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B-cell translocation gene 3 overexpression inhibits proliferation and invasion of colorectal cancer SW480 cells via Wnt/β-catenin signaling pathway

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Increasing evidences have shown that B-cell translocation gene 3 (BTG3) inhibits metastasis of multiple cancer cells. However, the role of BTG3 in colorectal cancer (CRC) and its possible mechanism have not yet been reported. In our study, we evaluated BTG3 expression in several CRC cell lines. Then, pcDNA3.1-BTG3 was transfected into SW480 cells. We found that BTG3 was upregulated in SW480 cells after overexpression plasmid transfection. BTG3 overexpression significantly inhibited cell growth and decreased PCNA (proliferating cell nuclear antigen) and Ki67 levels. BTG3 overexpression markedly downregulated Cyclin D1 and Cyclin E1 levels, whereas elevated p27. Overexpression of BTG3 arrested the cell cycle at G1 phase, which was abrogated by p27 silencing. Furthermore, migration, invasion and EMT of SW480 cells were significantly suppressed by BTG3 overexpression. Further investigations showed the inhibition of Wnt/β-catenin signaling pathway. We then used GSK3β specific inhibitor SB-216763 to activate the Wnt/β-catenin signaling pathway. We found that Wnt/β-catenin signaling pathway activation reversed the effect of BTG3 overexpression on cell proliferation, cell cycle progression, invasion and EMT. In conclusion, BTG3 overexpression inhibited cell growth, induced cell cycle arrest and suppressed the metastasis of SW480 cells via the Wnt/β-catenin signaling pathway. BTG3 may be considered as a therapeutic target in CRC treatment.

Key words: colorectal cancer, BTG3, metastasis, p27, SB-216762, Wnt/β-catenin signaling pathway

Colorectal cancer (CRC) is the third most prevalent malignancy and the fourth leading cause of cancer-related death worldwide [1, 2]. More than 1,200,000 new cases were diagnosed and approximately 608,000 patients died from CRC annually [3]. Metastasis contributes to the high mortality in CRC patients. When diagnosed, nearly 60% patients exhibit metastatic disease [4]. Environmental factors and genetic abnormalities in humans have been implicated in the pathogenesis of CRC [5]. Despite the development of new therapies for CRC patients, the overall survival rate has improved slightly [6]. Therefore, it is vital to explore the possible mechanisms involved.

B-cell translocation gene (BTG)/transducer of the ErbB2 (Tob) family comprises 6 members (BTG1, BTG2, BTG3, BTG4, Tob1 and Tob2) in mammalian cells, which possess anti-proliferative properties [7, 8]. BTG3 is a candidate tumor suppressor and a direct target of p53. BTG3 interacts with transcription factor E2F1 and inhibits its activity [9]. Previous findings have reported that BTG3 is downregulated in many cancers, including prostate cancer, renal carcinoma, gastric cancer and ovarian carcinoma [10-13]. BTG3 depletion increases the growth rate of HCT116 cells. Conversely, BTG overexpression inhibits cell proliferation [9, 14]. However, the role of BTG3 in CRC progression and the possible mechanism remain unknown.

In the present study, we cultured several CRC cell lines *in vitro* and the expression level of BTG3 was evaluated by Real-time PCR and Western blotting. BTG3 was overexpressed in CRC SW480 cells via transfection with pcDNA3.1-BTG3 plasmid to explore the role and mechanism of BTG3 in CRC cell proliferation, cell cycle distribution and metastasis.

Materials and methods

Cell culture. Human colorectal cancer (CRC) cell lines Caco-2, SW480 and COLO-205 were obtained from the cell

bank at Chinese Academy of Sciences (Shanghai, China). Caco-2 and SW480 cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA) in 5% CO_2 humidified atmosphere at 37°C. COLO-205 cells were maintained in RPMI-1640 medium (Gibco) containing 10% FBS (HyClone) at 37°C.

Overexpression plasmid construction and cell transfection. BTG3 gene was cloned using the specific primers that synthesized by Sangon Biotech (Shanghai, China) and subcloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) via the Hind III/Xho I restriction sites. The primer sequences were listed: BTG3-forward, 5'–GTCCAAGCTTATGAAGAATGAAATTGCTGC–3' and BTG3-reverse, 5'–CAATCTCGAGGTGAGGTGCTAACATGTGA–3'. SW480 cells were transfected with pcDNA3.1-BTG3 or pcDNA3.1 using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. At 24 h after transfection, G418 (100 μg/ml) was added to select the stable transfectants.

Quantitative Real-time PCR. The cells were harvested and the total RNA was extracted using the RNA extraction kit (BioTeke, Beijing, China). cDNA was synthesized and the mRNA levels of BTG3, PCNA, Ki67, Cyclin D1, Cyclin E1 and p27 were quantified using SYBR Green I (Solarbio, Beijing, China) on BIONEER Exicycler™96 Real-Time Quantitative PCR system (Daejeon, Korea). The obtained cDNAs were denatured for 10 min at 95°C followed by 40 cycles of amplification reactions (95°C for 10 s, 60°C for 20 s and 72°C for 30 s). The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers were: BTG3-forward, 5'–ATGAAATTGCTGCCGTTGTCT–3' and BTG3-reverse, 5'–GCCTGTCCTTTCGATGGTTTT–3'; PCNA-forward, 5'–CCTGTAGCGGCGTTGTTGC–3' and PCNA-reverse, 5'–GTCGCAGCGGTAGGTGTCG–3'; Ki67-forward, 5'–ATCGAACACCAGCTAAAGT–3' and Ki67-reverse, 5'–CAGGTAACCCAGAGCACAT–3'; Cyclin D1-forward, 5'–CCCGCACGATTTCATTGAAC–3' and Cyclin D1-reverse, 5'–AGGGCGGATTGGAAATGAAC–3'; Cyclin E1-forward, 5'–GCCTTGTATCATTTCTCGTCA–3' and Cyclin E1-reverse, 5'–TGGGTCTGTATGTTGTGTGC–3'; p27-forward, 5'–ACCGACGATTCTTCTACTC–3' and p27-reverse, 5'–ATTCCATGAAGTCAGCGATA–3'; β-actin-forward, 5'–CTTAGTTGCGTTACACCCTTTCTTG–3' and β-actin-reverse, 5'–CTGTCACCTTCACCGTTCCAGTTT–3'.

Western blotting. The protein levels of BTG3, PCNA, Ki67, Cyclin D1, Cyclin E1, p27, E-cadherin, Vimentin, Wnt1, Wnt3a, active-β-catenin, p-GSK3β and GSK3β were evaluated by Western blotting. Briefly, the cells were lysed using lysis buffer (Wanleibio, Shenyang, China) and kept on ice for 15 min. After centrifuged at 12000 rpm for 10 min at 4°C, the total proteins in the supernatant of the cell lysates were obtained. The total proteins were diluted in PBS and the protein concentration was quantified using BCA protein assay kit (Wanleibio). The protein sample (40 μg/lane) was separated by 6%/9%/12% SDS-PAGE (Wanleibio) and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with BTG3 (1:200, sc-18545, Santa cruz, Dallas, Texas, USA), PCNA (1:500, bs-2006R, Bioss, Beijing, China), Ki67 (1:500, bs-2130R, Bioss), Cyclin D1 (1:400, BM0771, Boster, Wuhan, China), Cyclin E1 (1:400, BA0774, Boster), p27 (1:200, sc-393380, Santa cruz), E-cadherin (1:400, BA0474, Boster), Vimentin (1:500, bs-8533R, Bioss), Wnt1 (1:200, sc-5630, Santa cruz), Wnt3a (1:400, BA2628-2, Boster), active-β-catenin (1:1000, 05-665, Millipore), GSK3β (1:200, sc-9166, Santa cruz) and p-GSK3β (Ser 9) (1:200, sc-11757, Santa cruz) antibodies at 4°C overnight after blocking with non-fat milk/BSA, followed by incubation with the corresponding horseradish peroxidase (HRP)-labeled secondary antibody (1:5000) (goat anti-rabbit IgG-HRP, WLA023,Wanleibio; goat anti-mouse IgG-HRP, WLA024,Wanleibio; and donkey anti-goat IgG-HRP, A0181, Beyotime, Haimen, China) at 37°C for 45 min. The bands were visualized using enhanced chemiluminescent (ECL) reagent (Wanleibio) and quantified using Gel-Pro-Analyzer 4.0 (Media Cybernetics, Rockville, MD, USA).

MTT assay. When growing to 90% confluence, the cells were seeded onto 96-well plates at a density of 3000 cells/well. The plates were cultured at 37°C for 0, 12, 24, 48, 72, 96 and 120 h, followed by incubation with 5 mg/ml MTT solution (Sigma-Aldrich, St. Louis, MO, USA) for 4 h. The supernatant was discarded and 200 μl DMSO (Sigma-Aldrich) was added to dissolve the insoluble formazan crystals. The absorbance was measured at 490 nm in an ELX-800 Absorbance Microplate Reader (BioTek, Winooski, VT, USA).

Bromodeoxyuridine (BrdU) incorporation assay. Cell proliferation was investigated using BrdU ELISA Kit (Maibio, Shanghai, China) according to the manufacturer's instructions. Briefly, the cells cultured in 96-well plates were incubated with 10 μl BrdU for 2 h at 37°C. The supernatant was discarded and 200 μl FixDenat was added into each well to incubate with the cells for 30 min at room temperature. Then, the cells were stained with antibody against BrdU-POD at room temperature for 90 min. After washing with PBS, the cells were treated with 100 μl substrate (TMB) and then with 25 μl stop solution. The absorbance was measured at 450 nm using Absorbance Reader (BioTek).

Colony formation assay. The cells were harvested by centrifugation (800 rpm for 3 min) and resuspended in 1 ml DMEM. Then, the cells were seeded into 35 mm culture dishes (200 cells in each culture dish) and incubated at 37°C for nearly 14 d. After being fixed with 4% paraformaldehyde (Sinopharm, Shenyang, China) for 20 min, the cells were stained with Wright-Giemsa stain (Nanjing Jiancheng, Nanjing, China) for 5–8 min. The colonies containing ≥50 cells were counted under the inverted microscope (Motic, Xiamen, China).

Flow cytometric analysis. Cell cycle distribution was evaluated using Cell Cycle and Apoptosis Analysis Kit according to the manufacturer's instructions (Beyotime) by flow cytometer (BD, Franklin Lakes, NJ, USA). Briefly, the cells were washed

with PBS and fixed with 70% ethanol for 2 h at 4°C. The cells were harvested by centrifugation. Subsequently, the cell pellets were resuspended in 500 µl staining buffer containing 25 µl propidium iodide (PI) and 10 µl RNase A and incubated for 30 min at 37°C in the dark.

siRNA transfection. SW480 cells overexpressing BTG3 were seeded into 6-well plates and transfected with p27 siRNA or control siRNA (GenePharma, Shanghai, China) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The siRNA sequences were: p27 siRNA, 5'–CCUGCAACCGACGAUUCUUTT–3'; control siRNA: 5'–UUCUCCGAACGUGUCACG–3'. The expression of p27 was evaluated by Real-time PCR and Western blotting. Cell cycle distribution was determined by flow cytometry.

Transwell migration and invasion assays. The cells were digested with trypsin into single cell suspension and 200 µl (2×10^4 cells) was added into the upper part of the chamber (Corning, Corning, NY, USA) coated with (for invasion assay) or without Matrigel (for migration assay) (BD). Medium containing 20% FBS (800 µl) was added into the lower part. After 24 h of cell culture at 37°C, the cells were washed with PBS, fixed in 4% paraformaldehyde (Sinopharm) for 20 min and stained with 0.5% crystal violet (Amresco, Solon, OH, USA). The invaded and migrated cell numbers were counted under an inverted microscope (AE31; Motic).

Immunofluorescence assay. The cells that cultured on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, followed by blocking with serum (Solarbio) at room temperature. Then, the slides were incubated with primary antibodies against E-cadherin (1:200, BA0474, Boster) and Vimentin (1:200, bs-8533R, Bioss) at 4°C overnight. After washing with PBS, the slides were incubated with Cy3-labeled goat anti-rabbit IgG (H+L) (1:200, A0516, Beyotime) for 60 min at room temperature. Nuclei were stained with DAPI and images were captured under an Olympus BX53 fluorescence microscope (Tokyo, Japan).

SB-216763 treatment and groups. The cells were divided into 5 groups. (1) control group, the cells cultured in DMEM were served as the controls. (2) DMSO group, the cells were incubated with DMSO before analysis. (3) BTG3+DMSO group, SW480 cells overexpressing BTG3 were treated with DMSO. (4) SB-216763 group, the cells were incubated with GSK3β specific inhibitor SB-216763 (20 µM). (5) BTG3+SB-216763 group, the cells that transfected with pcDNA3.1-BTG3

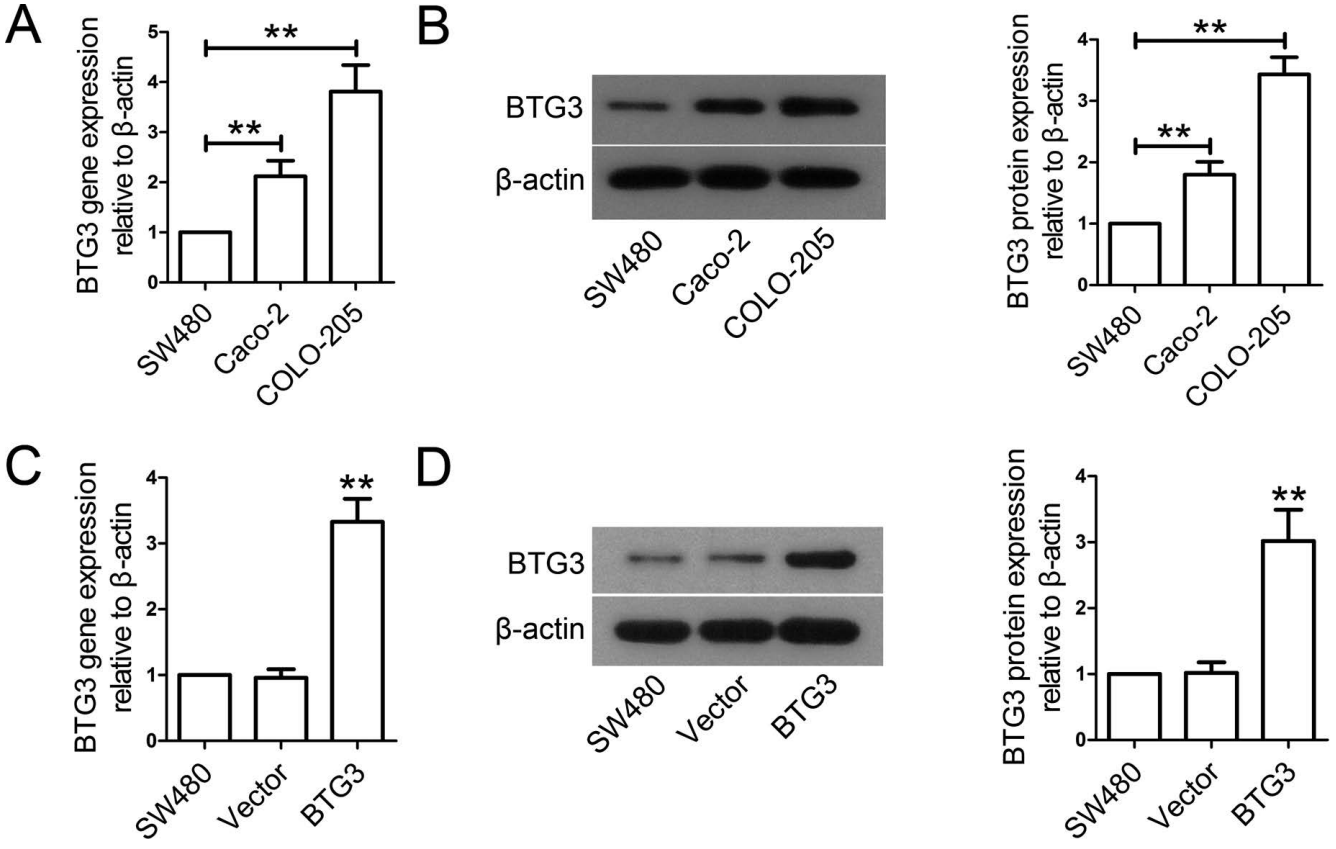

Figure 1. BTG3 expression in CRC cell lines and the establishment of BTG3 overexpression cell line. (A) The relative expression of BTG3 in SW480, Caco-2 and COLO-205 was measured by Real-time PCR. (B) BTG3 expression was examined by Western blotting. (C) SW480 cells were transfected with pcDNA3.1-BTG3. The mRNA level of BTG3 was measured by Real-time PCR. (D) After transfection, the BTG3 protein level was determined by Western blotting. Data are expressed as Mean±SD. ** *P*<0.01 compared to SW480 or Vector.

were incubated with GSK3β specific inhibitor SB-216763. Cell proliferation was examined by MTT assay. Cell cycle distribution and invasion ability were evaluated. The protein levels of active-β-catenin, p-GSK3β, GSK3β, PCNA, Ki67, Cyclin D1, Cyclin E1, p27, E-cadherin and Vimentin were quantified by Western blotting.

Statistical analysis. Data are expressed as Mean±SD. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Differences were analyzed by One-way ANOVA followed by Bonferroni post-hoc test. $P<0.05$ was considered statistically significant.

Results

BTG3 expression in CRC cell lines. Initially, we evaluated the expression levels of BTG3 in SW480, Caco-2 and

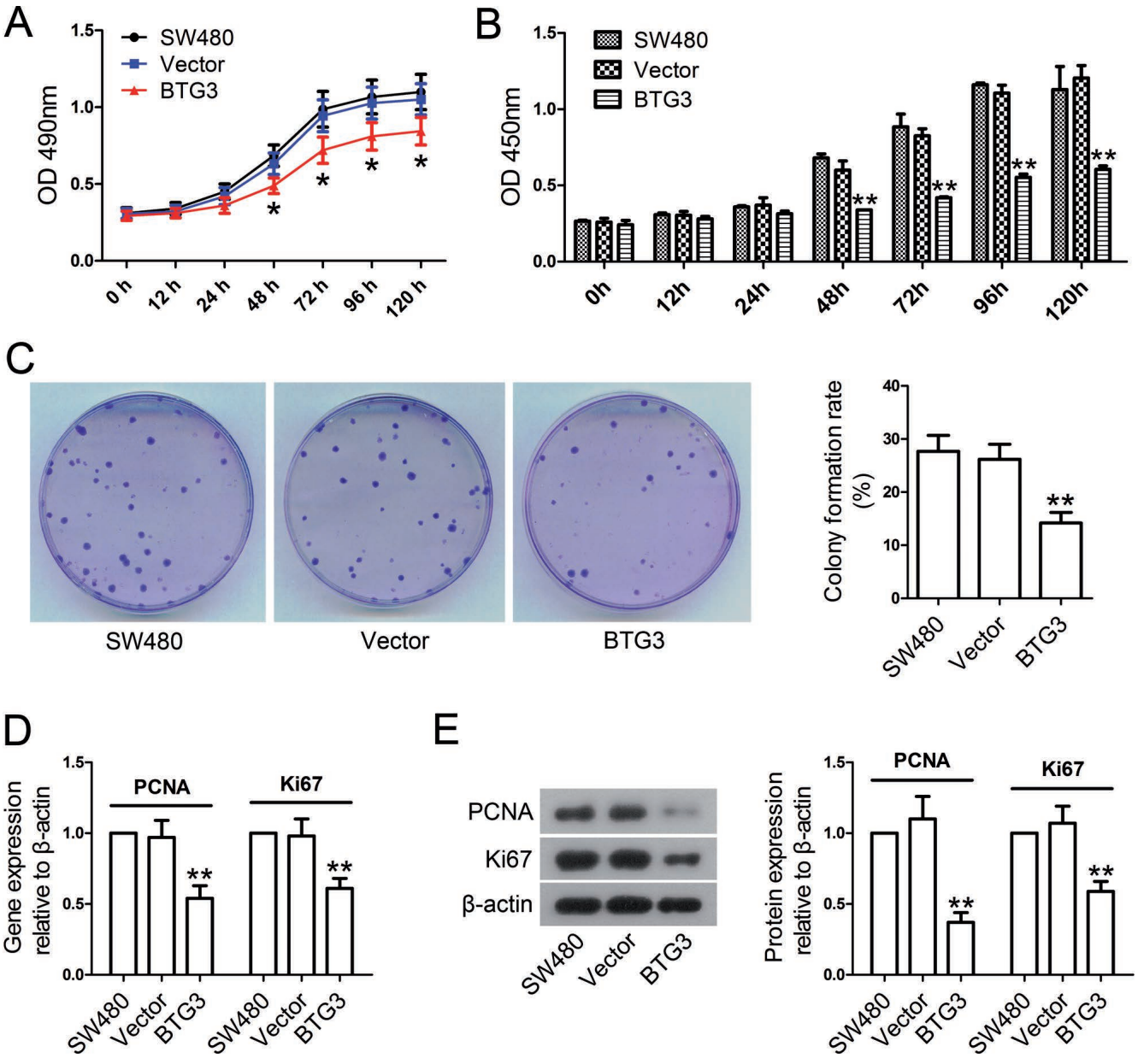

Figure 2. Effect of BTG3 overexpression on proliferation and colony formation in SW480 cells. (A) Cell viability was determined by MTT assay. (B) Cell proliferation was evaluated by BrdU incorporation assay. (C) Colony formation assay was performed using SW480 cells that transfected with pcDNA3.1-BTG3 or pcDNA3.1 plasmid. (D) PCNA and Ki67 mRNA levels were measured by Real-time PCR. (E) PCNA and Ki67 protein levels were measured by Western blotting. Data are expressed as Mean±SD. * $P<0.05$ and ** $P<0.01$ compared to Vector.

COLO-205 using quantitative Real-time PCR. As shown in Figure 1A, BTG3 gene expression was 2.12-fold higher in Caco-2 and 3.81-fold higher in COLO-205 as compared to that in SW480 cells. We then examined BTG3 expression in these CRC cell lines using Western blotting. The protein expression levels of BTG3 in Caco-2 and COLO-205 were 1.8-fold and 3.43-fold higher than that in SW480 cells (Figure 1B). Thus, SW480 cells were used in subsequent experiments.

BTG3 overexpression inhibits cell growth and colony formation. To evaluate the role of BTG3 in CRC cells, SW480 cells were transfected with pcDNA3.1 or pcDNA3.1-BTG3 and stable transfectants were selected by G418. Real-time PCR analysis showed that BTG3 was overexpressed in SW480 cells that transfected with pcDNA3.1-BTG3 (Figure 1C). Western blotting analysis confirmed the overexpression of BTG3 in SW480 cells (Figure 1D).

As shown in Figure 2A, BTG3 overexpression significantly suppressed cell proliferation at 48, 72, 96 and 120 h. BrdU incorporation assay further confirmed the anti-proliferation effect of BTG3 overexpression in SW480 cells (Figure 2B). Next, colony formation ability of the cells in each group was examined. The results showed that colony formation capacity of the cells in the BTG3 group was greatly reduced as compared to that in the Vector group (Figure 2C). Real-time PCR and Western blotting results demonstrated that the expression levels of PCNA and Ki67 were markedly downregulated after BTG3 overexpression (Figure 2D and 2E).

BTG3 overexpression arrests the cell cycle at G1 phase via p27. To investigate how BTG3 inhibits cell growth, we examined the effect of BTG3 overexpression on cell cycle distribution in SW480 cells. As shown in Figure 3A, the percentage of cells in G1 phase in the BTG3 group was significantly higher than that in the Vector group, while the proportions of S and G2 phase cells were decreased. We then measured the expression levels of cell cycle regulators using Real-time PCR and Western blotting. We found that Cyclin D1 and Cyclin E1 were significantly decreased at both mRNA and protein levels in the cells transfected with BTG3 overexpression plasmid, whereas p27 expression was elevated during this process (Figure 3B and 3C).

To further verify if p27 is involved in the regulation of cell cycle, we silenced p27 using siRNA. The results showed that p27 mRNA and protein levels were efficiently suppressed by p27 knockdown (Figure 3D). Flow cytometry results showed that p27 silencing greatly decreased the number of cells overexpressing BTG3 in G1 phase and increased the percentage of cell population in G2 phase (Figure 3E).

BTG3 overexpression inhibits cell migration, invasion and EMT. The effect of BTG3 overexpression on migration and invasion of SW480 cells was explored by Transwell assays. The number of migrated cells or invaded cells in the BTG3 group was significantly lower than that in the Vector group (Figure 4A and 4B). The expression of E-cadherin was markedly upregulated in the cells transfected with pcDNA3.1-BTG3 compared to that in the Vector-transfected cells, but the expression of Vimentin was downregulated (Figure 4C and 4D).

BTG3 overexpression inhibits the activation of Wnt/β-catenin signaling pathway. We then measured the activation of Wnt/β-catenin signaling pathway. As shown in Figure 5, the levels of Wnt1, Wnt3a and active-β-catenin and p-GSK3β (Ser 9)/GSK3β ratio were significantly reduced in the cells after transfection with pcDNA3.1-BTG3.

SB-216763 treatment induces the activation of Wnt/β-catenin signaling pathway. To further confirm the involvement of Wnt/β-catenin signaling pathway, SB-216763 was used to activate Wnt/β-catenin signaling pathway. As shown in Figure 6A, DMSO had no effect on the expression levels of Wnt/β-catenin signaling pathway members compared with the control group. SB-216763 significantly activated the Wnt/β-catenin pathway compared with the DMSO group. The active-β-catenin level and p-GSK3β/GSK3β ratio in the BTG3+SB-216763 group were significantly higher than those in the BTG3+DMSO group.

The activation of Wnt/β-catenin pathway reverses the effect of BTG3 overexpression on cell proliferation, cell cycle progression, invasion and EMT. DMSO did not change the cell properties. However, SB-216763 significantly promoted cell proliferation, invasion and EMT of SW480 cells. Moreover, SB-216763 reversed the effect of BTG3 overexpression on cell proliferation (Figure 6B), cell cycle progression (Figure 6C), invasion (Figure 6D) and EMT (Figure 7). We then detected the expression levels of several markers in BTG3-overexpressing SW480 after incubation with SB-216763. We demonstrated the upregulation of PCNA, Ki67, Cyclin D1, Cyclin E1 and Vimentin and the downregulation of E-cadherin and p27.

Discussion

BTG3 is a tumor suppressor and can inhibit cell growth and invasion of several tumors [15]. However, the expression level and the biological function of BTG3 in colorectal cancer (CRC) cell line remain unknown. In the present study, we compared the expression of BTG3 in several CRC cell lines and found that SW480 expressed lower levels of BTG3 than the other two cell lines Caco-2 and COLO-205. BTG3 expression was upregulated in SW480 cells. We then evaluated the role and the underlying mechanism of BTG3 in cell proliferation, cell cycle progression, migration and invasion.

The occurrence and progression of CRC are associated with the abnormal proliferation of tumor progenitor cells [16]. Previous literatures have shown that upregulation of BTG3 suppresses the growth of tumor cells, including gastric cancer, esophageal adenocarcinoma (EAC) and hepatocellular carcinoma (HCC) [12, 15, 17]. We consistently found that BTG3 overexpression significantly inhibited cell proliferation and colony formation of CRC cells SW480. The anti-proliferation effect was further examined by measuring

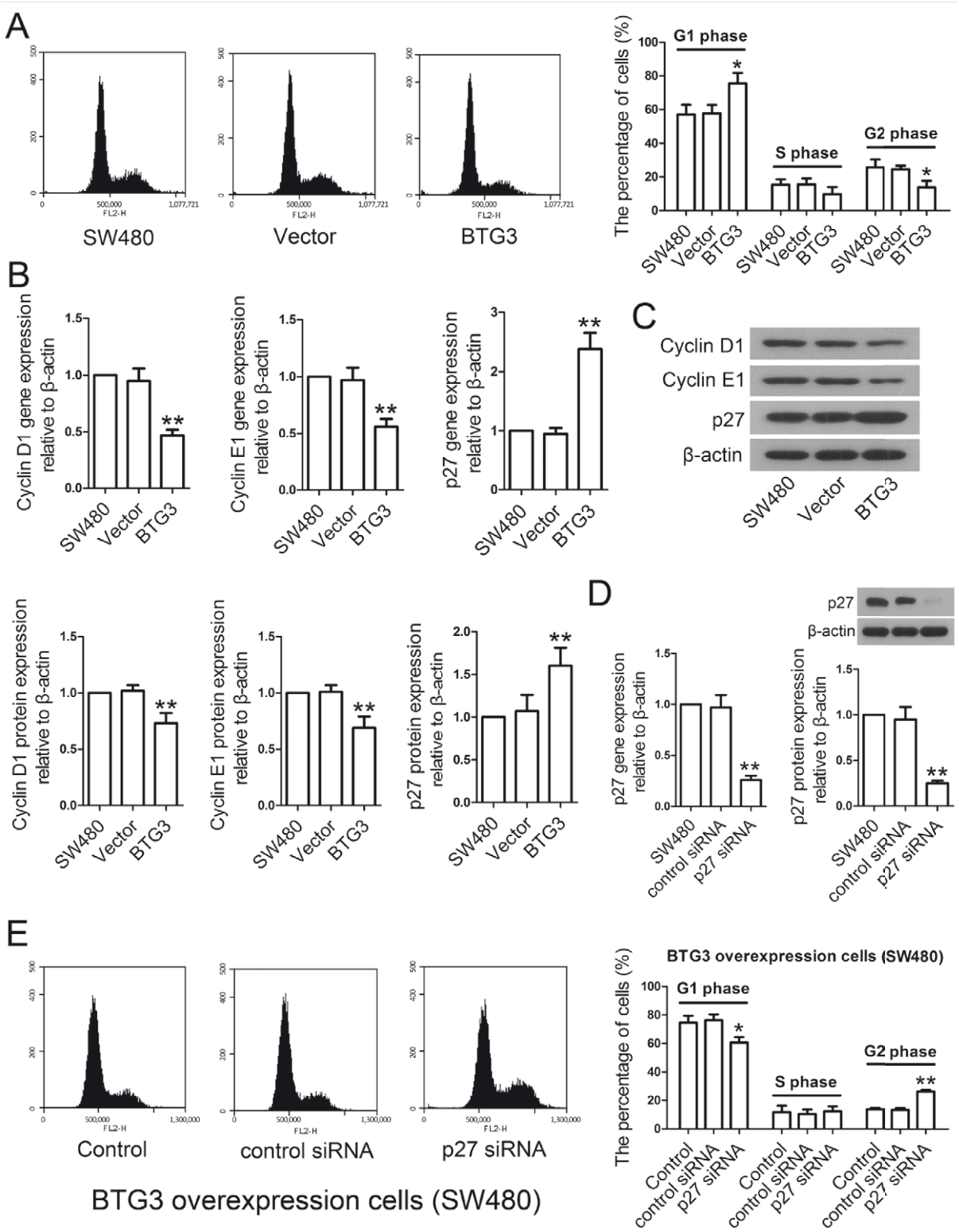

Figure 3. Effect of BTG3 overexpression on cell cycle progression. (A) Cell cycle distribution of SW480 cells overexpressing BTG3 was analyzed by flow cytometry. (B) The mRNA levels of Cyclin D1, Cyclin E1 and p27 were detected by Real-time PCR. (C) Cyclin D1, Cyclin E1 and p27 protein levels were measured by Western blotting. (D) BTG3-overexpressing SW480 cells were transfected with p27 siRNA or control siRNA. Real-time PCR and Western blotting were performed to examine p27 levels. (E) After p27 knockdown, BTG3 overexpression cells were subjected to cell cycle analysis by flow cytometry. Data are expressed as Mean±SD. * $P<0.05$ and ** $P<0.01$ compared to Vector or control siRNA.

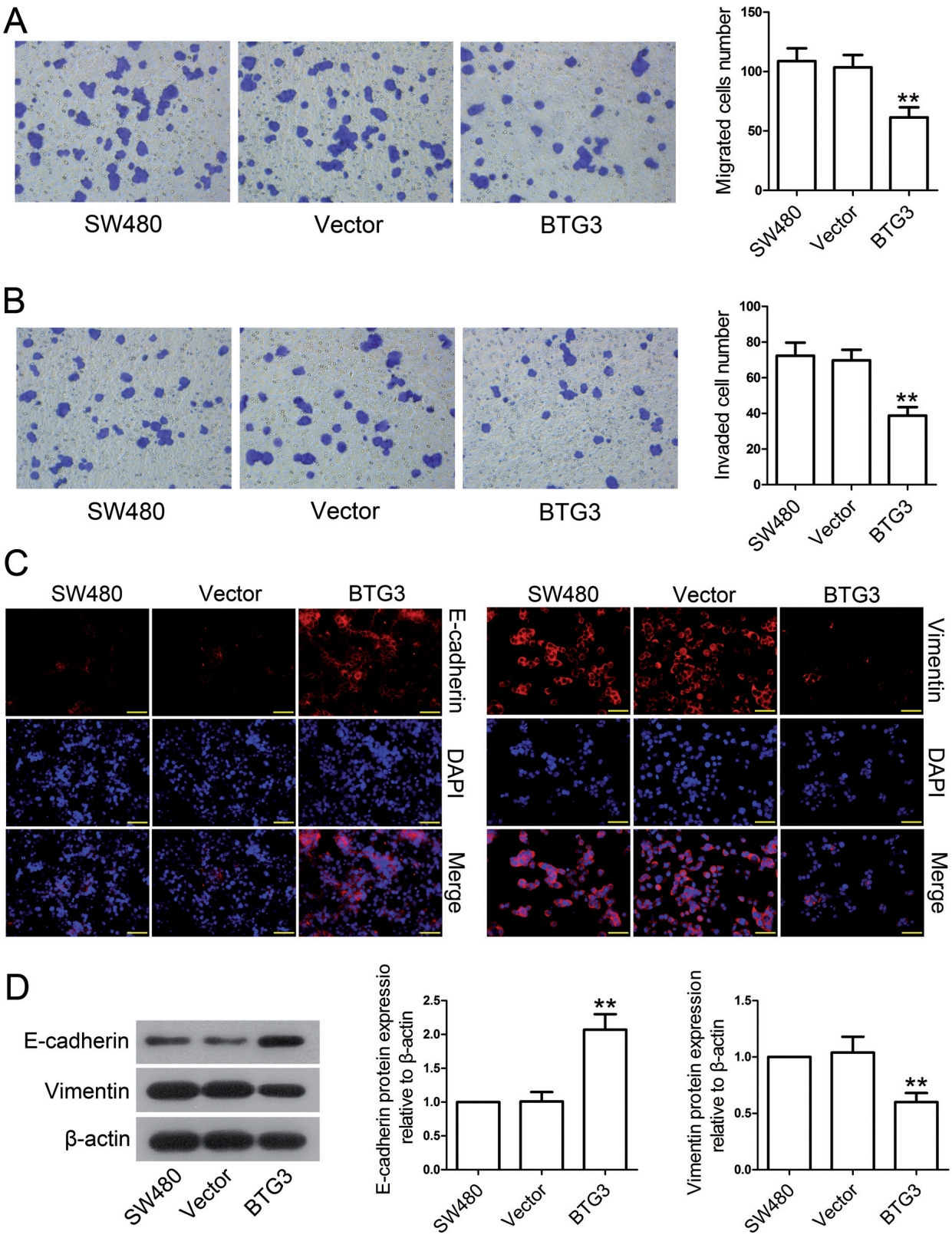

Figure 4. Effect of BTG3 overexpression on migration and invasion of SW480 cells. (A) Cell migration was evaluated by Transwell migration assay. (B) Cell invasion was evaluated by Transwell invasion assay. (C) E-cadherin and Vimentin levels were measured by Immunofluorescence staining. (D) E-cadherin and Vimentin levels were confirmed by Western blotting. Data are expressed as Mean±SD. ** $P<0.01$ compared to Vector.

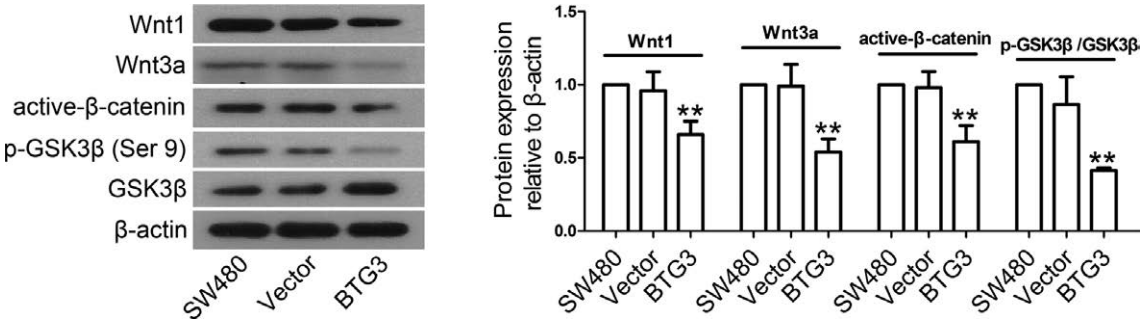

Figure 5. Effect of BTG3 overexpression on the Wnt/β-catenin signaling pathway. SW480 cells were transfected with pcDNA3.1-BTG3 or pcDNA3.1. Wnt1, Wnt3a, active-β-catenin, p-GSK3β (Ser 9) and GSK3β protein levels were analyzed by Western blotting. Data are expressed as Mean±SD. ** *P*<0.01 compared to Vector.

the expression of proliferation makers, PCNA and Ki67. PCNA, synthesized in the early stage of G1 to S phase, is involved in DNA replication, cell cycle progression and DNA repair [18]. Ki67 is a cell proliferation marker and is expressed in G1, S and G2/M phases except G0 phase [19, 20]. The results showed that BTG3 overexpression greatly downregulated PCNA and Ki67 levels in SW480 cells. Accordingly, our results suggested that BTG3 overexpression might inhibit CRC cell proliferation by reducing PCNA and Ki67 expression.

Cell cycle arrest induced by cell cycle regulators can inhibit cell proliferation and tumor growth [21]. Cyclin D1 and Cyclin E1 are crucial regulators of the G1/S checkpoint of the cell cycle progression [22]. We found that overexpression of BTG3 induced the cell cycle arrest at G1 phase, accompanied with the downregulation of Cyclins and upregulation of p27 expression. p27 is an inhibitor of CDKs (cyclin-dependent kinases) and can prevent the G1/S transition. Therefore, p27 is usually used as a tumor suppressor [23]. We silenced p27 expression in SW480 cells to investigate whether p27 was involved in cell cycle regulation. The results showed that p27 knockdown significantly alleviated BTG3 overexpression-induced cell cycle arrest at G1 phase. The above results suggested that BTG3 overexpression might arrest the cells at G1 phase via p27.

Cell migration and invasion are essential for tumor metastasis, which lead to high morbidity and mortality in patients [24]. A recent research has demonstrated that elevation of BTG3 expression in esophageal adenocarcinoma (EAC) cells results in the impaired migration and invasion capabilities. Downregulation of BTG3 correlates with lymph node metastases in EAC patients [15]. Additionally, BTG3 inhibits invasion of gastric cancer (GC) and hepatocellular carcinoma (HCC) cells [17, 25]. However, the role of BTG3 in CRC progression has not yet been reported. In our study, we found that BTG3 overexpression suppressed migration and invasion abilities of SW480 cells *in vitro*, as evaluated by Transwell migration and invasion assays. Epithelial-mesenchymal transition (EMT) is a process in which the cells lost cell-cell adhesion and cell motility is increased [26]. EMT plays a critical role in fibrosis, wound healing and cancer metastasis [27]. EMT is characterized by the downregulation of epithelial markers such as E-cadherin and the upregulation of mesenchymal markers, including N-cadherin and Vimentin [28]. We demonstrated that overexpression of BTG3 elevated E-cadherin expression and decreased Vimentin expression in SW480 cells. Our results suggested that BTG3 overexpression might inhibit migration and invasion of CRC cells by regulating the expression of EMT-related proteins.

Wnt/β-catenin signaling pathway is associated with proliferation, differentiation, cell death and tumorigenesis [29, 30]. Abnormal activation of Wnt/β-catenin pathway is a contributor to the development of malignancy, including CRC [31, 32]. Wnt/β-catenin signaling pathway is an oncogenic pathway that initiated by secreted glycoproteins Wnt1 and Wnt3a [33]. Wnt proteins bind to their receptors and lead to the release of β-catenin from the destruction complex. β-catenin translocates into the nucleus and interacts with transcription factors to induce the transcription of the downstream target genes [34, 35]. A recent research has shown that upregulation of BTG3 reduces the phosphorylation levels of GSK3β in osteosarcoma U2OS cells and thus decreases β-catenin. Conversely, BTG3 knockdown elevates the levels of p-GSK3β and nuclear β-catenin [36]. In our present study, we demonstrated that BTG3 overexpression decreased the levels of Wnt1, Wnt3a, active-β-catenin and p-GSK3β (Ser 9)/GSK3β. The results indicated that BTG3 overexpression might suppress the Wnt/β-catenin signaling pathway by activating GSK3β, a negative regulator of this signaling pathway.

To further investigate the involvement of Wnt/β-catenin signaling pathway, SB-216763 (a specific GSK3β inhibitor) was used to activate Wnt/β-catenin signaling pathway. We found that Wnt/β-catenin signaling pathway activation attenuated the effect of BTG3 overexpression on SW480 cell proliferation, cell cycle progression, invasion and EMT. Our results suggested that the Wnt/β-catenin pathway was involved in the antitumor effect of BTG3 in CRC.

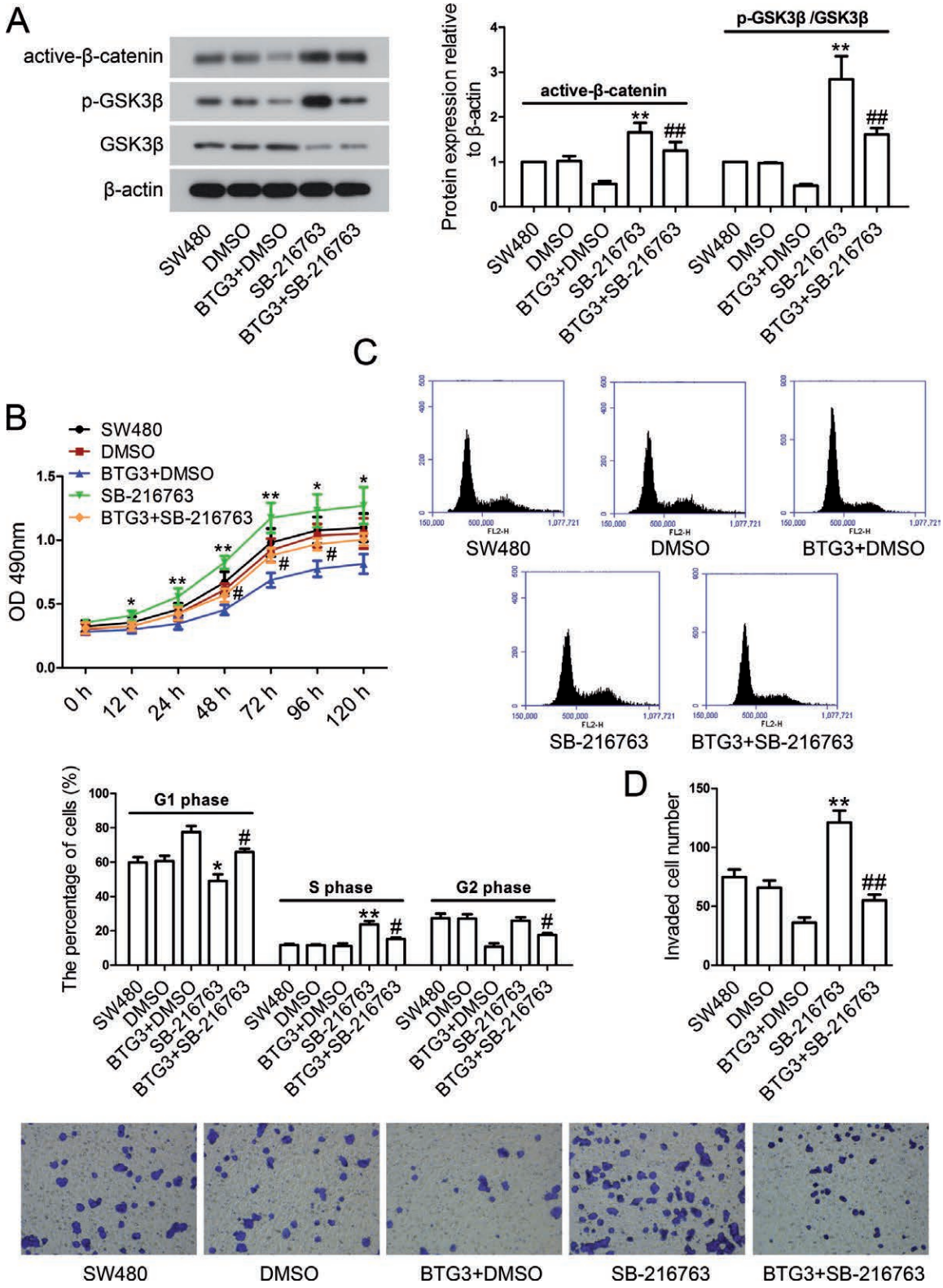

Figure 6. Effect of SB-216763 incubation on SW480 cell properties. (A) The levels of active-β-catenin, p-GSK3β and GSK3β were quantified by Western blotting. (B) MTT assay was performed to measure cell viability. (C) Flow cytometric analysis of cell cycle distribution. (D) Transwell invasion assay was performed to measure cell invasion ability. Data are expressed as Mean±SD. * *P*<0.05 and ** *P*<0.01 compared to DMSO. # *P*<0.05 and ## *P*<0.01 compared to BTG3+DMSO.

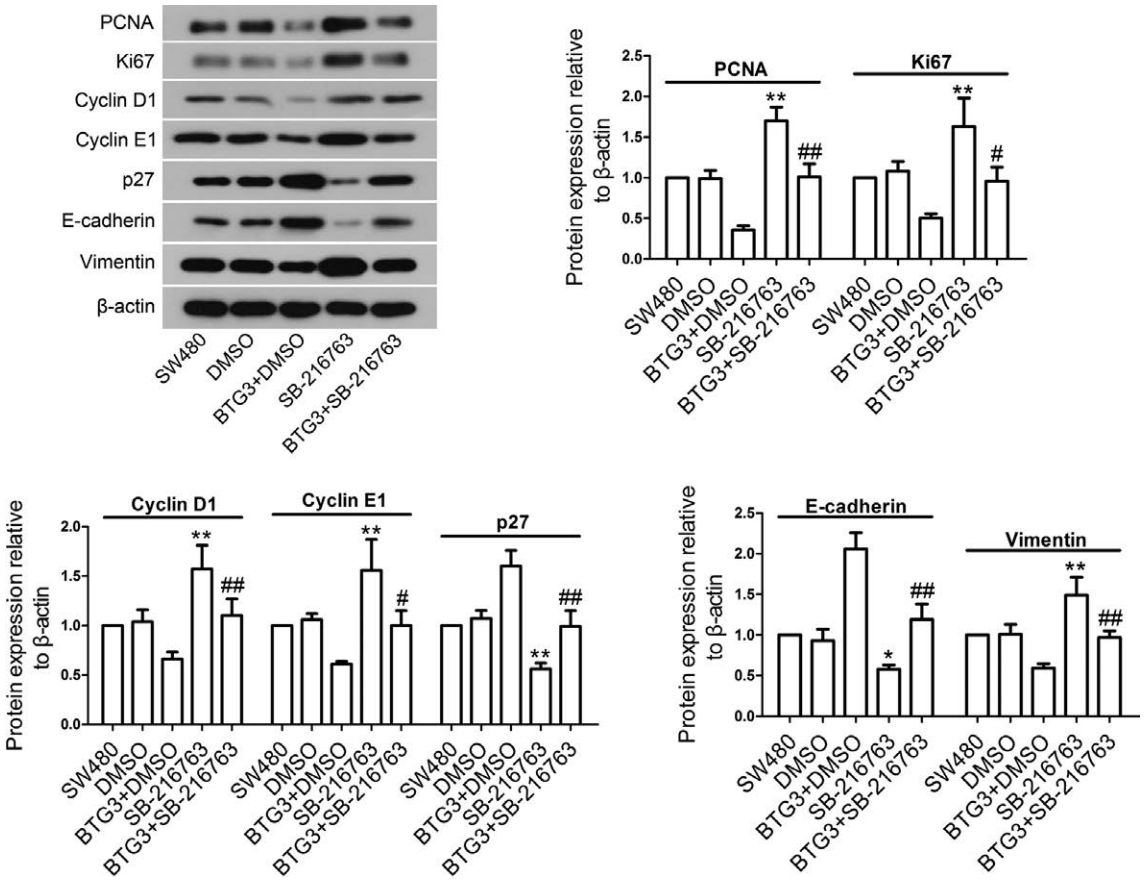

Figure 7. Effect of SB-216763 incubation on the expression of markers. The expression of PCNA, Ki67, Cyclin D1, Cyclin E1, p27, E-cadherin and Vimentin were determined by Western blotting. Data are expressed as Mean±SD. * *P*<0.05 and ** *P*<0.01 compared to DMSO. # *P*<0.05 and ## *P*<0.01 compared to BTG3+DMSO.

In conclusion, we demonstrated BTG3 overexpression inhibited SW480 cell proliferation, migration, invasion and EMT and induced cell cycle arrest at G1 phase via the Wnt/β-catenin signaling pathway. BTG3 may be a potential molecular target for the treatment of CRC.

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