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Original Article

Effect of overexpression of activating transcription factor 3 on biological behaviors of human colorectal cancer HCT116 cells

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Abstract: Colorectal cancer is the third most common cancer and the fourth leading cause of cancer-related mortality worldwide. Activating transcription factor 3 (ATF3) has been identified as both an oncogene and a tumor suppressor in colorectal cancer, which encourages further studies to validate the exact role of ATF3 in colorectal cancer development and progression. The aim of this study was to examine the effect of ATF3 overexpression on biological behaviors of human colorectal cancer HCT116 cells. A stable ATF3-overexpressed HCT116 cell line was established by transfecting HCT116 cells with lentivirus carrying a full-length of ATF3. Cell proliferation was measured with MTS and colony-formation assays, and cell migration and invasion was detected using Transwell migration/invasion assays. In addition, a sphere-forming assay was performed to assess the cancer-initiation capacity, while the gene and protein expression was quantified with qRT-PCR, Western blotting and flow cytometry. ATF3 overexpression was found to suppress proliferation, migration and invasion of HCT116 cells as compared with the control cells, while down-regulation of Survivin and PCNA expression was detected in HCT116 cells at both translational and transcriptional levels, and a reduction was measured in the CyclinD1 protein expression in HCT116 cells overexpressing ATF3 than in the control cells. In addition, MMP2 and MMP9 expression was found to be down-regulated in HCT116 cells overexpressing ATF3 at both translational and transcriptional levels, and Western blotting detected up-regulation of TIMP-2 and TIMP-1 expression in HCT116 cells overexpressing ATF3 relative to the control cells. Overexpression of ATF3 resulted in reversal of epithelial-mesenchymal transition (EMT), as revealed by changes of cell morphology and altered expression of E-cadherin, Snail, Slug and Twist. Most importantly, ATF3-overexpressed HCT117 cells produced less and smaller spheres as compared with the control cells. The expression of multiple markers associated with colorectal cancer-initiation including CD24, CD133, CD166, EpCAM, EZH2 and ID1 were down-regulated in HCT116 cells overexpressing ATF3 at both translational and transcriptional levels, while lower β-catenin protein expression was detected in ATF3-overexpressed HCT116 cells relative to the control cells. The results of this study demonstrate that ATF3 overexpression inhibits colorectal cancer progression and suggest that ATF3 exhibits antitumor activity through reversing EMT and impairing cancer-initiation ability.

Keywords: ATF3, proliferation, migration, invasion, epithelial-mesenchymal transition, self-renewal, cancer-initiation capacity

Introduction

Colorectal cancer is the third most common cancer and the fourth leading cause of cancer-related mortality worldwide [1]. There are over 1.2 million people diagnosed with colorectal cancer every year, and more than 600,000 patients are estimated to die from this malignancy [2]. The great achievements in diagnosis and treatments result in a high increase of the 5-year survival rate in colorectal cancer patients [3]; however, recurrence remains a major problem for improving overall survival after surgical interventions [4-6]. A search for the molecular

biomarkers for early diagnosis and prognosis prediction, and elucidation of the exact mechanisms underlying disease progression are therefore of great significance in the era of precision medicine [7], which may be useful to identify novel targets and design more effective treatments [8, 9].

Activating transcription factor 3 (ATF3), a member of the mammalian activation transcription factor/cAMP responsive element-binding (CREB) protein family of transcription factors [10], has been found to be linked with the biological behaviors of multiple cancers, such as breast cancer [11], diffuse large B-cell lymphoma [12], prostate cancer [13, 14], esophageal squamous cell carcinoma [15], esophageal cancer [16], and Hodgkin lymphoma [17]; however, the exact role of ATF3 remains controversial in cancer development and progression, since this adaptive-response gene has been identified as both an oncogene and a tumor-suppressor gene in cancers [18]. In human colorectal cancer cell lines, ATF3 was reported to promote cell migration and metastasis [19, 20], and function as a tumor suppressor [21], suggesting the dual role in human colorectal cancer cell apoptosis and metastasis [22], which encourages further studies to validate the exact role of ATF3 in colorectal cancer development and progression. The present study was therefore designed with aims to examine the effect of ATF3 overexpression on biological behaviors of human colorectal cancer HCT116 cells, so as to provide new insights into the role of ATF3 in colorectal cancer.

Materials and methods

Ethical statement

The study was approved by the Ethics Review Committee of Fujian Provincial Cancer Hospital (permission no. FJZLYY2012-00171). All experimentations performed in this study were in compliance with the national and local laws and regulations.

Cell culture

Human colorectal cancer HCT116 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). HCT116 cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum at 37°C containing 5% CO_2. Cells growing to 60-70% confluence in complete medium were harvested for the subsequent experiments.

ATF3 overexpression

A lentiviral vector control and a construct containing the entire full-length coding region of the *ATF3* gene were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polybrene (Sigma-Aldrich; St. Louis, MO, USA) at a concentration of 6 μg/ml was added to the medium for viral infection. For generating stable clones, cells infected with virus were selected by 1.5 μg/ml puromycin (Merck KGaA, Darmstadt, Germany) for three weeks. The overexpression efficiency of ATF3 was checked by Western blotting and qRT-PCR assays.

MTS assay

HCT116 cells overexpressing ATF3 were seeded onto 96-well plates at a density of 4×10^3 cells per well and cultured for 72 h. Cell proliferation was determined using the MTS assay (Promega; Madison, WI, USA) following the manufacturer's instructions.

Flow cytometry analysis

HCT116 cells overexpressing ATF3 were labeled with mouse anti-human CD24-Percp-Cy 5.5 (BD Pharmingen; San Jose, CA, USA) and mouse anti-human CD133-2-PE (Miltenyi Biotec; Bergisch Gladbach, Germany), while mouse Percp-Cy 5.5-IgG1 K (BD Pharmingen; San Jose, CA, USA) and PE-IgG2b antibodies (Miltenyi Biotec; Bergisch Gladbach, Germany) served as isotype controls. After staining, cells were washed and then analyzed on a FACS Caliber flow cytometer (BD Biosciences; San Jose, CA, USA). All experiments were performed in triplicate and repeated three times.

Colony-forming and sphere-forming assays

HCT116 cells overexpressing ATF3 and control cells were seeded onto 6-well plates at a density of 500 cells per well, and cultured for a week. Cells were fixed in 70% ethanol and stained with 10% (*v/v*) Giemsa's staining solution (Merck; Damstadit, Germany), and cell colonies were counted. For sphere-forming assay, cells were cultured in serum-free DMEM/F12 medium containing 20 ng/ml basic fibroblast growth factor (Millipore; Bedford, MA, USA), 10 ng/ml recombinant human epidermal growth

Table 1. Primers for the qRT-PCR assay used in this study

Gene	Sequence (5'-3')	
ATF3	Forward:	CGTGCTGCTCTACGACATGA
	Reverse:	GCTCCAACTGAAGGTCCCTG
Survivin	Forward:	TCTCTACATTCAAGAACT
	Reverse:	TTGAAGCAGAAGAAACAC
CyclinD1	Forward:	CCATGAACTACCTGGACCG
	Reverse:	GATGGAGTTGTCGGTGTAGAT
PCNA	Forward:	AACCTGCAGAGCATGGACTC
	Reverse:	TCATTGCCGGCGCATTTTAG
MMP2	Forward:	TCTTCCCCTTCACTTTCCTG
	Reverse:	ACTTGCGGTCATCATCGT
MMP9	Forward:	GCAGAGATGCGTGGAGAGT
	Reverse:	CCCTCAAAGGTTTGGAATC
TIMP1	Forward:	TTCTGGCATCCTGTTGTTG
	Reverse:	AACGCTGGTATAAGGTGGTGGTC
TIMP2	Forward:	AAGATGCACATCACCCTCTG
	Reverse:	TCTGTGACCCAGTCCATCC
ID1	Forward:	CGTGCTGCTCTACGACATGA
	Reverse:	GCTCCAACTGAAGGTCCCTG
CD24	Forward:	GGTTGGCCCCAAATCCAACT
	Reverse:	GACCACGAAGAGACTGGCTG
CD133	Forward:	CAACGAGTCCTTCCTATA
	Reverse:	CTCTCCAACAATCCATTC
CD166	Forward:	ACCTCAGAATCTCATGTTTGG
	Reverse:	ATCACTGATCCTTGCATTACTG
EpCAM	Forward:	TCGTCAATGCCAGTGTACTTC
	Reverse:	ATCGCAGTCAGGATCATAAAG
EZH2	Forward:	TGATGACGATGATGATGATGGAGAC
	Reverse:	TGTGCCCTTATCTGGAAACATTGAG
β-catenin	Forward:	TTAAGTCTGGAGGCATTC
	Reverse:	GGTTGTGGAGAGTTGTAA
β-actin	Forward:	TGGCACCACACCTTCTACA
	Reverse:	AGCACAGCCTGGATAGCA

factor (R&D Systems; Minneapolis, MN, USA) and 2% B27 supplement (Gibco; Carlsbad, CA, USA). Then, cells were seeded onto 96-well plates at a density of 100 cells per well and cultured for 5 to 7 days, and the mean size and number of spheres were evaluated.

Wound healing assay

HCT116 cells were seeded onto 6-well plates in duplicate at approximately 80% confluence and allowed to grow overnight. Then, a scratch wound was made through the center of each well with a 10 μL pipette tip. Plates were washed with PBS and fresh medium was added to remove loose cells. Following 48 h culture, cells were examined under a light microscope to determine the resealing of the cell monolayer.

Transwell migration assay

A transwell migration assay was performed on 24-well plates with 8.0 μm pore inserts (Becton Dickinson AG; Allschwil, Switzerland). Transwell chambers were pre-coated with 1 μg/ml fibronectin on the lower membrane, while invasion assay was done with the membrane insert (8.0 μm in pore size) coated with Matrigel (BD Biosciences; San Jose, CA, USA) that was diluted in medium (1:5 dilution). HCT116 cells were seeded onto 24-well plates at a density of 1×10^5 cells per well containing 100 μL of FBS-free media. Inserts were then placed in the well containing the culture medium supplemented with 500 μL 20% FBS. Following 48 h culture, the medium and loose cells in the culture insert were removed, and cells at the bottom of the insert were methanol-fixed and stained with 0.1% crystal violet. Five random fields were selected and counted at a 100 × magnification for estimating the mean number of cells in each insert.

qRT-PCR assay

Total RNA was extracted from HCT cells and using the RNeasy Mini Kit (Qiagen; Valencia, CA, USA) according to the manufacturer's protocol, and reversely transcribed into cDNA by using Reverse Transcription System A3500 (Promega; Madison, WI, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) assay was run on a LightCycler 480 Real-Time PCR System (Roche; Branford, CT, USA) with a DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific; Waltham, MA, USA USA). Primers for genes of interest were described in **Table 1**, and β-actin served as an internal control. For the relative quantification of gene expression, three independent amplifications were performed for each target gene, with triplicate samples. The relative *mRNA* expression was quantified with the $2^{-\Delta\Delta Ct}$ method.

Western blotting analysis

Total protein was extracted from HCT116 cells, and the protein concentration was quantified with the BCA assay (Pierce; Rockford, IL, USA). Total protein was then electrophoresed on poly-

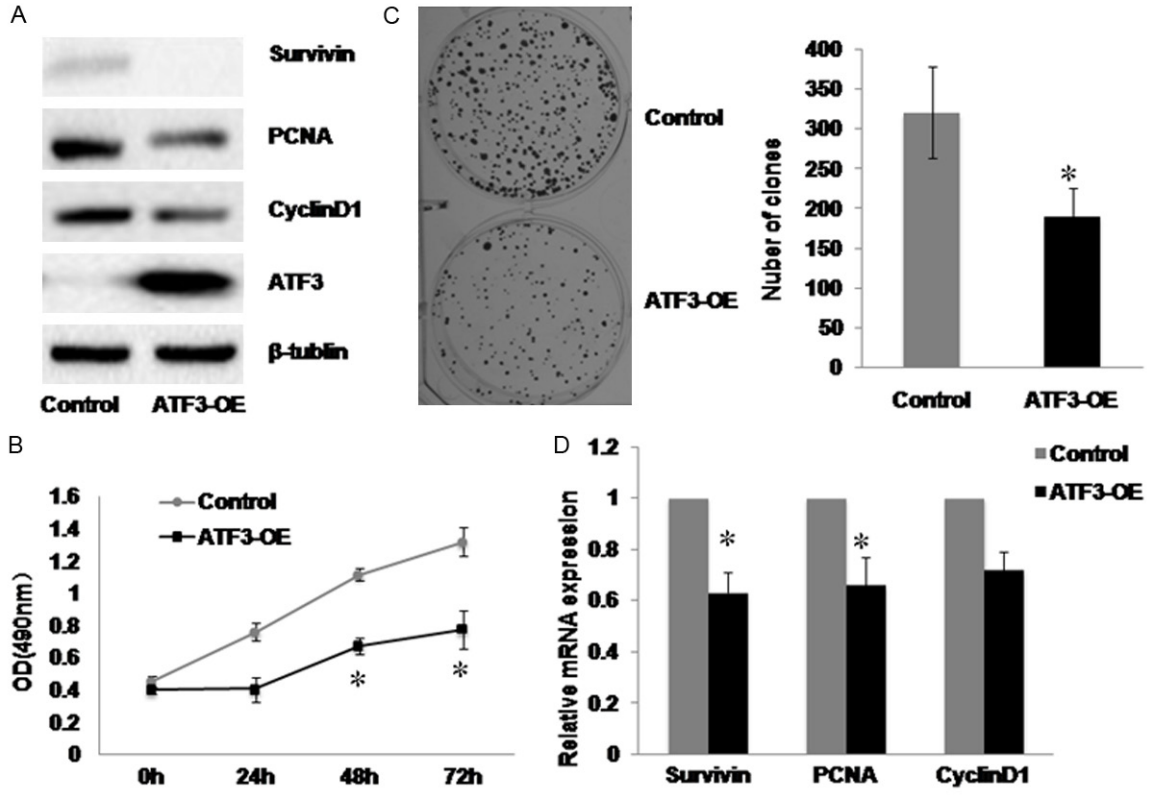

Figure 1. Effect of ATF3 overexpression on the HCT116 cell proliferation. A. Western blotting assay detects ATF3, PCNA, Survivin and CyclinD1 protein expression in the ATF3-overexpressed HCT116 cells (ATF3-OE cells) and control cells; B. MTS assay measures the HCT116 cell proliferation; C. Colony-forming assay observes formation of the HCT116 cell colonies. A total of 200 ATF3-OE HCT116 and control cells were cultured per well in the 6-well plates, and the number of cell colonies was counted after 7 days of culture; D. qPCR assay determines PCNA, survivin, CyclinD1 mRNA expression. Data are expressed as mean ± SD. *$P < 0.05$ vs. the control cells. All data are captured from three independent experiments.

acrylamide gels, and the blots were transferred to nitrocellulose (NC) membranes. Subsequently, the membranes were blocked in 3% bovine serum albumin and incubated with the anti-CD24, anti-CD133-2, anti-Lgr5 (Abcam, Inc.; Cambridge, MA, USA), anti-Id1, anti-Twist, anti-EpCAM, anti-CD166, anti-CyclinD1 (Santa Cruz Biotechnology; Santa Cruz, CA, USA), anti-EZH2, anti-Snail, anti-OCT4 (Cell Signaling Technology; Beverly, MA, USA) primary antibodies overnight at 4°C, while β-tublin (Santa Cruz Biotechnology; Santa Cruz, CA, USA) served as a loading control. The binding of HRP-conjugated secondary antibodies was visualized with enhanced chemiluminescence (ECL) reagents (Perkin Elmer; Waltham, MA, USA).

Data management

All measurement data were described as mean ± standard deviation (SD), and all statistical analyses were performed using the statistical software SPSS version 20.0 (SPSS, Inc.; Chicago, IL, USA). Differences of means were tested for statistical significance with Student *t* tests. A *P* value < 0.05 was considered statistically significant.

Results

Effect of ATF3 overexpression on HCT116 cell proliferation

qRT-PCR analysis showed a 65-fold increase of ATF3 mRNA expression in the HCT116 cells overexpressing ATF3 relative to the control cells, and Western blotting assay determined significantly higher ATF3 protein expression in the HCT116 cells overexpressing ATF3 than in the control cells (**Figure 1A**). MTS and colony-forming assays showed that ATF3 overexpression remarkably inhibited the HCT116 cell

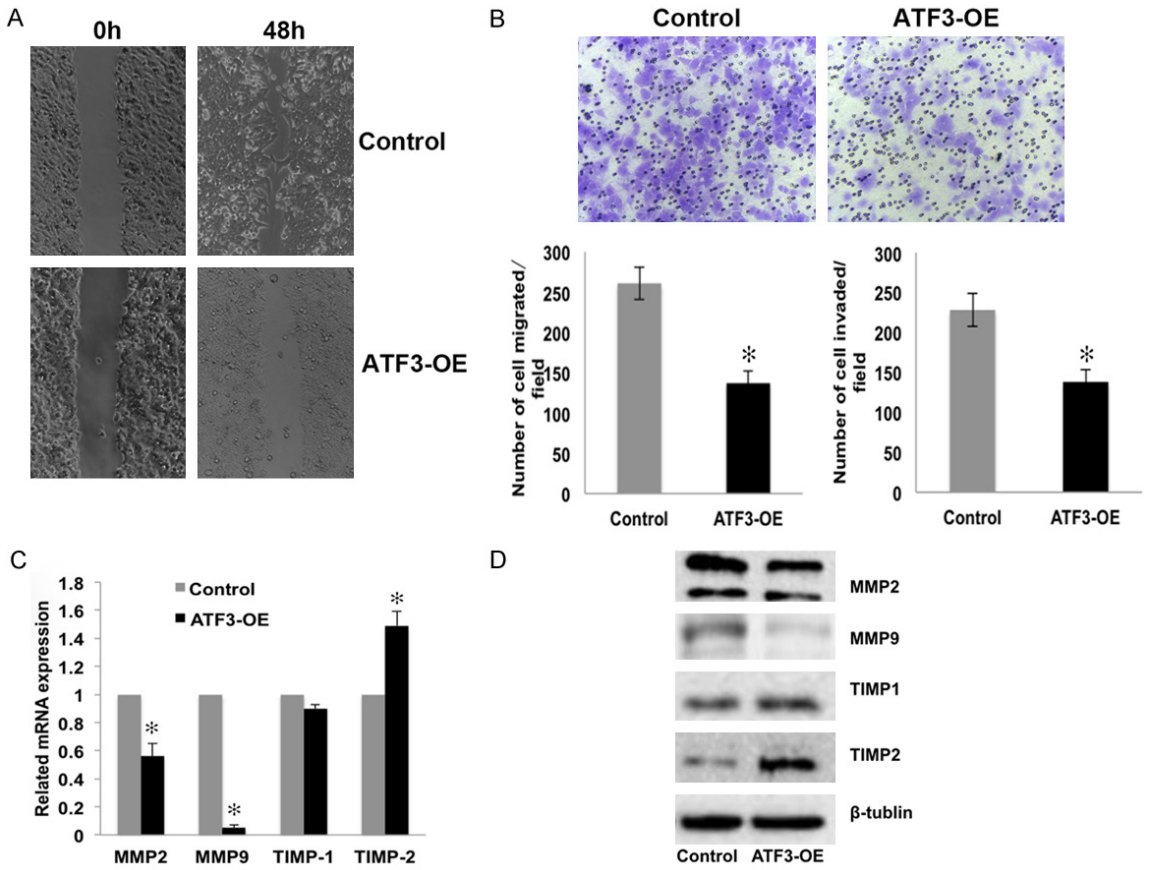

Figure 2. Effect of ATF3 overexpression on the HCT116 cell migration and invasion. A. Wound healing assay detects the HCT116 cell migration, and cells were observed under a light microscope at a magnification of × 100 after 0 and 48 h of scratching the cell monolayer; B. Transwell migration/invasion assay detects the HCT116 cell invasion. Cells were incubated in the Transwell chambers and after 24 hours, cells at the bottom of the insert were stained and counted; C. qRT-PCR assay quantifies the MMP2, MMP9, TIMP1 and TIMP2 mRNA expression; D. Western blotting analysis determines the MMP2, MMP9, TIMP1 and TIMP2 protein expression. Data are expressed as mean ± SD. *$P < 0.05$ vs. the control cells. All data are captured from three independent experiments.

growth and colony formation in relative to the control cells (**Figure 1B** and **1C**). In addition, ATF3 overexpression was found to down-regulate Survivin and PCNA expression in the HCT116 cells at both translational and transcriptional levels (**Figure 1A** and **1D**), and a reduction was measured in the CyclinD1 protein expression in the HCT116 cells overexpressing ATF3 than in the control cells. Taking together, our findings suggest that ATF3 overexpression may suppress HCT116 cell proliferation.

Effect of ATF3 overexpression on HCT116 cell migration and invasion

The HCT116 cells overexpressing ATF3 were found to move slowly towards the gap as compared to the control cells (**Figure 2A**), and significantly less cells overexpressing ATF3 migrated through the chambers than the control cells (**Figure 2B**). Our findings suggest that ATF3 overexpression results in a reduction in migratory and invasive potential of the HCT116 cells. In addition, the MMP2 and MMP9 expression was found to be down-regulated in the HCT116 cells overexpressing ATF3 at both translational and transcriptional levels, as determined by Western blotting and qRT-PCR assays (**Figure 2C**), and Western blotting detected up-regulation of TIMP-2 and TIMP-1 expression in the HCT116 cells overexpressing ATF3 relative to the control cells (**Figure 2D**). The results indicate that ATF3 overexpression inhibits HCT116 cell migration and invasion.

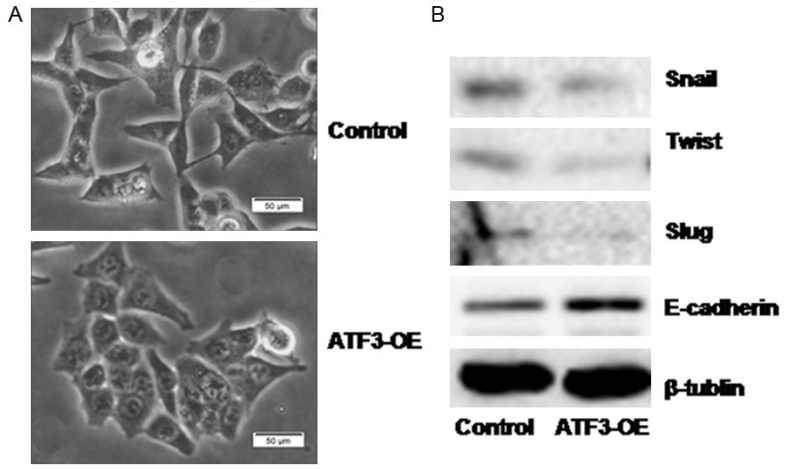

Figure 3. Effect of ATF3 overexpression on the epithelial-mesenchymal transition (EMT) features of the HCT116 cells. A. Light microscopy shows epithelial-like appearance of the ATF3-overexpressed HCT116 (HCT-OE) cells (× 200); B. Western blotting assay determines the E-cadherin, Snail, Slug and Twist protein expression in the HCT116 cells.

Effect of ATF3 overexpression on HCT116 cell epithelial-mesenchymal transition (EMT)

The HCT116 cells overexpressing ATF3 had changes of morphology from scattering to aggregation and showed epithelial-like appearance (**Figure 3A**). Western blotting analysis determined up-regulation of E-cadherin expression and down-regulation of Snail, Slug and Twist expression in the HCT116 cells overexpressing ATF3 relative to the control cells (**Figure 3B**). Our findings suggest that ATF3 overexpression reverses the EMT potential of the HCT116 cells.

Effect of ATF3 overexpression on cancer-initiating cell feature of HCT116 cells

ATF3-overexpressed cells were found to form smaller spheroids, and less spheroids were observed in the serum-free cell cultures as compared with the controls, while the control HCT116 cells formed spheroids with clear and complete boundaries in the serum-free culture (**Figure 4A**). To examine the association of stem cell markers expression with ATF3 overexpression in HCT116 cells, qPT-PCR, Western blotting and flow cytometry analysis were performed. qRT-PCR assay showed a decrease of CD24 and CD133 mRNA expression in the ATF3-overexpressed cells than in the control cells (**Figure 4B**), which was consistent with the results detected by flow cytometry (**Figure 4C**) and Western blotting (**Figure 4D**) that reporting lower CD24 and CD133 expression in the ATF3-overexpressed cells than in controls, while the CD166, EpCAM, ID1 and EZH2 expression was found to decrease in the ATF3-overexpressed HCT116 cells at both translational and transcriptional levels (**Figure 4C** and **4D**). In addition, the expression of β-catenin, a key factor linked to self-renewal in colorectal cancer, was detected to appear a reduction tendency in the ATF3-overexpressed HCT116 cells relative to the control cells (**Figure 4C**), and the β-catenin protein expression was significantly reduced in the ATF3-overexpressed HCT116 cells (**Figure 4D**). The results demonstrate that ATF3 overexpression impairs the self-renewal of the HCT116 cells.

Discussion

In molecular biology, ATF is a group of bZIP transcription factors, which act as homodimers or heterodimers with a range of other bZIP factors [24]. Currently, totally seven members have been identified in the ATF family, namely ATF1, ATF2, ATF3, ATF4, ATF5, ATF6, and ATF7 [24]. ATF1 has been linked to multiple cancers, such as clear cell sarcoma [25], melanoma [26] and angiomatoid fibrous histiocytoma [27]. ATF2 has been found to play a dual role in tumorigenesis [28]. ATF4, which is highly expressed in cancers than in normal tissues and regulates processes relevant to cancer progression, has been identified as a potential therapeutic target in cancers [29]. ATF5, a transcription factor closely related to cell apoptosis, differentiation and development, may be promising biomarkers for rectal cancer [30] and neural tumors [31] and a therapeutic target for pancreatic cancer [32], and ATF5 was reported to enhance radioresistance and malignancy in cancer cells [33]. ATF6, a constitutively expressed, endoplasmic reticulum (ER) membrane-anchored transcription factor, is associated with liver cancer [34], breast cancer [35], prostate cancer [36], colonic neoplasm [37] and soft tissue

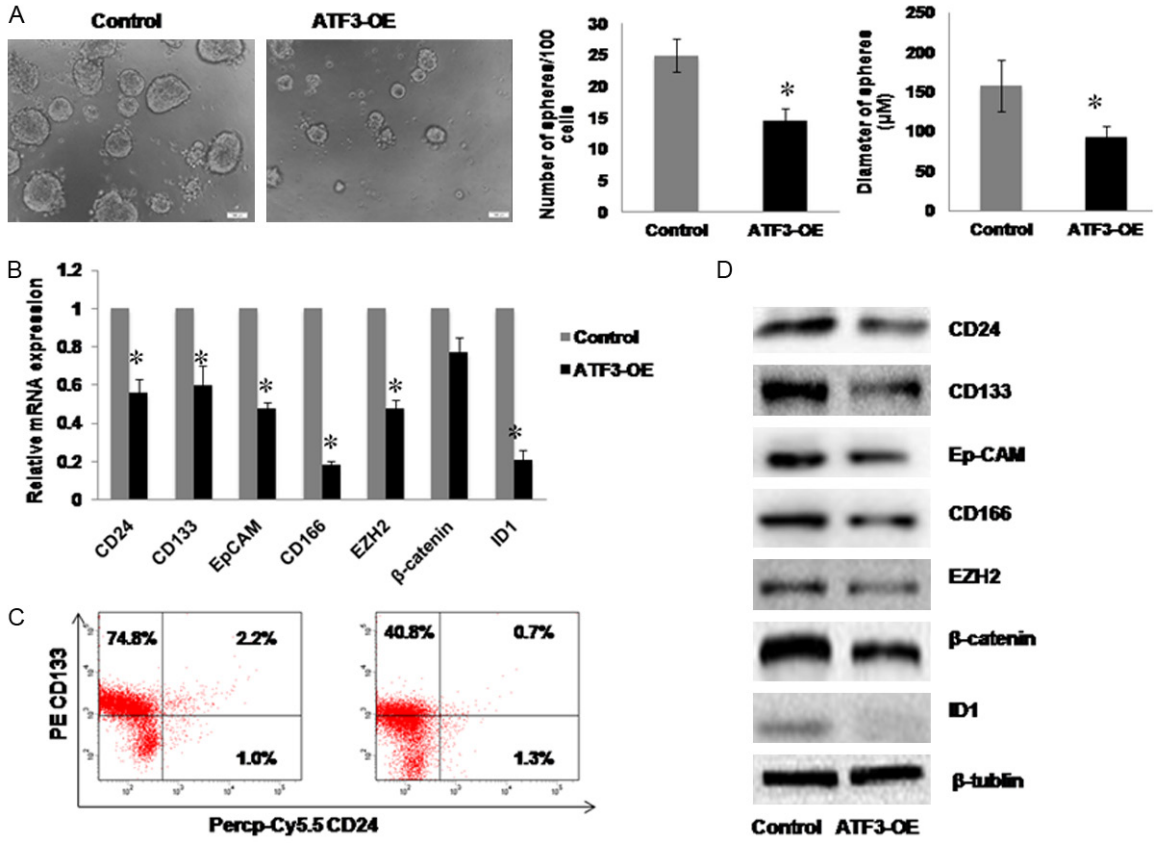

Figure 4. Effect of ATF3 overexpression on the cancer-initiation features of the HCT116 cells. A. Light microscopy displays a sphere formation capacity of the HCT116 cells cultured in the serum-free medium for 7 days (× 100). The number and diameter of the spheres are assessed; B. qRT-PCR assay quantifies the *mRNA* expression of the colorectal cancer stem cell markers (CD24, CD133, CD166, EpCAM, ID1, EZH2 and β-catehin) in the HCT116 cells; C. Flow cytometry detects the positive CD24 and CD133 populations; D. Western blotting assay determines the protein expression of the colorectal cancer stem cell markers (CD24, CD133, CD166, EpCAM, ID1, EZH2 and β-catehin) in the HCT116 cells. Data are expressed as mean ± SD. *$P < 0.05$ vs. the control cells. All data are captured from three independent experiments.

sarcoma [38]. ATF7, a novel bZIP protein that interacts with PTP4A1 [39], was recently identified as a favorable factor for survival of patients with colorectal cancer [40].

Like ATF2 [28], ATF3 was found to act as an oncogene as well as a tumor suppressor [41]. Many studies support an oncogenic role of ATF3 in breast cancer, prostate cancer, Hodgkin lymphoma, colon cancer; however, there is also much evidence proving the inhibition of ATF3 on the development of prostate cancer, colorectal cancer and ovarian cancer [41]. Due to the depute on the exact role of ATF3 in the development of colorectal cancer, this *in-vitro* assay was designed, aiming to investigate the effects of ATF3 overexpression on the biological behaviors of human colorectal cancer HCT116 cells, so as to provide new insights into the role of ATF3 in colorectal cancer.

In this study, we established a human colorectal cancer HCT116 cell line overexpressing ATF3, and *in-vitro* experiments were performed to examine the effect of ATF3 overexpression on the biological behaviors of the HCT116 cells. MTS and colony-forming assays showed suppression of the HCT116 cell proliferation following ATF3 overexpression, and Transwell migration assay and wound healing assay measured inhibition of HCT116 cell migration and invasion after overexpressing ATF3. In addition, ATF3 overexpression was found to reverse the EMT potential and impair the self-renewal of the HCT116 cells. Our data provide additional evidence supporting ATF3 as a tumor suppressor in colorectal cancer [21].

Previous studies have demonstrated that overexpression of ATF3 inhibited proliferation of HCT116 cells; however, the underlying mecha-

nisms remain unknown [42]. In this study, the expression of PCNA, a representative marker for cell proliferation, was detected to be down-regulated in the HCT116 cells overexpressing ATF3, which was in agreement with previous reports [43]. Survivin, a member of inhibitors of apoptosis proteins (IAPs), is a functional protein that promotes cell proliferation in colon cancer [44, 45], and we also detected repressed Survivin expression in the ATF3-overexpressed HCT116 cells. In addition, the expression of CyclinD1, a well-established positive regulator of early cell-cycle progression, was found to be down-regulated at a translational level following ATF3 overexpression. It was reported that ATF3 could suppress cyclinD1 dependent on the cAMP response element (CRE) in the cyclinD1 promoter [46]. In the current study, we did not detect a significant change in the CyclinD1 expression at a transcriptional level in the ATF3-overexpressed HCT116 cells relative to the control cells; however, the underlying mechanisms are unclear and remain to be further investigated. Based on these findings, it is hypothesized that ATF3 may inhibit colorectal cancer cell proliferation through multiple pathways.

In colorectal cancer, the potential role of ATF3 in metastasis may depend on cell genetic background [21]. In the current study, our findings confirmed that ATF3 overexpression could suppress the migration and invasion of the HCT116 cells [42]. ATF3 expression has been shown to alter the *mRNA* expression of invasion-related genes, such as maspin, PAI-1, β-catenin, and MTA-1 [42], and ATF3 was reported to repress MMP2 expression through decreasing the transactivation of this gene by p53 [47]. In this study, we detected suppression of MMP2 and MMP9 expression in ATF3-overexpressed HCT116 cells at both protein and *mRNA* levels. In addition, ATF3 overexpression resulted in up-regulated expression of TIMP-2 and TIMP-1, the corresponding inhibitors of MMP2 and MMP9, respectively. MMPs are zinc-dependent endopeptidases involved in the degradation of extracellular matrix (ECM), which promote tumor metastasis [48], while TIMPs function as endogenous MMPs regulators, and interact with other cell adhesion molecules, cytokines and growth factors to exhibit a critical impact on ECM [48]. It is indicated that the MMP2-TIMP2 and MMP9-TIMP1 complexes in tumor environments destroy the integrity of the basement membranes [48]. Taking these results together, it is suggested that ATF3 overexpression inhibits HCT116 cell invasion dependent, at least in part, on the decrease of MMPs expression.

Cancer stem cells (CSCs) have shown infinite self-renewal ability and capability of differentiation into diverse populations comprising a tumor and thus promoting cancer initiation and metastasis [49]. Cells that have undergone EMT were found to display enhanced cancer-initiating cell features [50]. Yin and colleagues [11] identified ATF3 as an oncogene in breast cancer, and found that ectopic expression of ATF3 led to increased $CD24^{low}$-$CD44^{high}$ population of the breast-cancer-initiating cells. The present study, for the first time, examined the correlation between ATF3 overexpression and stemness features in human colorectal cancer HCT116 cells. Unlike breast cancer, we detected that ATF3 overexpression reversed EMT features of the HCT116 cells and resulted in altered expression of EMT-related markers (Snail, Twist and E-cadherin), and ATF3 overexpression was found to suppress spheroid formation in the HCT116 cells. In addition, the expression of β-catenin, a representative self-renewal protein, was found to be decreased after ATF3 overexpression. To date, the major molecular markers to identify colon CSCs (such as CD44, CD133, CD24, CD166, and EpCAM) remain controversial [51], and it is considered that a combination of multiple makers seems more reasonable to evaluate the self-renewal features in colon cancer. In this study, ATF3 overexpression was found to result in a reduction in the expression of stemness markers, including CD24, CD133, CD166, and EpCAM at both translational and transcriptional levels. As a transcription factors, ATF3 may bind to these genes promoter. However, we found a reduction in the ID1 and EZH2 expression in the ATF3-overexpressed HCT116 cells. It has been shown that ATF3 in conjunction with SMAD complex binding to ID1 promote can repress ID1 transcription [52]. In addition, ID1 and ID3 are able to function together to govern colon cancer-initiating cell self-renewal through restricting cell cycle via p21 [53]. As an essential stemness marker for glioblastoma cancer [54], EZH2 expression was found to correlate with ID1 expression [55]. Combining previous findings and our data together, it is assumed that overexpression of ATF3 decreases HCT116 cancer

cell-initiation feature through down-regulating ID1 expression in part.

In conclusion, the results of this study demonstrate that ATF3 overexpression inhibits colorectal cancer progression and suggest that ATF3 exhibits antitumor activity through reversing EMT and impairing cancer-initiation ability. These findings are of great significance for the effective targeting of EMT and cancer-initiation cells via induction of ATF3 expression, which may offer new hopes for anti-cancer therapy. Further studies to elucidate the signaling pathway and the mechanisms through which ATF3 controls the multiple biological features of colorectal cancer cells seem justified.

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Disclosure of conflict of interest

None.

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