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BTG3 Overexpression Suppresses the Proliferation and Invasion in Epithelial Ovarian Cancer Cell by Regulating AKT/GSK3β/β-Catenin Signaling

Qi An, MM[1], Yan Zhou, MM[2], Chao Han, MD[1], Yubing Zhou, PhD[1], Feng Li, MM[1], and Duolu Li, MD[1]

Abstract

Epithelial ovarian cancer (EOC) is the leading cause of cancer-related death among all the gynecological malignancies of the female genital system, and its incidence and mortality rates continue to rise. B-cell translocation gene 3 (BTG3) plays an important role in the occurrence and development of numerous cancers. However, the role of BTG3 in EOC remains poorly understood. In this study, we aimed to investigate the biological role and potential molecular mechanism of BTG3 in EOC. We found that BTG3 protein expression was significantly lower in human EOC cell lines. Next, BTG3 upregulation by transfection with pcDNA3.1-BTG inhibited cell proliferation and invasion but promoted cell apoptosis in 2 human EOC cell lines, SKOV-3 and HO-8910 cells. In addition, BTG3 knockdown by small interfering RNA promoted cell proliferation and invasion, but inhibited cell apoptosis in 2 human EOC cell lines, SKOV-3 and HO-8910 cells. Importantly, several proteins, including phosphorylation serine/threonine kinase (p-AKT), phosphorylated glycogen synthase kinase 3β (p-GSK3β), and β-catenin, were markedly decreased by BTG3 upregulation, whereas increased by BTG3 knockdown. Taken together, the results of our study suggest that BTG3 overexpression could inhibit cell proliferation and invasion and promotes cell apoptosis in EOC cell, possibly by regulating the AKT/GSK3β/β-catenin signaling pathway, providing novel insights into the treatment of EOC through BTG3 overexpression.

Keywords

BTG3, proliferation and invasion, AKT/GSK3β/β-catenin signaling, epithelial ovarian cancer

Introduction

Epithelial ovarian cancer (EOC) is the most common histological type and accounts for 80% to 90% of ovarian cancer.[1] The EOC is also the leading cause of cancer-related death among all the gynecological malignancies of the female genital system.[2] Due to the lack of effective methods of early diagnosis, approximately 70% of patients with EOC are diagnosed at a late stage.[3] Although surgical therapies and platinum-based chemotherapy drugs have improved ovarian cancer survival in recent decades, the 5-year survival rate is still only 30%.[4,5] Additionally, the detailed pathogenesis of EOC is still unclear. Thus, studying the molecular mechanisms of EOC and searching for promising novel treatments may have an impact on the development of EOC.

The protein encoded by B-cell translocation gene 3 (BTG3) is a member of the BTG gene family/transducer of human epidermal growth factor receptor 2 (ErbB2 or HER2) gene family (BTG/Tob), which also includes BTG1, BTG2/Mouse 12-O-tetradecanoyl phorbol-13-acetate inducible sequence 21 (TIS21)/proprotein convertases 3 (PC3), BTG3, BTG4/PC3B, Tob, and Tob2 in human cells.[6] All family members contain a conserved N-terminal domain spanning 104 to 106 amino acids and a variable C-terminal domain. Overexpression of these proteins not only suppresses cell proliferation but also regulates differentiation and cell cycle progression in a variety of cell types.[7] The BTG3 has been reported to be a tumor suppressor gene and is downexpressed in several malignancies such as ovarian carcinoma,[7] gastric cancer,[8] lung cancer,[9] esophageal adenocarcinoma,[10] and prostate cancer.[11] In addition, BTG3 functions as a barrier to tumor progression and is associated with regulating the AKT–glycogen synthase kinase 3β (GSK3β) signal pathway.[12] However, the mechanistic details as to how BTG3 exerts its function in human EOC are still unknown.

[1] Department of Clinical Pharmacology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China
[2] Department of Obstetrics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Corresponding Author:
Qi An, Department of Clinical Pharmacology, The First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe East Road, Zhengzhou, Henan 450052, China.
Email: qiansstt@163.com

Considering the important role of BTG3 in tumorigenesis and tumor progression, we investigated the biological functions of BTG3 in human EOC cell lines and explored its potential underlying mechanism. We found that the protein levels of BTG3 in human EOC cells were significantly decreased, as compared with human normal ovarian cells. Then, we demonstrated that BTG3 overexpression in 2 human EOC cell lines, HO-8910 and SKOV-3, promoted apoptosis, inhibited cell proliferation and invasion, possibly by regulating AKT/GSK3β/β-catenin signaling pathways. Taken together, the results of our study provide evidence that BTG3 is a novel and promising molecular target for human EOC therapy.

Materials and Methods

Cell Culture

Human EOC cell lines, including OVCAR-3, SKOV-3, and HO-8910, and a normal human ovarian cell line, IOSE80, were obtained from the cell resource center of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Other human EOC cell lines, OV-1063 and CAOV-4, were purchased from American Type Culture Collection (ATCC). All cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, Maryland), 100 U/mL penicillin, and 100 mg/mL streptomycin. These cell lines were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Plasmid Construction and Stable Cell Line Generation

The pcDNA3.1-BTG3 vector was constructed according to the previous reports. Briefly, the BTG3 complementary DNA was generated using sense 5′-ATGAAGAATGAAATTGCTG-CCGTTG-3′ and antisense 5′-GTGAGGTGCTAACATGT-GAGGATT-3′ from HO-8910 cells. Next, the expression sequences encoding BTG3 were subcloned into the pcDNA3.1 vector. In addition, the BTG3 small interfering RNA (siRNA) and small interfering negative control (siNC) were purchased from GenePharma (Shanghai, China). For transfection experiments, HO-8910 and SKOV-3 cells were plated into 6-well plates and incubated for 24 hours. Then, the cells were transfected with pcDNA3.1-BTG3 or BTG3 siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. Next, clones reexpressing BTG3 were selected by G418 for further study. As an NC, cells stably transfected with the empty pcDNA3.1 vector were also generated.

Cell Counting Kit-8 Assay

As described in detail previously,[13] cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) (Beyotime, Nantong, China). Briefly, cells were cultured into 96-well plates and then transfected with pcDNA3.1-BTG3 or BTG3 siRNA using Lipofectamine 2000 according to the manufacturer's instructions. After a 48-hour transfection, CCK-8 solution was added to each well and incubated for 3 hours, and the absorbance was read at 450 nm. This assay was performed in triplicate and repeated 3 times.

Flow Cytometric Analysis of Apoptosis

After culturing HO-8910 and SKOV-3 cells transfected with pcDNA3.1-BTG3 or BTG3 siRNA for 48 hours, apoptosis was detected by annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining (BD Biosciences, San Jose, California) according to the supplier's instructions. As described previously, cells were collected and washed with ice-cold phosphate-buffered saline. The binding buffer was added to resuspend cells, and then cells were stained with 10 μL of FITC-annexin V buffer at room temperature for 15 minutes. Next, 5 μL of PI was added and kept under dark incubation for another 5 minutes. Finally, cells were analyzed using a flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey). The percentage of cell numbers in each quadrant was calculated using Cellquest software (version 4.0.2).

Cell Invasion Assay

As described in detail previously,[14] Transwell inserts were used to assess the cell invasion ability of HO-8910 and SKOV-3 cells. Briefly, for the invasion assay, pcDNA3.1-BTG3- or BTG3-siRNA–transfected cells in serum-free RPMI-1640 were seeded in the upper chambers of Matrigel-coated Transwell plates. In the lower chamber, RPMI-1640 containing 10% FBS (600 μL) was added as a chemotactic factor. All of the Transwell chambers were then incubated at 37°C for 48 hours. Each subclone was seeded in the triplicate. The migrated cells were observed under a Leica inverted microscope (Deerfield, Illinois) and counted.

Protein Extraction and Western Blotting

Total proteins from treated and untreated HO-8910 and SKOV-3 cells were extracted using radioimmunoprecipitation (RIPA) lysis buffer containing phenylmethylsulfonyl fluoride. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Boster Biology Co, Wuhan, China). Equal amounts of protein were separated from sodium dodecyl sulphate (SDS)-polyacrylamide gels and electrotransferred to a polyvinylidene fluoride membrane. Membranes were blocked with 5% skim milk for 1 hour at room temperature and probed at 4°C overnight with primary antibodies: anti-BTG3, anti-AKT, anti-GSK3β, anti-β-catenin, anti-β-actin, anti-p-AKT, and anti-phosphorylated GSK 3β (p-GSK3β; Santa Cruz Biotechnology, Santa Cruz, California). The appropriate anti-mouse or anti-rabbit immunoglobulin G (IgG) horseradish peroxidase-linked secondary antibody (Cell Signaling Technology, Beverly, Massachusetts) was applied at room temperature for 1 hour. An electrogenerated chemiluminescence (ECL) detection method (Pierce Biotechnology, Rockford, Illinois) was subsequently used to visualize the protein band of interest.

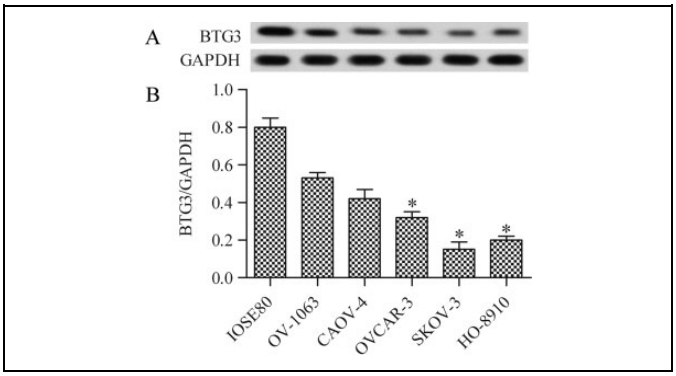

Figure 1. B-cell translocation gene 3 (BTG3) protein expression is decreased in human epithelial ovarian cancer (EOC) cell lines. A, The protein expression of BTG3 was analyzed by Western blotting. B, The relative protein expression of BTG3 was analyzed by Image J software. *$P < .05$ as compared with IOSE80.

Densitometric analysis was performed using Image J software (version 10.2, Bethesda, MD, USA).

Statistical Analysis

Data are summarized as mean (standard deviation) from at least 3 independent experiments. Statistical analysis was carried out using SPSS version 17.0 software. Statistical differences were processed using 1-way analysis of variance. A P value of $<.05$ was considered statistically significant.

Results

B-Cell Translocation Gene 3 Protein Expression is Decreased in Various Human EOC Cell Lines

To investigate the function of BTG3 in human EOC cell lines, we evaluated BTG3 expression in 5 EOC cell lines (OV-1063, CAOV-4, OVCAR-3, SKOV-3, and HO-8910) and a normal human ovarian cell line (IOSE80) by Western blotting. As shown in Figure 1A and B, BTG3 protein expression was downregulated in human epithelial ovarian cell lines, especially in HO-8910 and SKOV-3 cells, when compared with that of the normal human ovarian cell line. The results reveal that low BTG3 protein expression is clearly involved in human EOC development.

B-Cell Translocation Gene 3 Overexpression Inhibits the Proliferation of Human EOC Cell Lines

To investigate the potential function of BTG3 in suppressing cell proliferation, the BTG3 stable overexpression or knockdown cells was constructed in human EOC cell lines, HO-8910, and SKOV-3 (Figure 2A-D). Furthermore, cell proliferation of HO-8910 and SKOV-3 cells transfected with pcDNA3.1-BTG3 was markedly inhibited when compared to the corresponding pcNC group (the pcNC group: cells transfected with the empty vector pcDNA3.1). The BTG3 knockdown promoted cell proliferation when compared with siNC group (Figure 2E and F). These results suggest that BTG3 overexpression inhibits human epithelial ovarian cell proliferation.

B-Cell Translocation Gene 3 Overexpression in Human EOC Induces Cell Apoptosis

To further investigate the role of BTG3 in epithelial ovarian cells, we next assessed the effect of BTG3 overexpression on apoptosis in both HO-8910 and SKOV-3 cells. Annexin V/PI double staining showed that the apoptosis rate was significantly increased in HO-8910 and SKOV-3 cells transfected with pcDNA3.1-BTG3 when compared to wild-type (WT) and pcNC cells (Figure 3A-D) and decreased by BTG3 knockdown (Figure 3E-H). These results suggested that BTG3 overexpression promotes apoptosis in human EOC cells.

B-Cell Translocation Gene 3 Overexpression Affects Cell Invasion in Human EOC Cells

To further investigate the effect of BTG3 on cell invasion in human EOC cells, the Transwell invasion assay was performed on both HO-8910 and SKOV-3 cells. The results show that the number of invading cells was dramatically decreased in cells transfected with pcDNA3.1-BTG3 when compared with WT and NC cells and increased by BTG3 knockdown (Figure 4), suggesting that BTG3 upregulation significantly decreases the invasive ability of EOC cells.

B-Cell Translocation Gene 3 Overexpression Regulates the AKT/GSK3β/β-Catenin Signaling Pathway in Human EOC Cells

To explore the underlying molecular basis of BTG3 in regulating EOC cell proliferation, apoptosis, and invasion, the expression of several proteins, including AKT, GSK3β, and β-catenin, as well as the phosphorylation of AKT (p-AKT) and GSK3β (p-GSK3β) was examined by Western blotting. The results show that the protein expression levels of these markers were significantly decreased after transfection with pcDNA3.1-BTG3 in HO-8910 cells in comparison with WT and pcNC cells but increased by BTG3 knockdown (Figure 5A and C). Furthermore, similar data were obtained using SKOV-3 cells (Figure 5B and D). These results imply that BTG3 overexpression regulates the AKT/GSK3β/β-catenin signaling pathway in human EOC cells.

Discussion

In this study, we evaluated BTG3 protein expression in human EOC cell lines (OV-1063, CAOV-4, OVCAR-3, SKOV-3, and HO-8910) and a normal human ovarian cell line (IOSE80) using Western blot analysis. We found that BTG3 was significantly downexpressed in human EOC cells. Furthermore, BTG3 overexpression by pcDNA3.1-BTG3 suppressed cell proliferation and invasion of SKOV-3 and HO-8910 cells and promoted EOC cell apoptosis. Most importantly, our findings showed that the underlying mechanism may be associated with

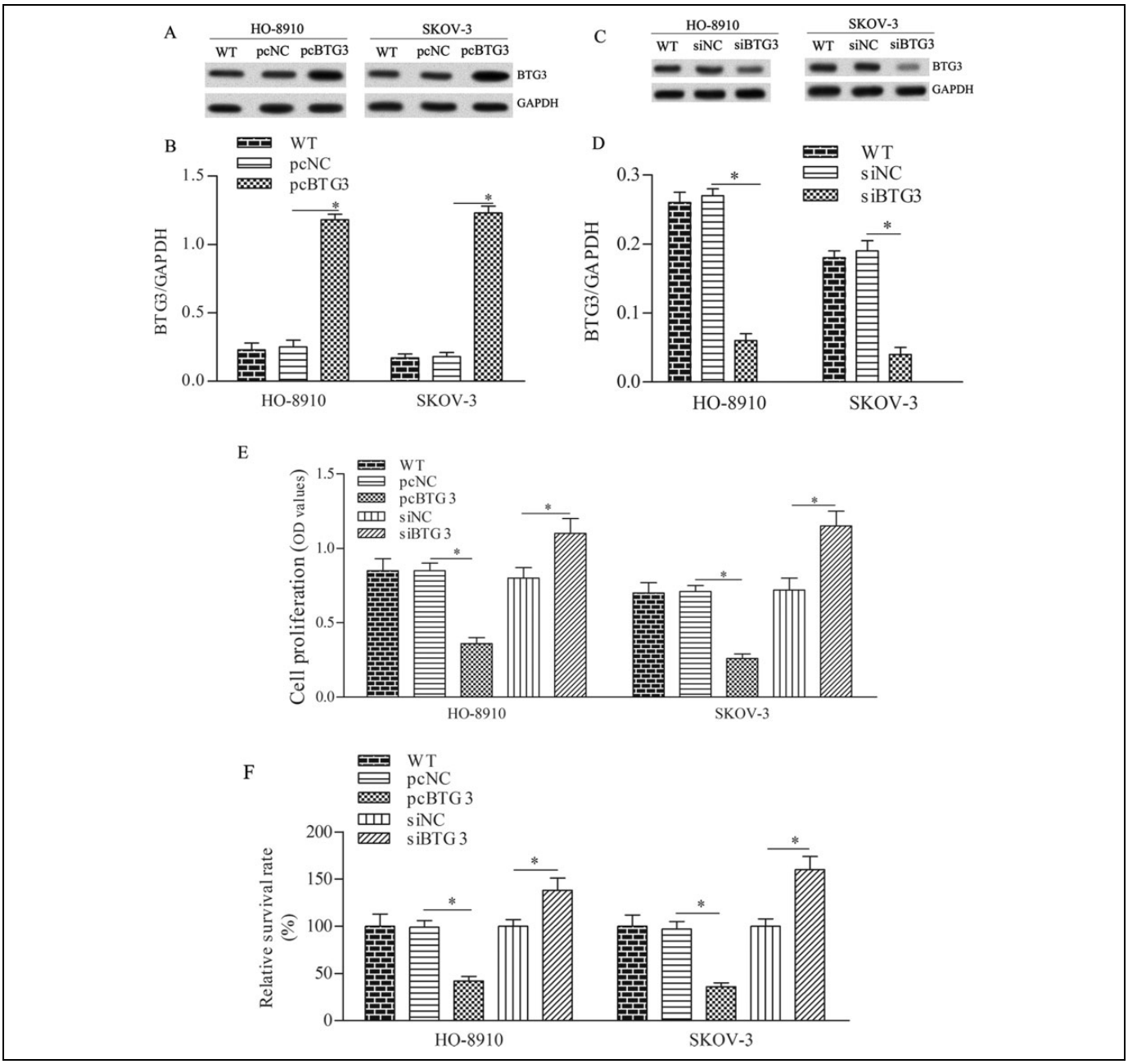

Figure 2. B-cell translocation gene 3 (BTG3) overexpression inhibits the proliferation in human epithelial ovarian cancer (EOC) cells. A and C, The protein expression level of BTG3 was analyzed by Western blotting. B and D, The relative protein expression of BTG3 was analyzed by Image J software. E and F, Cell proliferation was examined by the Cell Counting Kit-8 (CCK-8) assay. pcBTG3 indicates cells transfected with pcDNA3.1-BTG3; pcNC, cells transfected with the empty vector pcDNA3.1; siBTG3, cells transfected with BTG3 siRNA; siNC, cells transfected with siRNA negative control; siRNA, small interfering RNA; WT, wild-type cells. *$P < .05$.

regulating the AKT/GSK3β/β-catenin signaling pathway in human EOC cells.

Increasing evidence has shown that the mRNA and protein expression of BTG3 in gastric cancer tissues are downregulated compared to adjacent normal gastric tissue.[8] Moreover, in lung cancer, reverse transcription-polymerase chain reaction and Western blot were used to assess BTG3 expression, which is significantly diminished compared with that of nonmalignant lung tissues.[9] Also, in esophageal adenocarcinoma, BTG3 expression was downregulated at the mRNA and protein levels in vivo and in vitro.[10] Here, our findings indicate that BTG3 protein expression is also markedly downregulated in human EOC cell lines when compared with a normal epithelial ovarian cell line (Figure 1), which is consistent with the results obtained by Western blot and immunohistochemistry, showing that BTG3 expression is significantly lower in ovarian cancer tissues than normal ovarian tissue.[7]

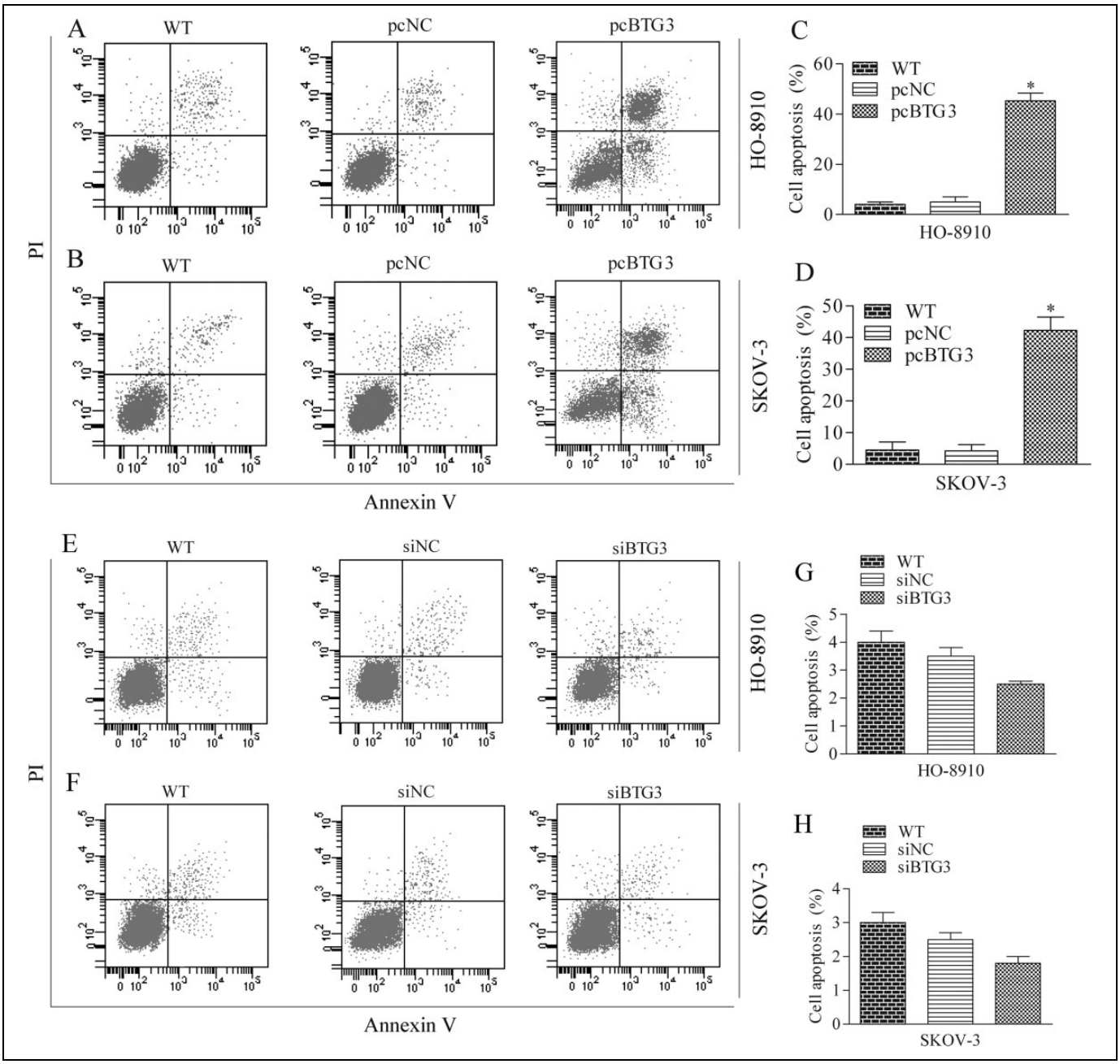

Figure 3. B-cell translocation gene 3 (BTG3) overexpression induces apoptosis. Apoptosis was detected using the Annexin V/PI assay in HO-8910 (A and E) and SKOV-3 (B and F) cells. Quantitative analysis of apoptosis in HO-8910 (C and G) and SKOV-3 (D and H) cells. **P* < .05 as compared with pcNC or siNC. PI indicates propidium iodide; siNC, small interfering negative control.

The BTG3 is known to be a member of the antiproliferative BTG/Tob gene family. The BTG3 can bind and inhibit E2F1, a transcription factor important for S-phase entry and thereby suppress cell cycle progression.[15] Recent evidence has shown that overexpression of BTG3 in gastric cancer inhibits cell proliferation and induces apoptosis in vitro and in vivo.[16] The BTG3 upregulation also induces apoptosis and suppresses colony formation and proliferation in esophageal adenocarcinoma.[10] The BTG3 downregulation in gastric cancer promotes cell proliferation.[8] In line with these reports, BTG3 overexpression in human EOC cells, as found in this study to significantly inhibit cell proliferation, and BTG3 knockdown promoted cell proliferation (Figure 2). Moreover, our findings show that BTG3 overexpression in SKOV-3 and HO-8910 cells significantly promoted apoptosis, but BTG3 knockdown inhibited cell apoptosis (Figure 3), which might have contributed to the inhibition of proliferation.

Invasion/metastasis is the main feature of cancer cells, which can acquire the ability to overcome cell–cell adhesion, breach the basement membrane barrier, and invade surrounding tissue and vessels.[17] Recently, BTG3 expression was reported to be related with the invasive capabilities of cancer

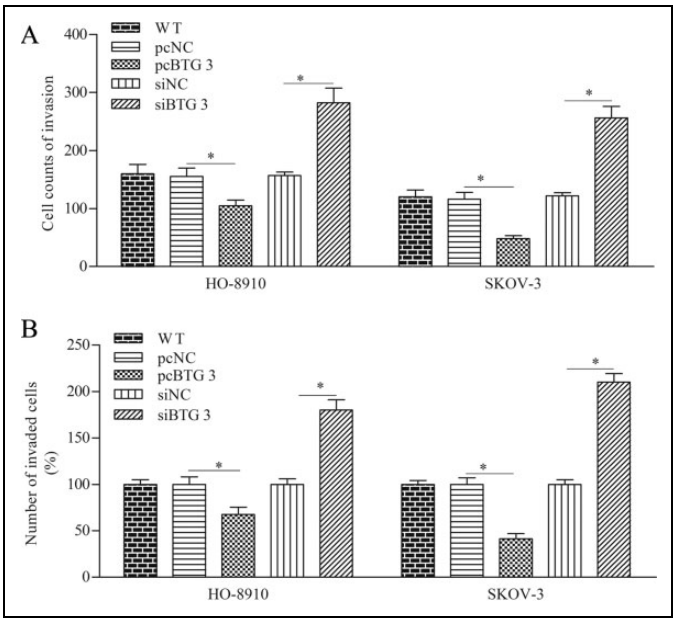

Figure 4. B-cell translocation gene 3 (BTG3) overexpression inhibits cell invasion in human epithelial ovarian cancer (EOC). Cell invasion was assessed using Matrigel-coated Transwell chambers (A and B). *$P < .05$.

cells such as esophageal carcinoma,[10] gastric cancer,[8] and lung cancer.[9] Consistent with these reports, the Transwell invasion assay performed in the present study showed that BTG3 overexpression can suppress the invasive ability of human EOC cells, but BTG3 knockdown promoted cell invasive (Figure 4).

To elucidate the potential molecular mechanisms involved in cell proliferation, apoptosis, and invasion by BTG3 overexpression in human EOC cells, we investigated the expression of several proteins, including AKT, GSK3β, and β-catenin, in SKOV-3 and HO-8910 cells transfected with pcDNA3.1-BTG3 or BTG3 siRNA. Akt, a serine/threonine kinase, includes Akt1 (protein kinase B α [PKBα]), Akt2 (PKBβ), and Akt3 (PKBγ) and is involved in the initiation of many signaling pathways in both normal and cancer cells.[18] The Akt activation by phosphorylation promotes cell proliferation, inhibits cell apoptosis, and increases tumor metastasis in various cancers.[19,20] In addition, GSK3β has been reported to be directly phosphorylated by PI3K/AKT signaling, which inhibits its activation.[21] The GSK3β, a multifunctional serine/threonine kinase, functions in diverse cellular processes including proliferation, differentiation, motility, and survival.[22] The GSK3β knockdown suppresses cell proliferation, promotes cell apoptosis, and inhibits cell motility in human nonsmall lung cancer cell.[23] Phosphorylation of GSK3β by Akt/PI3K inhibits its ability to phosphorylate and target β-catenin for degradation by the ubiquitin-proteasome pathway.[24,25] β-Catenin is a component of the E-cadherin/cell–cell adhesion complex and is an essential molecule in the wingless and INT-1 (Wnt)/β-catenin signal pathway regulating cell proliferation, apoptosis, and metastasis in EOC cells.[25] Recent evidence has shown that the AKT/GSK3β/β-catenin signaling pathway plays key roles in cell proliferation, apoptosis, and cell adhesion/invasion in ovarian cancer.[26] Consistent with a recent report showing that BTG3 overexpression can inhibit the AKT/GSK3β/β-catenin signaling pathway in osteosarcoma U2OS cells,[12] our findings show that BTG3 regulates the AKT/GSK3β/β-catenin signaling pathway in human EOC cells, suggesting that AKT/GSK3β/β-catenin signaling may be associated with cell proliferation, cell apoptosis, and invasion in EOC.

In summary, this report shows for the first time that BTG3 overexpression inhibits cell growth by reducing proliferation and inducing apoptosis and suppresses human EOC cell

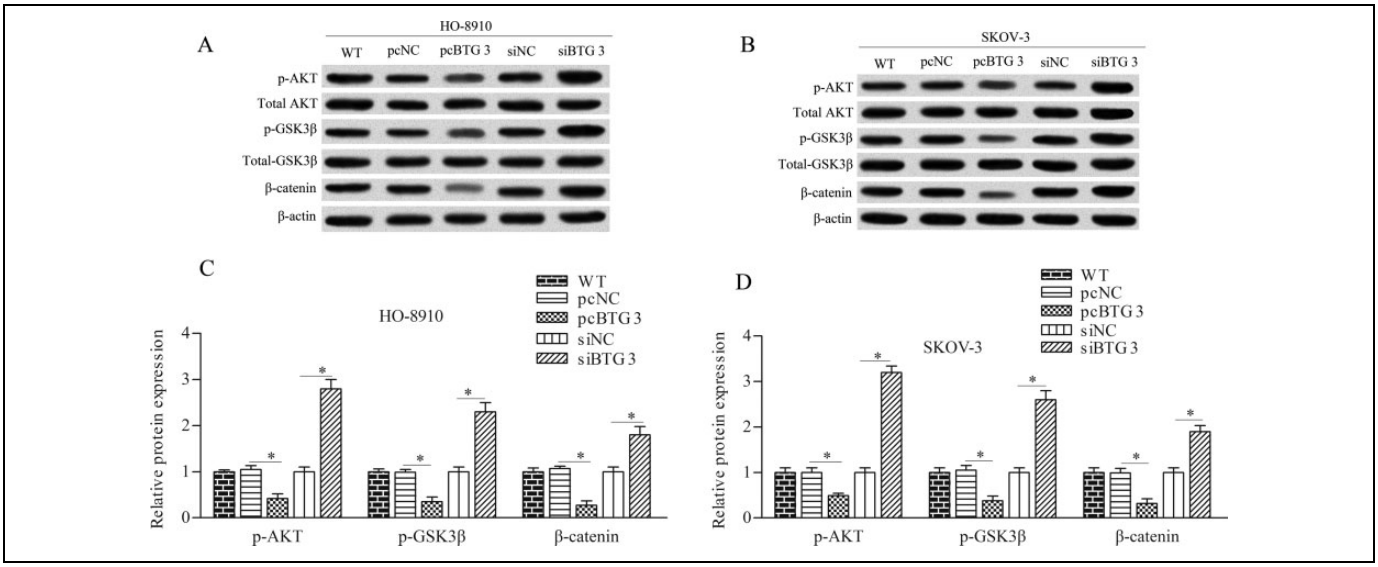

Figure 5. B-cell translocation gene 3 (BTG3) regulates the AKT/GSK3β/β-catenin signaling pathway in human epithelial ovarian cancer (EOC) cells. The protein expression levels of p-AKT, p-GSK3β, and β-catenin were examined by Western blot in HO-8910 (A) and SKOV-3 (B) cells. The relative protein expression of p-AKT, p-GSK3β, and β-catenin in HO-8910 (C) and SKOV-3 (D) cells was analyzed by Image J software. *$P < .05$. p-AKT indicates phosphorylated AKT; GSK3β, glycogen synthase kinase 3β.

invasion in vitro, which may be associated with regulation of the AKT/GSK3β/β-catenin signaling pathway by BTG3. Our results suggest that BTG3 is a promising therapeutic molecular target for the treatment of human EOC. Further studies are needed to clarify the molecular mechanisms of BTG3 activity in EOC in vivo.

Authors' Note

Qi An and Yan Zhou contributed equally to this work.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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