

# Basic transcription factor 3 expression silencing attenuates colon cancer cell proliferation and migration *in vitro*

XU LI, JINKE SUI, JUNJIE XING, FUAO CAO, HAO WANG, CHUANGANG FU and HANTAO WANG

Department of Colorectal Surgery, Changhai Hospital, Shanghai 200433, P.R. China

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**Abstract.** Basic transcription factor 3 (BTF3) is an RNA polymerase II transcription factor that also regulates apoptosis. Numerous studies have identified that BTF3 is aberrantly expressed in several types of tumor. However, the function of BTF3 in colorectal cancer remains unknown. The aim of the present study was to assess the function of BTF3 during colon cancer tumorigenesis. Applying a lentivirus-transfected short hairpin RNA approach, expression of BTF3 was dysregulated in the colon cancer HCT116 and HT-29 cell lines; knockdown efficiency was verified using the quantitative polymerase chain reaction and western blotting. To determine the function of BTF3 in colon cancer, cell proliferation was assessed using an MTT assay, cell apoptosis and the cell cycle were assessed using flow cytometry, and cell migration was assessed using a Transwell assay. Knockdown of BTF3 inhibited cell proliferation, possibly because BTF3 knockdown induced cell early apoptosis and arrested cells in G<sub>0</sub>-G<sub>1</sub> phase. BTF3 knockdown also inhibited cell migration. The results of the present study identified that BTF3 expression is associated with colon cancer progress, and BTF3 may therefore be a molecular marker for diagnosis and treatment outcomes of human colon cancer.

## Introduction

Different transcription factors serve various functions in multiple physiological mechanisms, including cell cycle progression, cell metabolism, growth and development (1). Basic transcription factor 3 (BTF3) is involved in various biotic and abiotic stress processes, as well as different physiological and developmental mechanisms (2,3).

BTF3 is encoded by the human BTF3 gene and is evolutionarily conserved in a range of organisms (4,5). BTF3 was initially described as a member of the general transcription

machinery and forms a stable complex with the RNA polymerases (6). BTF3 initiates transcription by binding to promoter elements such as the TATA and CAAT box sequences in the promoter region (7,8). Owing to alternative splicing, BTF3 is present in two different isoforms: BTF3a and BTF3b. BTF3a is the transcriptionally active form of BTF3, whereas the BTF3b isoform, which lacks the 44 N-terminal amino acids of BTF3a, is transcriptionally inactive, although it is able to bind to RNA polymerase II (9).

A previous mouse embryonic development study revealed that mice homozygous for a loss-of-function mutation in the BTF3 gene succumbed early in development, indicating its function in biological development (10). In addition to its functions as transcription regulator, BTF3 also aids the regulation of the cell cycle and apoptosis (11,12). Decreased BTF3 expression is associated with increased apoptosis in lymphocytes (13), and downregulation of BTF3 inhibits transcription and protein synthesis (14). In *Caenorhabditis elegans*, overexpression of BTF3 prevents cell apoptosis, whereas the RNA interference-mediated knockdown of BTF3 induces cell apoptosis (11). In human cancer, BTF3 is overexpressed in glioma (15), hepatocarcinoma (16) and pancreatic ductal adenocarcinoma (17). Downregulation of BTF3 decreases the expression of several cancer-associated genes, including ephrin receptor B2 (18), heparanase 2 (19) and the oncogene ABL proto-oncogene 2, non-receptor tyrosine kinase (20). In breast cancer, BTF3 interacts with either 17 $\beta$ -estradiol or estrogen receptor  $\alpha$  (ER $\alpha$ ) through its AF1 domain and upregulates the transcriptional response of ER $\alpha$  reporter genes (21,22). These results suggest that BTF3 also serves an important function in tumor occurrence and the development of tumor progression.

The molecular mechanism of BTF3 in colon cancer remains unclear. In order to investigate the function of BTF3 in colon cancer, the present study used a lentivirus-mediated short hairpin RNA (shRNA) approach to target BTF3 to knockdown its expression in human colon cancer cells. In addition, the effect of BTF3-knockdown on cell proliferation, the cell cycle, cell apoptosis and migration was investigated. The results of the present study provide information on a novel molecular target for the diagnosis and therapy of colon cancer.

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*Correspondence to:* Dr Hantao Wang, Department of Colorectal Surgery, Changhai Hospital, 168 Changhai Road, Shanghai 200433, P.R. China  
E-mail: hantaowang1234@163.com

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## Materials and methods

**Cell culture.** The colon cancer HCT116 and HT-29 cell lines, and 293 cells were purchased from the American Type Culture

Collection (Manassas, VA, USA). All cells were grown in complete Dulbecco's medium Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), and 100 U/ml penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, USA), and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere.

**Plasmid and vector construction.** The selected and optimized shRNA against human BTF3 (5'-GCAGCGAAC ACTTTCACCATT-3') was transfected into the lentiviral vector pLKO.1-pure (Addgene, Inc., Cambridge, MA, USA). The constructed shBTF3 plasmid and control vector were further co-transfected with Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) into 293 cells with packaging plasmids (preserved in our lab) to generate a shBTF3-expressing lentivirus or empty vector lentivirus. Cells (1x10<sup>6</sup>) were cultured in a 10-cm dish and incubated at 37°C for 24 h. Control lentivirus and BTF3-targeted shRNA lentivirus were then used to transfect the HCT116 and HT-29 cells. Following selection in 2 µg/ml puromycin (Thermo Fisher Scientific, Inc.) for 1 week, cells were collected to evaluate the knockdown efficiency.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from BTF3-knockdown cell lines (those transfected with BTF3-targeted shRNA), the empty vector control cell line (Vector) and non-transfected cells using an RNeasy mini kit (Qiagen China Co., Ltd., Shanghai, China), according to the manufacturer's protocol. cDNA was generated by reverse transcription of 1-µg aliquots of RNA using the Takara PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The cDNA was used for qPCR using the SYBR Premix Ex-Taq kit (Takara Biotechnology Co., Ltd.) on a CFX96 instrument qPCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR was performed according to the manufacturer's instructions: Initial denaturation was at 95°C for 10 min followed by 30 cycles at 95°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. All expression data were normalized to β-actin levels using 2<sup>-ΔΔC<sub>q</sub></sup> method (23). Primer sequences were as follows: β-actin, 5'-CGAGCGCGGCTA CAGCT-3' (forward) and 5'-TCCTTAATGTCACGCACG ATTT-3' (reverse); and BTF3 5'-AGCTTGGTGCGGATA GTCTGA-3' (forward) and 5'-GTGCTTTCCATCCACAG ATTG-3' (reverse).

**Western blotting.** Cells were lysed in radioimmunoprecipitation buffer (Cell Signaling Technology, Inc., Danvers, MA, USA), containing Complete protease inhibitors (Roche Applied Science, Penzberg, Germany), phosphatase inhibitors (Roche Applied Science), 5 mM dithiothreitol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA) for 15 min, and then centrifuged at 15,000 x g for 10 min at 4°C. The supernatant was collected and protein concentration was quantified using the Bio-Rad Protein assay kit II #5000002 (Bio-Rad Laboratories, Inc.). The proteins were separated by SDS-PAGE (10% gel), blotted onto a polyvinylidene fluoride membrane (Merck KGaA), and then blocked for 1 h at room

temperature in TBST with 2% non-fat milk, followed by overnight incubation at 4°C with anti-BTF3 (Abnova, Taipei, Taiwan; Catalog no. H00000689-R01) or anti-β-actin (Cell Signaling Technology, Inc.; Catalog no. 3700) antibodies (1:1,000). Membranes were rinsed with Tris-buffered saline containing Tween-20 (TBST) with 0.05% Tween-20 and incubated for 2 h with a 1:5,000 diluted HRP-conjugated goat anti-human IgG secondary antibody (Thermo Fisher Scientific, Inc.; Catalog no. A18847). Following another three washes with TBST, target proteins were detected using an Enhanced Chemiluminescence detection kit (Pierce; Thermo Fisher Scientific, Inc.). Quantification of the western blot analysis was performed using ImageJ software v1.8.0 (National Institutes of Health, Bethesda, MD, USA).

**MTT assay.** The shBTF3 shBTF3-expressing lentivirus or empty vector lentivirus was transfected into HCT116 and HT-29 cells the knockdown efficiency were examined. HCT116 and HT-29 cells were seeded at 3x10<sup>3</sup> cells per well at 37°C in 96-well plates and cell viability was assessed every day for 4 days. In brief, at the start of each assay, 20 µl MTT (with 5 mg/ml thiazolyl blue tetrazolium bromide; Sigma-Aldrich; Merck KGaA) was added to each well and then the plate was incubated for an additional 4 h. Culture medium was removed from the wells, 100 µl dimethylsulfoxide (Sigma-Aldrich; Merck KGaA) was added to each well and plates were incubated for 20 min at 37°C. Absorbance was determined using a microplate reader (Bio-Rad Laboratories, Inc.) at a wavelength of 495 nm. Each experiment was performed three times.

**Transwell assay.** The cell migration assay was carried out using a modified Boyden chamber plate with 8-µm pore size polycarbonate membrane filters (Corning Incorporated, Corning, NY, USA) (24). ShBTF3- and empty vector-transfected HCT116 and HT-29 cells, along with non-transfected HCT116 and HT-29 cells, were incubated at 37°C in DMEM for 6 h. Subsequently, 1x10<sup>4</sup> cells from each group and cell type were added to the upper part of the Boyden chamber, and the bottom chamber was filled with DMEM containing 20% serum. The cells were allowed to migrate to the underside of the membrane during incubation for 48 h at 37°C. Next, the cells on the membrane filter were fixed with 4% paraformaldehyde and stained with 0.05% Giemsa (Sigma-Aldrich; Merck KGaA). The migration index was defined as the number of cells that had migrated to the membrane filter by cell counting in at least three random fields using a light microscope (magnification, x200) per filter.

**Flow cytometric assays.** Cells were harvested using trypsin/EDTA (Gibco; Thermo Fisher Scientific, Inc.) from 6-cm-diameter dishes. Following centrifugation 1,200 x g for 15 min at 4°C, pellets were then washed with PBS and centrifuged again. Following resuspension in binding buffer (10 mmol/l HEPES, 140 mmol/l NaCl, 5 mmol/l CaCl<sub>2</sub>), cells were incubated with annexin V and propidium iodide (PI) according to the manufacturer's protocol (BioVision, Inc., Milpitas, CA, USA). Cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software v10.0 (Tree Star, Inc., Ashland, OR, USA). For cell cycle analysis, cells were washed twice with

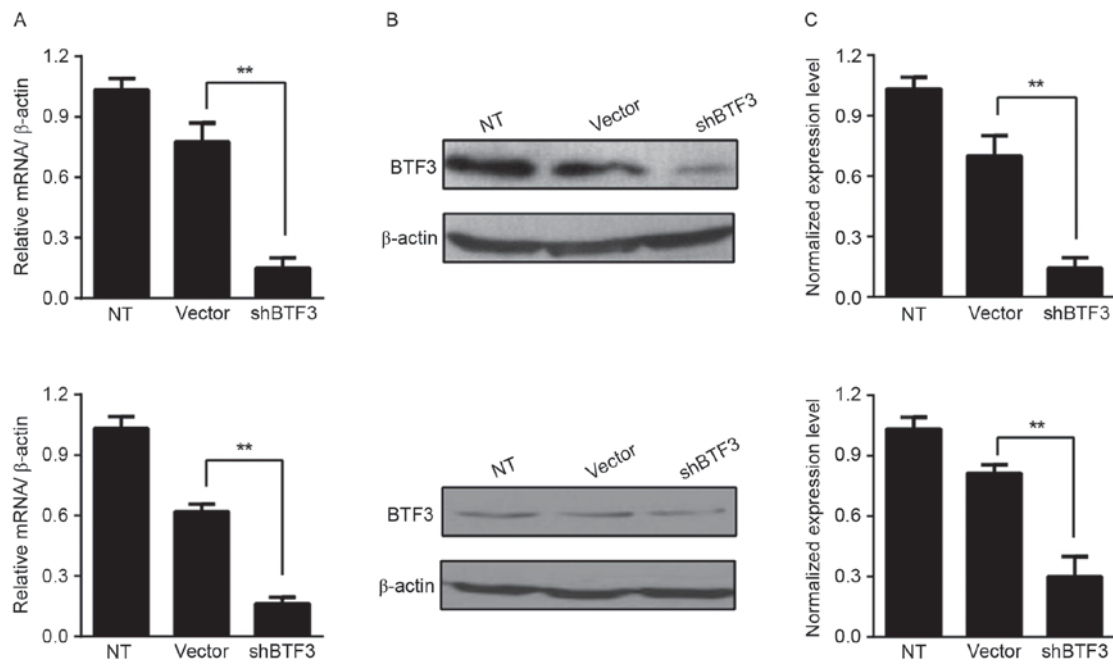


Figure 1. shRNA BTF3 knockdown in colon cancer HCT116 and HT-29 cells. (A) Quantitative polymerase chain reaction analysis detected BTF3 mRNA levels in empty vector control, shRNA-BTF3 lentivirus-transfected, and non-transfected HCT116 (top) and HT-29 (bottom) cells. (B) Western blot analysis of BTF3 protein expression in empty vector control and shRNA-BTF3 lentivirus-transfected, and in non-transfected HCT116 (top) and HT-29 (bottom) cells.  $\beta$ -actin was used as loading control. (C) Semi-quantitative densitometry analysis of BTF3 protein levels in HCT116 (top) and HT-29 (bottom) cells. \*\* $P < 0.01$ . NT, non-transfected; shRNA, short hairpin RNA; BTF3, basic transcription factor 3; shBTF3, short hairpin BTF3.

ice-cold PBS and fixed using 70% ethanol overnight at 4°C. The cells were then digested with 50  $\mu\text{g/ml}$  RNase A in 100  $\mu\text{l}$  PBS and stained with 20  $\mu\text{g/ml}$  PI for 30 min at 37°C. Cells were then analyzed using a FACSCanto II flow cytometer (BD Biosciences) and FlowJo software.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation. Statistical comparisons were made using one-way analysis of variance with a least significant difference post hoc test or an unpaired Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference. Each experiment was performed at least three times.

## Results

**Lentivirus-mediated knockdown of BTF3 in human colon cancer cells.** In order to investigate the function of BTF3 in colon cancer, a lentivirus vector was used to generate BTF3-knockdown stable HCT116 and HT-29 cell lines. Following selection in puromycin for 1 week and once stable cell proliferation was achieved, qPCR and western blot analysis were performed to assess the efficiency of BTF3-knockdown. As presented in Fig. 1A, expression of BTF3 mRNA in HCT116 cells transfected with shBTF3 lentivirus was 80% lower compared with that in non-transfected cells and 60% lower compared with that in cells transfected with the empty vector control ( $P < 0.01$ ). Similar results were obtained in the HT-29 cell line (Fig. 1A). Comparing BTF3-knockdown and empty vector-treated cells, the BTF3 protein level appeared to be markedly decreased in HCT116 and HT-29 cells (Fig. 1B). The semi-quantitative results of western blot densitometry analysis revealed that there was a significant difference in the

BTF3 protein expression level between shBTF3 and empty vector-treated cells ( $P < 0.01$ ; Fig. 1C).

**Knockdown of BTF3 inhibits proliferation of colon cancer cells.** To assess the effect of BTF3 on colon cancer cell proliferation, BTF3-knockdown, empty vector control- and non-transfected HCT116 and HT-29 cells were seeded in 96-well-plates and a series of MTT assays were performed. The results of these assays revealed that cell proliferation in BTF3-knockdown HCT116 and HT-29 cells was significantly decreased over the course of the 4 days, compared with controls ( $P < 0.01$ ; Fig. 2A and B). These results suggest that BTF3 knockdown significantly decreased the proliferation of colon cancer cells.

**BTF3 knockdown induces early apoptosis and arrests cells in  $G_0$ - $G_1$  phase.** To explore further the mechanism by which BTF3 knockdown inhibits cell proliferation, cell apoptosis was examined through annexin V/PI double staining for apoptotic cells. There was a significant increase in early apoptosis (annexin V-positive, PI-negative) in BTF3-knockdown compared with NT (non-transfected) control cells, from  $4.42 \pm 1.33$  to  $25.13 \pm 1.65\%$  ( $P < 0.01$ ; Fig. 3). This result indicated that BTF3 may be involved in colon cancer cell survival. In order to determine the function of BTF3 in cell-cycle progression, flow cytometry was used to assess the changes in cell cycle distribution prior to and following BTF3-knockdown in HCT116 cells. In vector control cells, the cell distribution was:  $G_0$ - $G_1$  phase,  $45.52 \pm 1.09\%$ ; S phase,  $3.08 \pm 0.27\%$ ;  $G_2$ -M phase,  $51.58 \pm 1.06\%$ . Following BTF3 knockdown, cell distribution altered to:  $G_0$ - $G_1$  phase,  $62.41 \pm 1.33\%$ ; S phase,  $1.95 \pm 0.32\%$ ;  $G_2$ -M phase,  $36.01 \pm 1.21\%$  (Fig. 3B). Comparing the control and

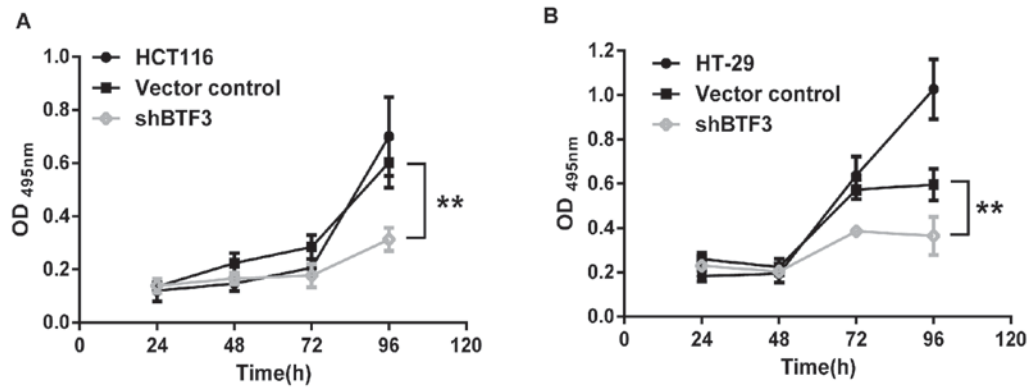


Figure 2. Knockdown of BTF3 inhibits cell proliferation in colon cancer cells. The proliferation of (A) HCT116 and (B) HT-29 cells following lentiviral transfection with empty vector control or shBTF3 was assessed using an MTT assay. \*\* $P < 0.01$ . shRNA, short hairpin RNA; BTF3, basic transcription factor 3; OD, optical density.

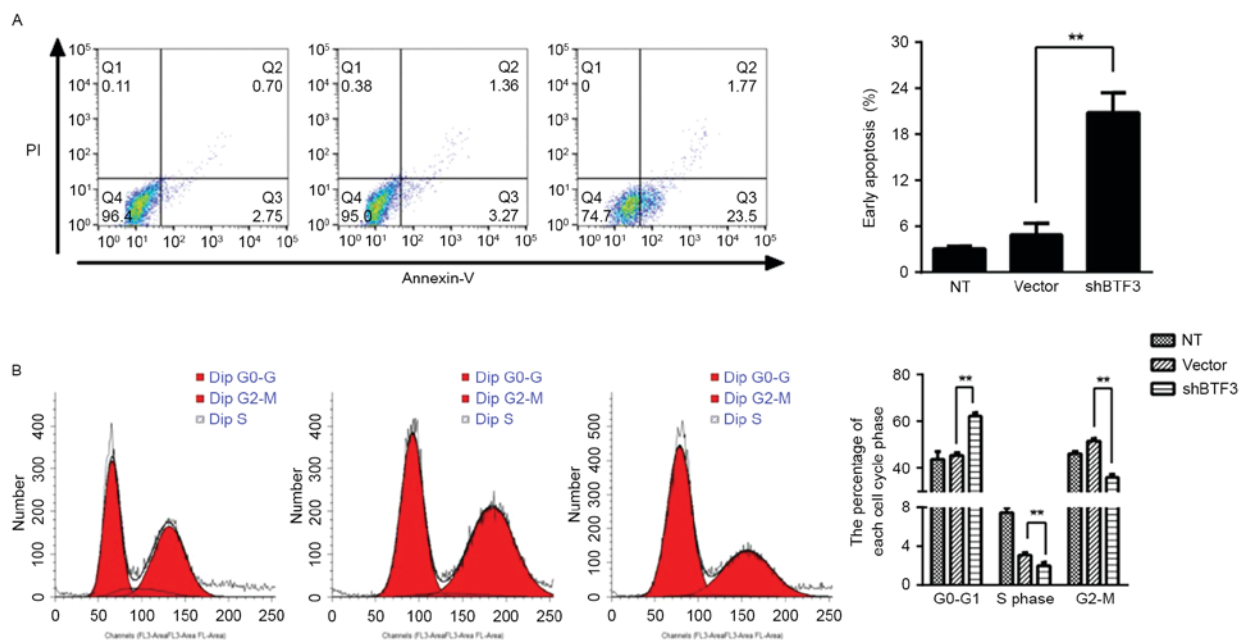


Figure 3. BTF3 knockdown induces HCT116 cells early apoptosis and arrested cells in  $G_0$ - $G_1$  phase of the cell cycle. (A) Flow cytometric analysis of empty vector control, shBTF3-transfected cells and non-transfected HCT116 cells apoptosis using annexin V and PI double staining. The proportion of early apoptotic cells (annexin-V-positive and PI-negative) was quantified. (B) Cell cycle distribution of empty vector control, shBTF3-transfected or non-transfected HCT116 cells determined by flow cytometry. \*\* $P < 0.01$ . BTF3, basic transcription factor 3; shRNA, short hairpin RNA; NT, non-transfected; PI, propidium iodide; shBTF3, short hairpin BTF3; Dip, diploid peak.

BTF3-knockdown cells revealed that cell distribution in the  $G_0$ - $G_1$  phase was significantly increased by BTF3-knockdown ( $P < 0.01$ ), and the proportion of cells in S and  $G_2$ -M phase was significantly decreased ( $P < 0.01$ ). These results indicated that BTF3 knockdown may be involved in cell-cycle regulation as the BTF3-knockdown cells were arrested at  $G_0$ - $G_1$  phase, decreasing the proportion of cells undergoing mitosis and therefore inhibiting cell proliferation.

**Knockdown of BTF3 inhibits colon cancer cell migration.** Cell proliferation and migration are two characteristics of tumor cells. The aforementioned results indicated that BTF3 knockdown is able to inhibit the proliferation of colon cancer cells. Whether BTF3 knockdown alters cell migration was assessed using a Transwell assay. A change in cell migratory ability was

observed following BTF3 knockdown in HCT116 and HT-29 cells (Fig. 4A). Compared with empty vector-transfected control cells, the migration index of BTF3-knockdown cells decreased significantly by 79.7 and 66.3%, in HCT116 and HT-29, respectively ( $P < 0.01$ ; Fig. 4B). These results indicated that BTF3 knockdown may significantly inhibit the migration of colon cancer cells.

## Discussion

BTF3, which functions as an additional transcription factor II-associated protein, does not bind to proximal promoter regions directly, but forms a stable complex with RNA polymerase II and is a part of the gene transcription initiation complex (4,7). Several studies have indicated that the expression

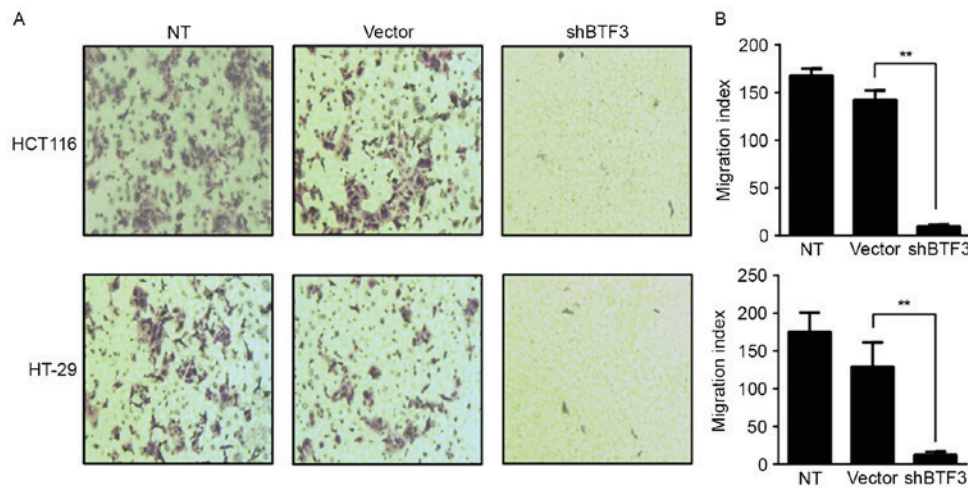


Figure 4. BTF3 knockdown inhibits human colon cancer cell motility. (A) The migratory ability of human colon cancer HCT116 (top) and HT-29 (bottom) cell lines transfected with an empty vector control or with shBTF3 was assessed using a Transwell assay, as was that of non-transfected cells. (B) Quantification of migration indices. Each experiment was performed in triplicate. \*\*P<0.01. BTF3, basic transcription factor 3; shRNA, short hairpin RNA; NT, non-transfected; shBTF3, short hairpin BTF3.

pattern of BTF3 is frequently disordered in tumors (13,17). On the other hand, BTF3 is also highly expressed in malignant tissue and affects the invasion and proliferation of tumor cells, which indicates that BTF3 may serve as an independent diagnostic factor (25). By analyzing clinical data from colorectal cancer patients, Wang *et al* (26) identified that BTF3 overexpression may be an early event in colon cancer and may be a useful biomarker for early-stage colon cancer. In addition, BTF3 expression is associated with the expression of nuclear factor  $\kappa$ B, RAD50 double-strand break repair protein, MRE11 homolog, double-strand break repair nuclease, nibrin and metadherin (26). This change in BTF3 expression may affect these signaling pathways, although the molecular mechanism of BTF3 in colon cancer remains unknown.

A shRNA targeting BTF3 was lentivirally transfected into HCT116 and HT-29 human colon cancer cells. The results demonstrated that knockdown of BTF3 significantly inhibited the proliferation of colon cancer cells, and the amount of early apoptotic cells was significantly increased. The cell-cycle distribution of BTF3-knockdown cells was also altered. The percentage of cells in  $G_0$ - $G_1$  phase was significantly increased in BTF3-knockdown colon cancer cells, whereas those in S and  $G_2$ -M phases was significantly decreased, which indicated that BTF3-knockdown cells underwent cell-cycle arrest in interphase  $G_0$ - $G_1$ . As a result of this arrest, cells could not enter mitosis and early apoptosis was induced, thus leading to a decrease in the proliferation of colon cancer cells. Previous studies revealed that regulation of apoptosis is associated with cell-cycle regulation (27,28). Although apoptosis may be induced at any point in the cell cycle, the propensity for apoptosis to be induced differs markedly depending location of the cell within the cell cycle (29). Progression through the cell cycle is also subject to a number of regulatory proteins. For example, the progression through  $G_1$  phase depends on the balance of cyclin D1 and cyclin-dependent kinase inhibitor 2A expression, owing to their identity as positive and negative regulators of progression through  $G_1$  phase, respectively (30). Therefore,

whether BTF3 caused cells to arrest at  $G_0$ - $G_1$  phase owing to its regulation of cyclins warrants further research.

BTF3 also has an important function in other types of cancer. Liu *et al* (31) identified that BTF3 is potentially associated with the development and progression of gastric cancer. BTF3 is expressed at different levels in different stages of gastric cancer; low expression or gene silencing of BTF3 inhibited tumor growth and may be beneficial for gastric cancer treatment (31). In pancreatic ductal carcinoma, overexpression of BTF3 may be involved in cell-cycle progression, cell proliferation and extracellular matrix degradation (17). Using an immunohistochemical tissue array for the diagnosis and stratification of prostate cancer, Symes *et al* (32) identified that BTF3 expression was significantly upregulated in malignant prostate cancer tissue compared with non-malignant tissue. Therefore, BTF3 has the potential to be used as a specific molecular marker for the diagnosis and stratification of prostate cancer (32). The results of the present study indicated that, in colon cancer cells, BTF3 knockdown inhibited cell proliferation and promoted early apoptosis, suggesting an association between BTF3 expression and colon cancer.

In conclusion, the results of the present study shed light on the biological function of BTF3 in colon cancer. The results of the present study demonstrated that BTF3 knockdown is able to inhibit the proliferation of colon cancer cells, suggesting that BTF3 may promote the occurrence of colon cancer. BTF3 may therefore serve as a biomarker for the diagnosis of colon cancer and provide a molecular target for tumor gene therapy.

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### Availability of data and materials

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

### Authors' contributions

XL carried out the design of the study, drafted the manuscript. JS conducted the cellular function experiments. JX performed molecular genetics examination. FC carried out the statistical analysis. HW helped the design the study and draft the manuscript. CF and HTW conceived the study, and participated in its design and coordination. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests

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