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Original Research

ATF3 inhibits the growth and stem cells-like features of SW620 colorectal cancer cells *in vitro*

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Abstract

Background and objective: Activating transcription factor 3 (ATF3) plays a crucial role in regulating tumor development depending on the cellular context or cancer cell type. However, the effect of ATF3 on stem cells-like features in colorectal cancer (CRC) has yet to be elaborated.

Methods: In this study, we overexpressed ATF3 in SW620 CRC cells to investigate its effects on stem cells-like features.

Results: Our results indicated that overexpressing ATF3 inhibited the proliferation, invasion, migration, and sphere formation capacity of SW620 CRC cells. ATF3 overexpression also decreased the size of tumorspheres and reduced expression of the cancer stem cell markers CD44 and CD133 in SW620 cells.

Conclusion: In summary, our study revealed that ATF3 suppresses CRC growth and stem cells-like features. ATF3 is considered a potential target in CRC therapy.

Keywords

ATF3; Migration; Invasion; Cancer stem cells

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies (second most common in women and third in men), accounting for approximately 10% of cancer-related deaths worldwide [1]. Thus, CRC represents a great challenge to human health. The treatments currently available for CRC include surgery, chemotherapy, radiotherapy, and immunotherapy, but the overall prognosis of patients with advanced-staged disease is extremely poor because of metastasis and recurrence [2]. Growing evidence suggests that cancer stem cells (CSCs), which are a minor subgroup of the bulk neoplasm, play important roles in tumor initiation, metastasis, and resistance to conventional therapies [3]. Therefore, it is critical to determine the molecular basis of CRC and identify therapeutic targets against CSCs to improve the clinical outcomes of CRC.

Activating transcription factor 3 (ATF3) is a member the basic leucine zipper (bZIP) protein superfamily of ATF/CREB transcription factors [4]. ATF3 has been demonstrated to be a stress response gene that can be induced by many factors, including genotoxic agents, ultraviolet radiation, and growth factors [5]. ATF3 is also an adaptive-response gene associated with various cancer processes through interactions with its bZIP domain [6, 7]. Preclinical studies have demonstrated that ATF3 can be either a tumor suppressor or an oncogene in different cancers [6]. For instance, ATF3 suppresses the growth of human melanoma, breast cancer, prostate cancer, and endometrial carcinoma [8–11]. In contrast, ATF3 was displayed to promote tumor growth and inhibit apoptosis in liver cancer and squamous cell carcinoma [12, 13]. In human CRC, most studies have suggested ATF3 is a tumor suppressor [14–18], although a few reports have indicated that ATF3 promotes

TABLE 1. RT-PCR primer sequences, amplification conditions and product length for genes.

Gene	Primer orientation	Primer sequence (5′-3′)	Annealing temperature (°C)	PCR cycles	Product size (bp)
ATT3	Forward	GTCCATCACAAAAGCCGAGGT	55	35	141
	Reverse	AGCTTCTCCGACTCTTTCTGC			
CD44	Forward	AGACCATCCAACAACTTCTACTCT	55	40	143
	Reverse	TCCTGCTTTCCTTCGTGTGT			
CD133	Forward	CCACAGATGCTCCTAAGGCT	55	35	91
	Reverse	AGAATGCCAATGGGTCCAGC			
β-actin	Forward	ACCGCGAGAAGATGACCCAG	56	35	80
	Reverse	GGATAGCACAGCCTGGATAGCAA			

tumor growth and migration [19, 20]. Finally, a recent study indicated that ATF3 can promote cancer progression by modulating the TGFβ signaling pathway [21]. Thus, there is a crucial need to confirm the role of ATF3 in CRC to determine if it could be targeted for treatment.

Although previous studies have shown that ATF3 can act through a variety of pathways, including NF-κB, TGFβ, JNK, p53, c-Myc, and Smad [6], the mechanism through which ATF3 alters CRC cells is unknown. We aimed to evaluate the potential relationship between ATF3 and features of cancer-initiating cells, particularly the effect of ATF3 on CRC stem cell characteristics.

2. Materials and methods

2.1 Cell culture

The human colon cell line NCM460 and the CRC cell lines SW620, SW480, HT29, HCT8, and HCT116 were all obtained from Immocell Biotechnology Co., Ltd. (Xiamen, China) and confirmed by short tandem repeat authentication (ATCC, Manassas, VA, USA). The product numbers of the commercial cell lines were IM-H445 (NCM460), IM-H112 (SW620), IM-H111 (SW480), IML-044 (HT29), IM-H099 (HCT8) and IM-H098 (HCT116). Characteristics of these CRC cell lines are described (written in Chinese) at http://immocell.com/index.html (doc 1457, doc 1124, doc 1123, doc 1564, doc 1111, and doc 1110). The updated ATCC STR profiling data are available in the following catalog numbers: CCL-227, CCL-228, HTB-38, CCL-244, and CCL-247. HT29, HCT-8, and SW620 cells were cultured in RPMI-1640 medium. HCT116 and NCM460 were cultured in McCoy's 5A medium (Immocell Biotechnology Co., Ltd., Xiamen, China), while SW480 cells were cultured in L-15 medium (Immocell Biotechnology Co., Ltd.). SW480, HT29, HCT8, and HCT116 cells were originally derived from a primary CRC. The SW620 cell line was originally derived from a CRC lymph node metastasis. All of these cell lines are now well established and widely commercially available.

The cell culture medium contained 10% fetal bovine serum (Immocell Biotechnology Co., Ltd.). The cell incubation condition was: 37 °C, 5% CO_2. CRC cells were lyzed and harvested for experiments when they reached at least 80% confluence.

2.2 RNA extraction and real-time reverse transcription PCR (RT-PCR)

Total RNA of cells or fresh tissue samples was extracted using the Superscript III transcriptase (Invitrogen Life Technologies, Inc., Waltham, MA, USA). Then 1 μg of total RNA was reverse-transcribed using the Promega Reverse Transcription System A3500 (Promega Corporation, Madison, WI, USA). qRT-PCR was performed through a Bio-Rad CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA). The levels of mRNA expression were normalized to β-actin. The gene specific primers are provided in Table 1. Three independent experiments were performed for each analysis.

2.3 Western blotting

The cells samples were lysed with ice-cold RIPA lysis buffer (Beyotime Biotechnology, Inc., Haimen, China) containing protease inhibitors. Briefly, 30 μg of total protein was loaded for electrophoresis on 10% denaturing SDS-PAGE gels (Beyotime Biotechnology, Inc.), and then transferred to membranes. The membranes were incubated with primary antibodies at 4 °C overnight, and then with the corresponding secondary antibody at room temperature for 1 h. The following antibodies were used for western blotting: anti-ATF3 (1 : 2000; Abcam, Cambridge, UK), anti-actin (1 : 5000; Promega), and HRP-conjugated goat anti-rabbit IgG (1 : 1000; Cell Signaling Technology, Danvers, MA, USA).

2.4 Transfections and establishment of stable cell lines

Full length of complementary DNAs (cDNAs) encoding ATF3 were isolated from an SW620 cDNA library with the forward primer: 5′-ATGATGCTTCAACACCCAG-3′ and the reverse primer: 5′-TTAGCTCTGCAATGTTCCTTC-3′. Expression plasmids for ATF3 were constructed in the pCDH-CMV-MCS-EF1a-GFP-T2A-puro vector. To generate ATF3-overexpressing stable cell lines, a lentivirus-mediated packaging system containing four plasmids, pCDH-ATF3 or control plasmid (AP178772), pMDL, REV, and VSVG (AP130393, AP130410, and AP130389, Xiamen Anti-Hela Biological Technology/Trade Co. Ltd., Xiamen, China) were transfected into HEK293T cells based on the manufacturer's protocol. After 48 h, lentiviral particles were harvested from the medium and used to infect SW620 cells. To genarate stable cell lines, culture medium containing 1

μg/mL puromycin was added for 2–3 weeks.

2.5 Proliferation assay

In total, 4×10^3 CRC cells/well were cultured for 48 h and then seeded in 96-well plates. Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Vazyme, Inc., Nanjing, China) for the indicated time points according to the manufacturer's recommendations (4 h, 37 °C). After treated the cells with the CCK-8 reagents, absorbance was measured at 450 nm, and each experiment was conducted in triplicate.

2.6 Colony formation assay

Cells were first inoculated at a density of 1×10^3/ well in 6-well plate. After culturing for 12–16 days, the medium was discarded. Subsequently, the cells were gently washed twice with PBS solution. Methanol was used to fix the cells for 30 min. The cells were then stained wells by 0.1% crystal violet for 10–20 min and rinsed twice with PBS. Finally, cell colonies were quantatively recorded.

2.7 Flow cytometric analysis

Apoptosis was determined by staining with Annexin V-fluorescein isothiocyanate (FITC) and 30 mg/mL propidium iodide (PI; Vazyme Biosciences, Inc.) according to the manufacturer's recommendations. Following staining, cells were detected using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo software. Annexin V-FITC- and PI-negative cells were alive; Annexin V-FITC-positive and PI-negative cells represented apoptotic cells. A few cells were positive for Annexin V-FITC and PI, indicating that they were late-stage apoptotic cells. Flow cytometry was also performed to analyze CD133 and CD44 expression using standard protocols. CRC cells were trypsinized, washed, and stained with mouse anti-human CD44-PE and CD133-Percp (both Biolegend Inc., San Diego, CA, USA). After staining, the cells were washed with PBS and analyzed on a flow cytometer.

2.8 Wound healing assay

Cells were all seeded in 6-well plates at approximately 100% confluence. The quantity of the wounds were made using a 200-μL pipette tip with a straight motion. After culturing with serum-free medium (24 h), the wounds were measured and photographed under a light microscope.

2.9 Transwell assay

Transwell plates (Corning Inc., Corning, NY, USA) with 8-μm pore size membranes were used for migration and invasion assays. Briefly, 5×10^5 cells were seeded into the upper chamber of the transwell plates. Cells that had migrated were stained with 0.5% toluidine blue, and six random fields were counted after 48 h. For the invasion test, the membrane of the upper chamber was coated with Matrigel before adding the cells. The migrating or invading cells were counted and photographed using an Olympus inverted microscope.

2.10 Sphere-forming assay

CRC cells were dissociated into single-cell suspensions in serum-free medium. The DMEM/F12 medium was supplemented with 2% B-27 supplement (Invitrogen, Life Technologies Inc.), 20 ng/mL basic fibroblast growth factor (bFGF; BD Biosciences), and 10 ng/mL recombinant human epidermal growth factor (rhEGF; BD Biosciences). After 5–7 days, the number of spheres floating above 50 μm were counted.

2.11 Statistical analysis

All experimental data were statistically analyzed by SPSS 20.0 statistical software (IBM Corp., Armonk, NY, USA). The measurement data are given as mean $\pm$ SEM. The Student's t test was used to analyze differences between two groups. Repeated measures ANOVA was performed for comparisons of cellular proliferation rates over time. Value of $p < 0.05$ was considered a statistically significant difference.

3. Results

3.1 ATF3 expression in CRC cell lines

ATF3 expression was evaluated in different cell lines by qRT-PCR. As shown in Fig. 1A, among the five cell lines, HT29 and HCT8 cells had higher ATF3 mRNA expression than the normal colon cell line (NCM460); however, these differences were not statistically significant. HCT116, SW480, and SW620 CRC cells had significantly lower mRNA expression than NCM460 cells. To generate a stable ATF3-overexpressing cell line, we chose SW620 cells as the optimal candidate for this study. Through constructing, packaging, and infecting with the lentivirus, we obtained stable cells following stringent puromycin selection, which was confirmed by western blot (Fig. 1B).

3.2 Effects of ATF3 on the proliferation and clonogenicity of SW620 cells *in vitro*

To evaluate the effect of ATF3 on the proliferation of SW620 cells, CCK-8 and colony formation assays were tested. As shown in Fig. 2A, overexpressing ATF3 decreased cell proliferation compared with control cells. Moreover, colony formation assays were conducted to test the long-term effects of increasing ATF3 levels. As shown in Fig. 2B, ATF3 overexpression remarkably decreased the quantity and size of SW620 stable cell colonies. These results suggested that ATF3 overexpression decreased CRC cell proliferation.

3.3 Effects of ATF3 on the apoptosis rates of SW620 cells *in vitro*

Annexin V-FITC/PI double staining was applied to examine the ATF3 on apoptosis rates. As shown in Fig. 3, compared with the control group, overexpressing ATF3 in SW620 cells induced a higher rate of apoptosis.

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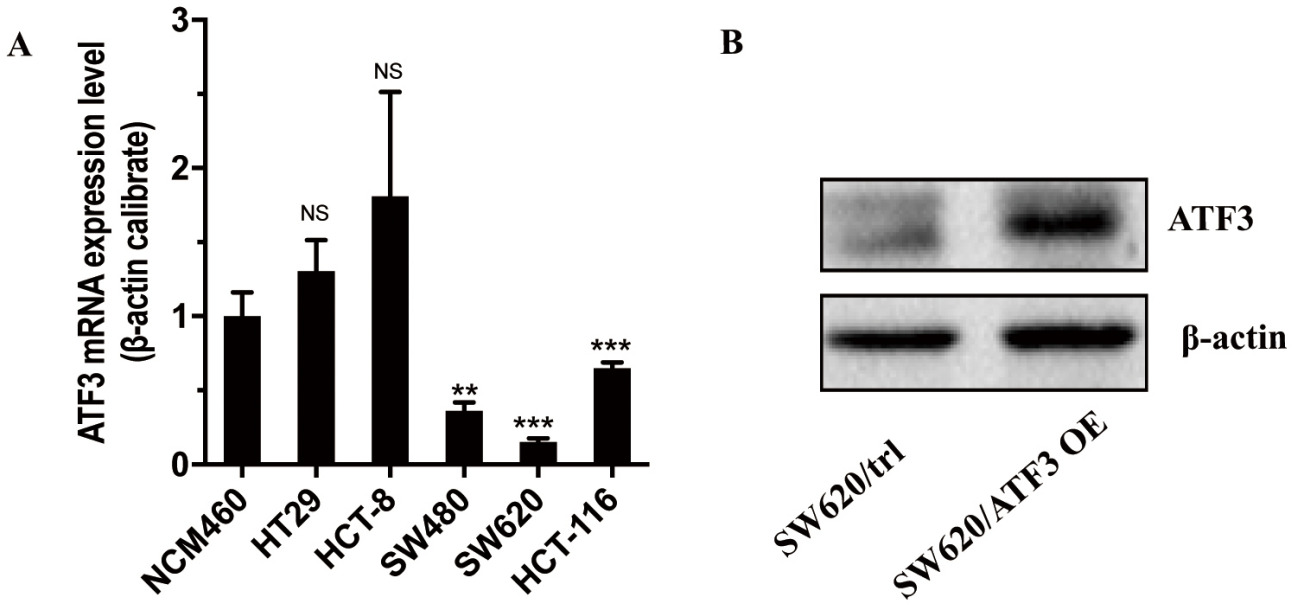

FIG. 1. Expression of ATF3 in colorectal cell lines. (A) RT-PCR was used to assess ATF3 mRNA levels in normal colon cells and colorectal cancer (CRC) cell lines. $^{**}p < 0.01$, $^{***}p < 0.001$, NS = not significant, compared with other CRC cell lines and NCM460 cells by independent Student's *t* test. (B) ATF3 overexpression in SW620 cells was confirmed by western blot analysis. Ctrl, control; OE, overexpression.

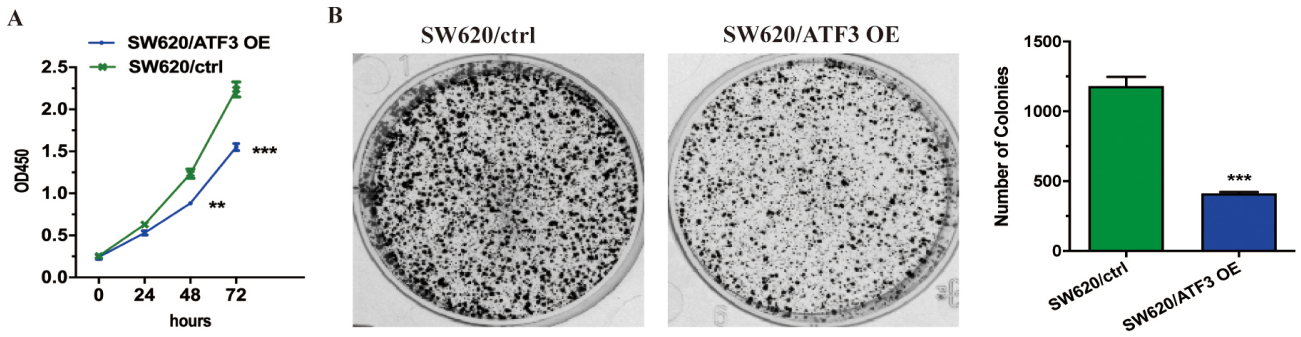

FIG. 2. Effect of ATF3 on the proliferation and clonogenicity of SW620 CRC cells. (A) CCK-8 proliferation assay showing that ATF3 overexpression in SW620 cells significantly decreased cell proliferation compared with the empty vector group (ctrl). $^{**}p < 0.01$, $^{***}p < 0.001$, between group comparison using repeated measures ANOVA. (B) Colony formation assay showing that ATF3 overexpression in SW620 cells led to decreased colony formation. $^{***}p < 0.001$, comparing the two groups with the independent Student's *t* test. Ctrl, control; OE, overexpression.

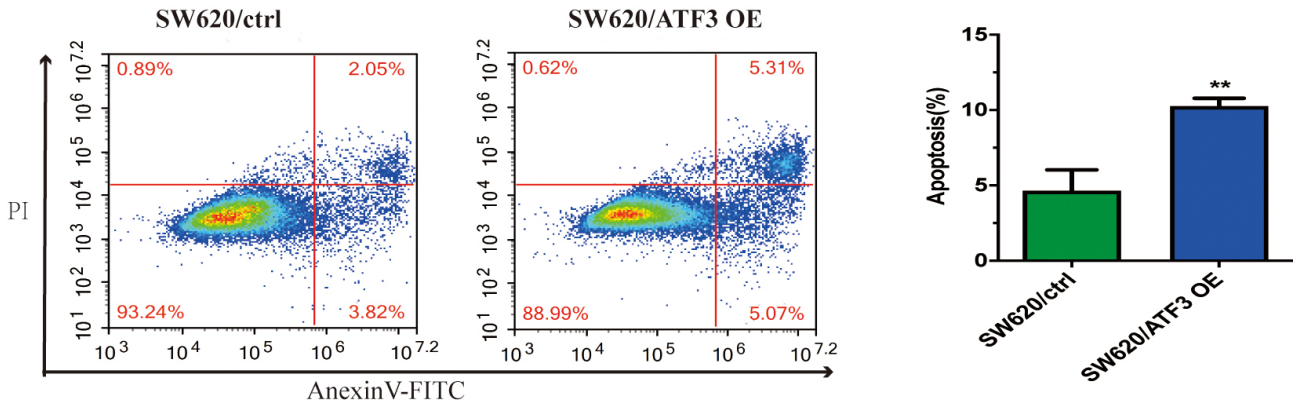

FIG. 3. Effect of ATF3 on apoptosis levels of SW620 CRC cells. Annexin V-FITC/PI double staining showing increased apoptosis levels in SW620 cells overexpressing ATF3 compared with control cells. $^{***}p < 0.001$, comparing the two groups using the independent Student's *t* test. Ctrl, control; OE, overexpression.

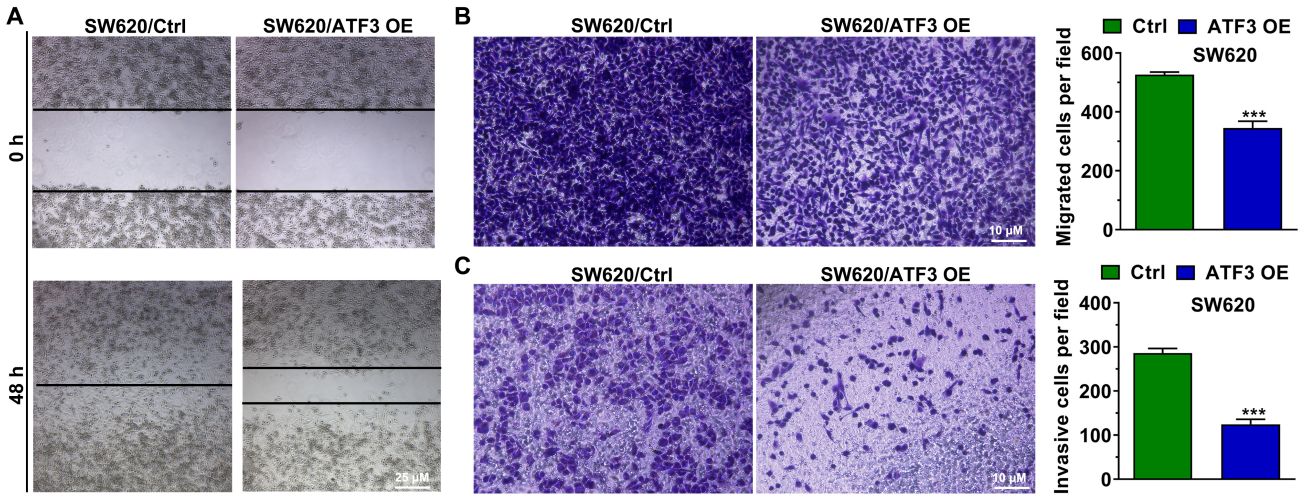

FIG. 4. Effects of ATF3 on the migration and invasion capacity of SW620 CRC cells. (A) Overexpressing ATF3 decreased the migration capacity of SW620 cells after 48 h as assessed by the wound healing assay. (B–C) Overexpressing ATF3 decreased the migration and invasion potential of SW620 cells compared with controls. Cells were counted under a light microscope at 100× magnification. ***$p < 0.001$, comparing between two groups using the independent Student's *t* test. Ctrl, control; OE, overexpression.

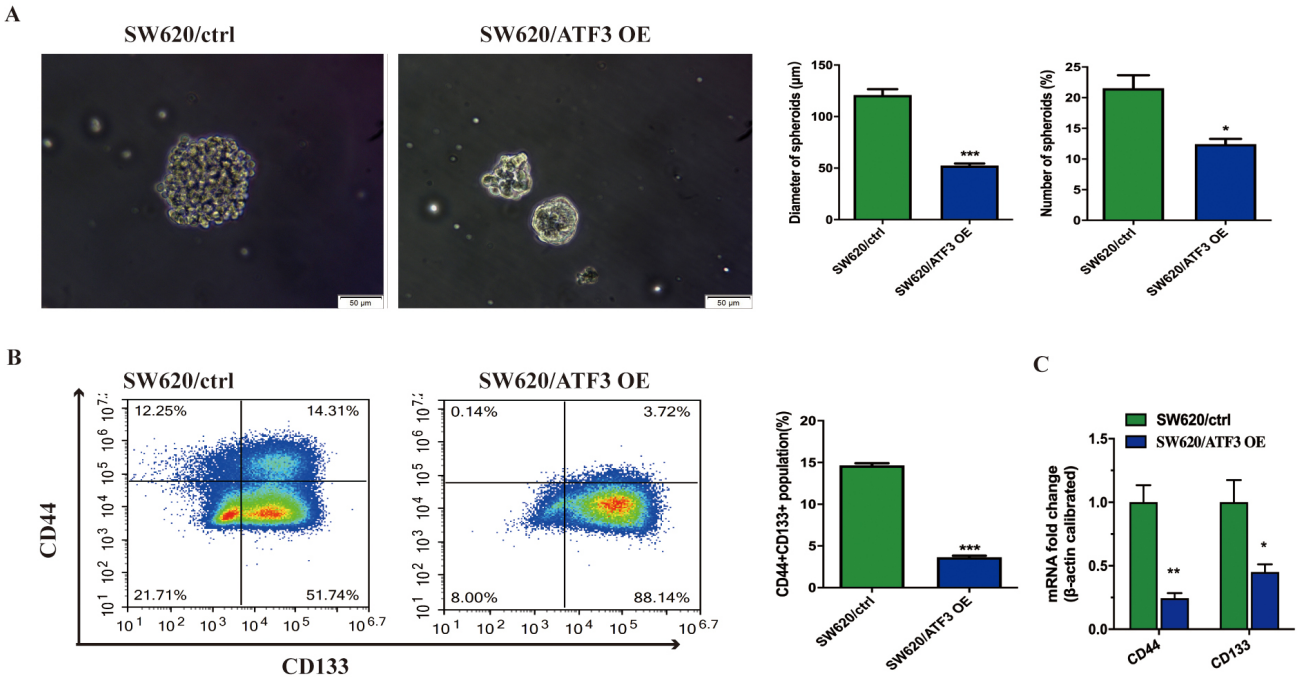

FIG. 5. Effects of ATF3 on the cancer-initiating cell features of SW620 CRC cells. (A) Overexpressing ATF3 in SW620 cells inhibited spheroid formation compared with the vector control. Cells were counted under a light microscope at 200× magnification. (B) The expression of cancer stem cell markers were measured at the mRNA level by qRT-PCR assay. (B) FACS analysis revealed that overexpressing ATF3 in SW620 cells led to decreased expression of the surface markers CD44 and CD133 compared with the vector control group. (C) CD44 and CD133 mRNA were downregulated along with ATF3 overexpression in SW620 cells compared with the vector controls. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, comparing the two groups using the independent Student's *t* test. Ctrl, control; OE, overexpression.

3.4 Effects of ATF3 on the migration and invasion of SW620 cells

As migration and invasion are key properties of cancer cells that are critical for tumor progression, it was necessary to determine the effects of ATF3 on the migration and invasion capacity of CRC cells. As shown in Fig. 4A, overexpressing ATF3 in SW620 cells inhibited their migration as evaluated through wound healing assays. Transwell migration assays performed without Matrigel also showed that overexpression ATF3 in SW620 cells inhibited cell migration (Fig. 4B). Finally, the effect of ATF3 on the invasion capacity of SW620 cells was evaluated by transwell invasion assays with Matrigel-coated upper chambers. ATF3 overexpression dramatically suppressed the invasion capacity of SW620 cells compared with controls (Fig. 4C).

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3.5 Effects of ATF3 on CRC stemness features in SW620 cells

To test whether ATF3 was related to cells with CRC-initiating capability, cells were cultured in non-adherent conditions and serum-free medium. CRC cells could form tumorspheres in serum-free media after 3–7 days. Overexpressing ATF3 in SW620 cells decreased the diameter and number of tumorspheres (Fig. 5A), indicating that ATF3 inhibited the self-renewal of CRC CSCs *in vitro*. To analyze whether ATF3 modulated the CSC subpopulation of CRC cells, flow cytometry was used to determine the expression of stem cell markers such as CD44 and CD133. As shown in Fig. 5B, overexpressing ATF3 in SW620 cells greatly decreased the $CD44^{+}/CD133^{+}$ subpopulation. Also, mRNA levels of CD44 and CD133 were detected by qRT-PCR. Both CD44 and CD133 mRNA levels were remarkably decreased in the ATF3-overexpression SW620 cells compared with control cells (Fig. 5C).

4. Discussion

Many studies have observed that the complex roles of ATF3 in tumor cells are associated with distinct cell subtypes [6]. ATF3 is involved in tumor processes including cell proliferation, migration, and CSC differentiation and self-renewal [21]. Understanding the biological role of ATF3 in CRC has been difficult. In human CRC specimens, ATF3 shows decreased expression compared with surrounding normal tissues, indicating that ATF3 may be a tumor suppressor in CRC [22, 23]. Studies have reported that ATF3 can decrease cell proliferation and promote apoptosis in CRC cells [15, 16, 22–25]. However, Tatsuaki *et al.* found higher ATF3 expression human colon cancer specimens than in normal tissues, which suggests that ATF3 may promote CRC [19, 20]. Yan *et al.* [26] also reported that increased ATF3 expression increased invasion and was linked to a poor prognosis in CRC. These opposing results may be due to different cellular contexts. In this report, we proposed two pieces of evidence that suggest tumor inhibitory effects of ATF3 in CRC: ATF3 inhibited epithelial-mesenchymal transition (EMT) and tumorigenesis, which is consistent with previous studies [15, 16, 22–25]. Furthermore, our results provide novel data suggesting that ATF3 suppresses tumorsphere formation capacity and therefore tumor-initiating frequency in CRC. CSCs are a rare subpopulation in a tumor but they have self-renewal, differentiation, and tumor initiation capacity and are less sensitive to chemotherapy and radiotherapy [3]. CD44 and CD133 expression are related to maintaining the pluripotency and self-renewal of CRC CSCs [27]. The hypothesis of this study was that ATF3-overexpression could suppress the malignancy of CRC by decreasing the CSC population, which were represented by CD44/CD133-double positive cells. Our results suggest that ATF3 could be a clinical target for tumor prevention and treatment.

To date, the exact molecular mechanisms of ATF3 in CRC have not been uncovered. Makoto Inoue *et al.* [28] showed that ATF3 is a direct binding target of TCF4/β-catenin, through which it could suppress cell invasion and migration in CRC. Other recent studies revealed that ATF3 is a stress-inducible transcription factor that induces DNA damage and mediates apoptosis following exposure to various anticancer compounds [17, 18]. Seong-Ho Lee *et al.* [29] suggested that the DNA damage induced by a high phenolic sorghum bran extract is associated with ATF3 overexpression in HCT116, HCT15, and SW480 CRC cells. Twigs of Cinnamomum cassia can inhibit the proliferation and induce apoptosis by inducing ATF3 overexpression in CRC cells [30]. Additionally, ATF3 may regulate several genes, including invasion-related genes (maspin and plasminogen activator inhibitor-1), metastasis-associated genes (matrix metalloproteinases), and death receptor 5 [15, 31].

The stemness of cancer cells and EMT play important and complex roles in promoting tumor's abilities in invasion and metastasis [32, 33]. It has been suggested that a few CSCs could self-renew and differentiate, resulting in tumor heterogeneity, over-proliferation, and invasion. EMT may affect morphological and functional changes in epithelial cells, which favors the migration of cancer cells from adjacent stromal tissues to target organs [32]. To our knowledge, evidence on the mechanisms underlying the effects of ATF3 on EMT and/or CSCs is poor. In breast cancer, increased ATF3 expression was associated with morphological changes and altered expression of E-cadherin, N-cadherin, vimentin, and fibronectin, which suggests cells underwent EMT [21]. Ectopic ATF3 expression led to increased CD24/CD44 expression, tumorsphere formation ability, and tumorigenesis in breast cancer [21], suggesting the capacity of ATF3 to promote self-renewal in cancer cells. Our findings complement the previously reported roles of ATF3, especially in CSC-mediated tumor invasion and metastasis. Our results suggest that ATF3 could be targeted to treat cancer-initiating cells and/or CSCs in the clinical care of CRC.

Certain limitations of this study should be acknowledged. First, the different CRC cell lines (e.g., SW480 and HCT-116) likely have distinct cellular contexts in which they develop the functional behaviors associated with ATF3. Because of the complexity of CRC, an integrative study of ATF3-associated genomic and proteomic events should be evaluated to determine the impact of ATF3 in different CRC microenvironments. Second, there was the possibility of a potential susceptibility mutation in the ATF3 gene in SW620 cells after transfection. In this study, we restricted our assessment to a limited set of direct sequencing and splice sites of ATF3 due to the COVID-19 pandemic. Further investigations are therefore needed to determine the coding exons to rule out the occurrence of a mutation that might subtly or substantially affect its gene regulatory functions.

5. Conclusions

In conclusion, our results indicated that ATF3 regulates stem cell features of CRC cells. Given that certain compounds with chemoprotective and/or chemotherapeutic function specifically activate ATF3 expression [29, 30], further investigations of these compounds as potential CRC treatments in

combination with traditional and/or innovative therapies is warranted. However, the precise underlying molecular mechanism through which ATF3 regulates cancer stemness remains to be elucidated.

Author contributions

XL and CQ designed the study, supervised the data collection. CD, FJ, XX, JF and WJ analyzed the data, interpreted the data. WJ, XL and CQ prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

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Conflict of interest

The authors declare no conflict of interest.

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