and silencing of SPIB was quantitatively analyzed (** $p < 0.01$, *** $p < 0.001$). (g) The tube formation assay was used to detect the ability of SPIB to inhibit tumor vascularization.

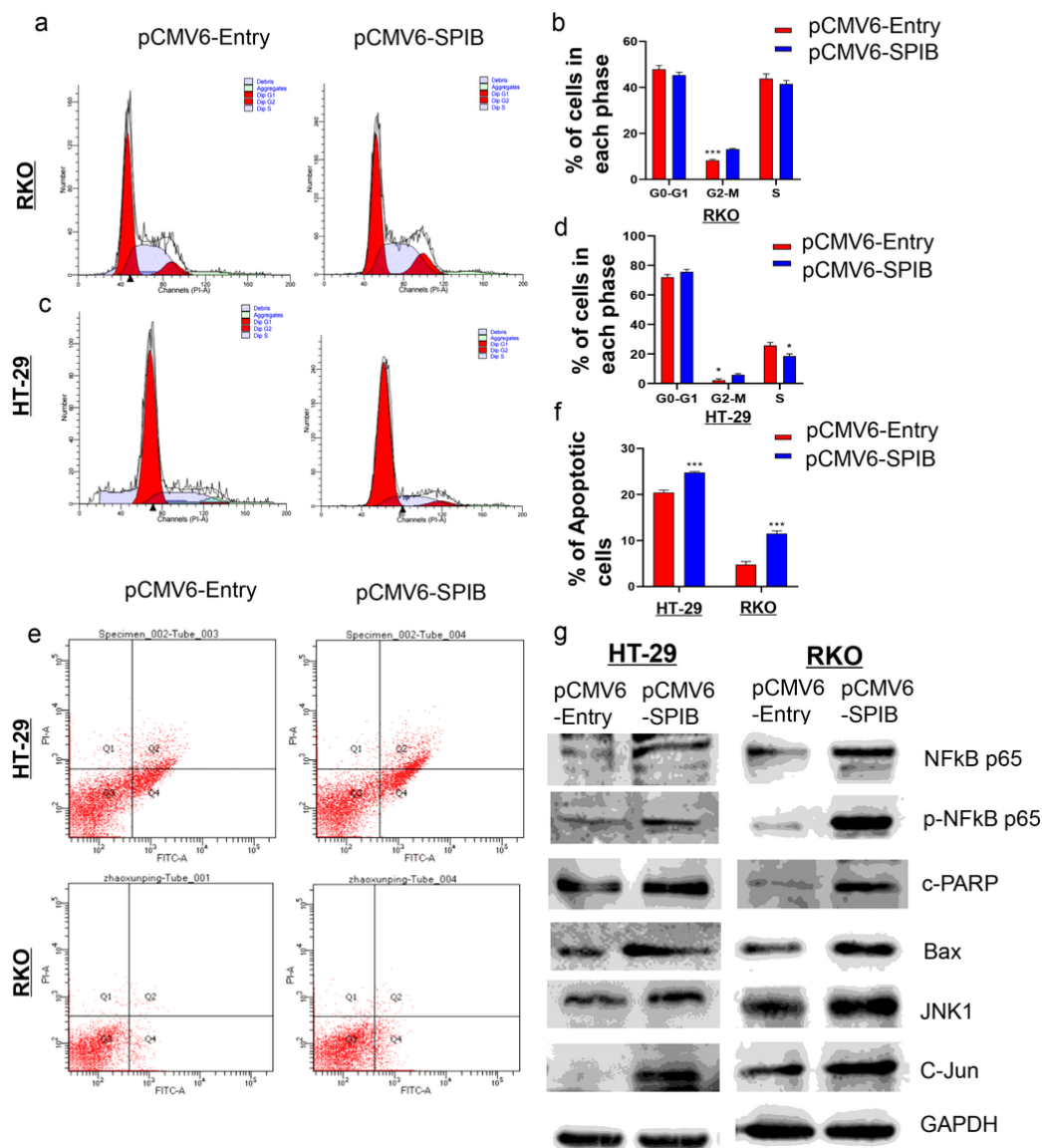


Figure 4

Overexpression of SPIB induced cell apoptosis and cell cycle arrest of CRC cells. SPIB induced the cell cycle of (a,b) RKO and (c,d) HT-29 cells arrest in G2/M phase. Students' t-test was used to evaluate values (** $p < 0.001$, * $p < 0.05$). (e,f) The effect of SPIB on apoptosis rate of HT-29 and RKO cells was detected by flow cytometry, students' t-test was used to evaluate values (** $p < 0.001$). (g) Western blot was used to detect the effects of SPIB on the protein expression level associated with apoptosis in HT-29 and RKO cells, GAPDH as control.

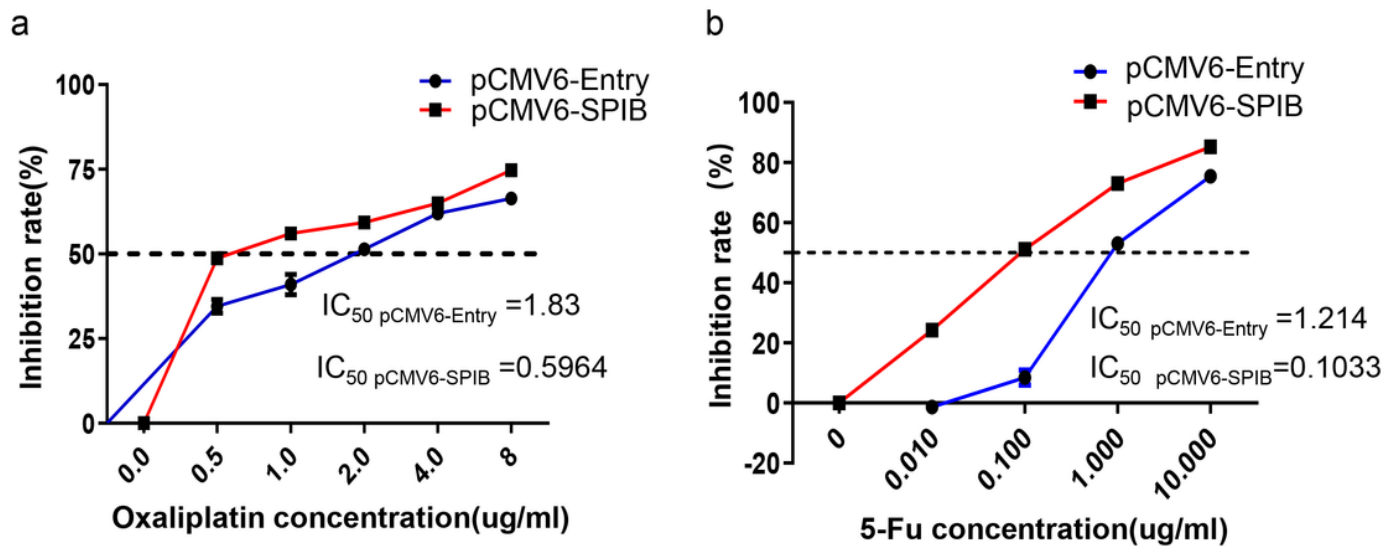


Figure 5

Overexpression of SPIB increased the sensitivity of CRC cells to oxaliplatin and 5-FU. The inhibitory effect of (a)oxaliplatin and (b)5-FU on the growth of RKO cells transfected with pCMV6-Entry or pCMV6-SPIB was detected by CCK-8, the IC_{50} was calculated by GraphPad software.

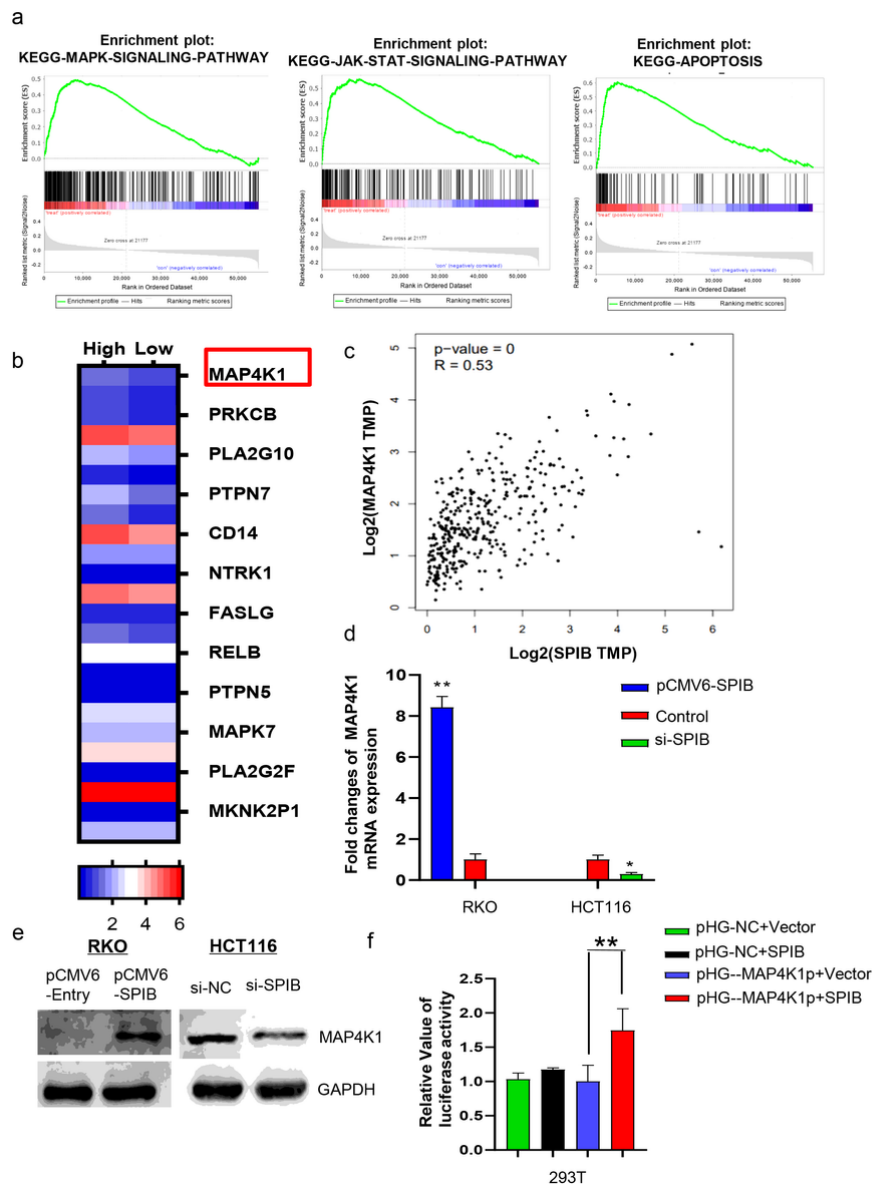


Figure 6

SPIB can activate the promoter of MAP4K1 and enhance the expression of MAP4K1. (a) SPIB-related signaling pathways in CRC were identified by gene set enrichment analysis. (b) The expression of a group of genes related to SPIB expression level in MAPK signal pathway. (c) The correlation between SPIB and MAP4K1 in GEPIA database (<http://gepia.cancer-pku.cn/detail.php>). (d) QRT-PCR and (e) western blot were used to detect the expression of MAP4K1 after SPIB overexpressing and silencing. (f) Double luciferase reporter assay was used to detect the interaction between SPIB and MAP4K1 promoter region.

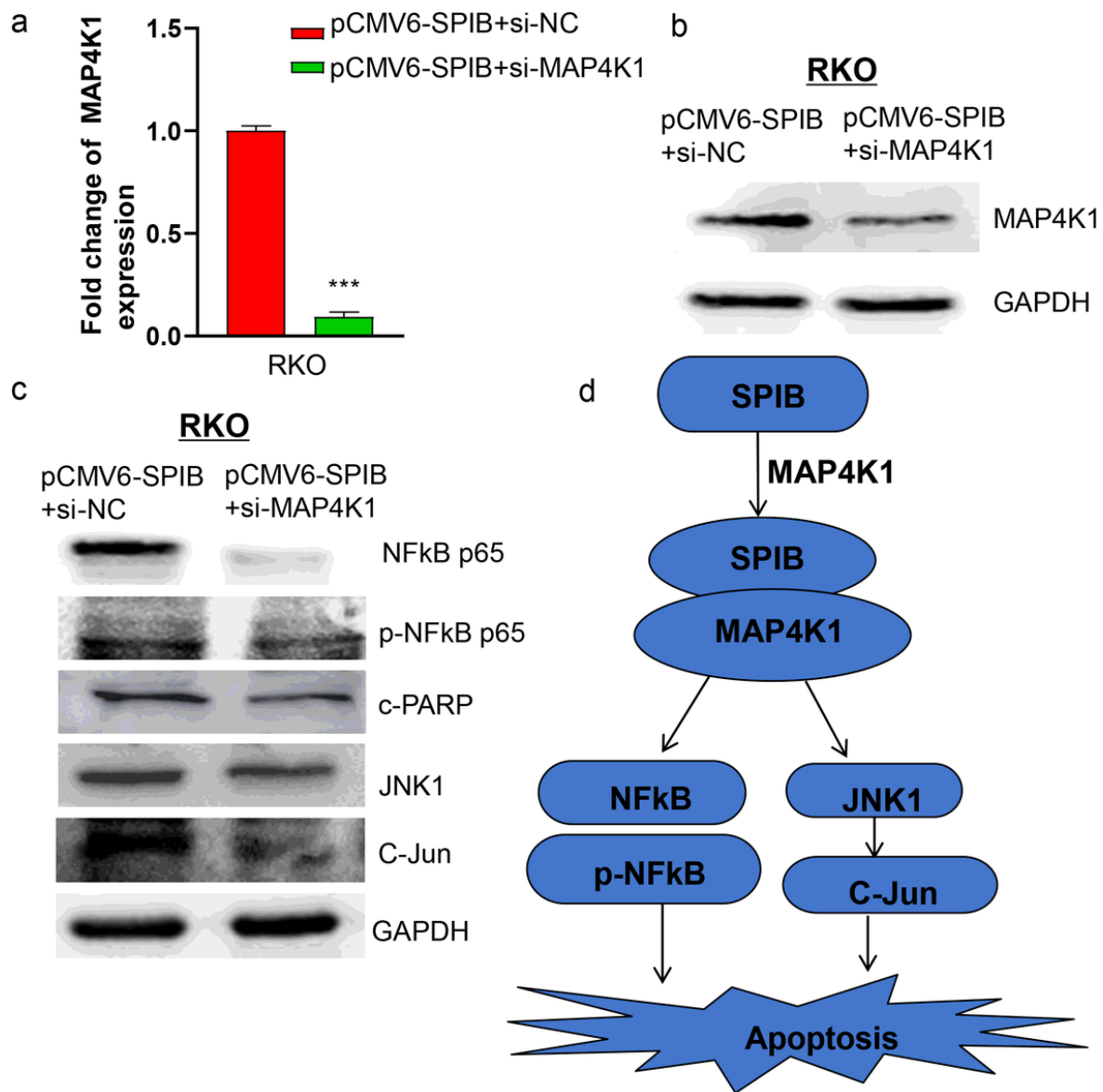


Figure 7

The effect of silencing MAP4K1 in SPIB overexpressed stable cell lines on the expression of apoptosis-related protein. (a) QRT-PCR and (b) western blot confirmed the expression of MAP4K1. (c) Western blot was used to detect the expression of NFkB P65, p-NFkB P65, c-PARP, JNK1, C-Jun after silencing MAP4K1, GAPDH as control. (d) Schematic diagram of SPIB promoting apoptosis.