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**Running title:** Cucurbitacin B suppresses colorectal cancer progression

# Cucurbitacin B inhibits cell proliferation and induces cell apoptosis in colorectal cancer by modulating methylation status of BTG3

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A previous report has revealed that cucurbitacin B (CuB) inhibits cancer cell proliferation and tumorigenesis in non-small cell lung cancer (NSCLC) through epigenetic modifications of several genes. However, whether CuB regulates cell proliferation and apoptosis by altering methylation status of BTG3 in colorectal cancer (CRC) remains unknown. In the present study, the results showed that BTG3 was downregulated in CRC tissues compared with adjacent normal tissues. CuB significantly increased BTG3 levels, induced promoter demethylation, and decreased the levels of DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) in both CRC cell lines (SW480 and Caco-2), and the effects of CuB were comparable with those of 5-Aza-dC. We also found that CuB inhibited cell proliferation, accompanied with decreased expression of Ki67. Furthermore, CuB treatment induced cell cycle arrest at G1 phase in SW480 and Caco-2 cells, as well as decreased levels of Cyclin D1 and Cyclin E1. Incubation with CuB promoted cell apoptosis in both CRC cell lines *in vitro*, accompanied with elevation of cleaved caspase-3 and cleaved PARP. BTG3 knockdown abolished the effects of CuB in CRC cells. In summary, CuB-induced proliferation inhibition and cell apoptosis may be due to the reactivation of BTG3 by promoter demethylation. CuB may be a promising agent for CRC therapy.

**Key words:** Cucurbitacin B, BTG3, colorectal cancer, 5-Aza-dC, promoter methylation

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in females and the third in males, with an estimated 1,360,600 new cases diagnosed in 2012 and 693,900 deaths [1]. Even after surgery, chemotherapy or radiotherapy, the 5-year survival rate of patients with stage I, II, III or IV CRC is approximately 93, 83, 60, or 8%, respectively [2]. More effective therapeutic strategies are needed for CRC treatment.

Epigenetics is defined as heritable changes in gene expression without alteration of DNA sequences [3]. DNA methylation is one of the epigenetic modifications in mammalian cells [4]. CpG islands in

the promoters of tumor-suppressor genes are usually unmethylated in normal cells, and hypermethylation of CpG islands is commonly correlated with malignancies [4]. B-cell translocation gene 3 (BTG3) has been reported to be a tumor suppressor in cancers [5]. In addition, BTG3 is downregulated in diverse human cancers, including renal carcinoma, non-small cell lung cancer (NSCLC), cervical cancer, gastric cancer, and esophageal adenocarcinoma [6-10], as well as in CRC [5]. The mechanism of BTG3 downregulation in tumor tissues may be associated with promoter hypermethylation [6, 11, 12], and this hypothesis need to be further verified.

Cucurbitacins, isolated from plants of the families *Cucurbitaceae* and *Cruciferae*, are a group of tetracyclic triterpenoids possessing anti-inflammatory, anti-cancer, and anti-diabetic activities [13, 14]. Cucurbitacin B (CuB), one of the most abundant members of cucurbitacins, has been reported to inhibit growth of numerous cancer cells [13]. Yar Saglam *et al.* have shown that treatment with CuB alone or in combination with gefitinib results in cell proliferation inhibition and apoptosis in CRC cells [15]. In addition, Shukla *et al.* have revealed that CuB inhibits NSCLC cell proliferation and tumorigenesis through modulating the expression of tumor-related gene by epigenetic modifications [16]. However, whether CuB regulates CRC cell proliferation and apoptosis by inducing promoter demethylation of BTG3 remains unknown.

In the present study, we firstly examined BTG3 expression levels in CRC tissues and adjacent normal tissues, and then tested the hypothesis that CuB can induce expression of BTG3 in CRC by DNA demethylation. Next, we investigated whether CuB affects cell proliferation, cell cycle distribution, and apoptosis via BTG3 in CRC.

## Materials and methods

**Tissue samples and cell culture.** Tissue samples were obtained from patients at The First Affiliated Hospital of Jinzhou Medical University. Written consent was obtained from all patients, and the protocols were approved by the Ethics Committee of Jinzhou Medical University. Tissue samples were immediately frozen in liquid nitrogen post-surgery and stored at -80 °C.

SW480, Caco-2, HCT116, and Colo205 cells were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co.,Ltd. (Shanghai, China). SW480 cells were cultured in L-15 medium (Zhong Qiao Xin Zhou Biotechnology) containing 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit-Haemek, Israel). Caco-2 and Colo205 cells were maintained in RPMI 1640 medium (Hyclone, Logan, Utah, USA) supplemented with 10% FBS. HCT116 cells were cultured in DMEM

(Hyclone) with 10% FBS (Biological Industries). The cells were cultured at 37 °C in a 5% $CO_2$ incubator.

**Cell Counting Kit-8 (CCK-8) assay.** Cells were seeded at 3000 cells/well in 96-well plates and then treated with increasing concentrations of CuB (0.1, 0.5, 1, 5, 10, 25, or 50 μM) (Selleck Chemicals, Houston, USA) for 48 h. After discarding the cell supernatant, culture medium containing 10 μl CCK-8 solutions (Sigma, St. Louis, USA) was added into each well and then the plates were placed at 37 °C for 1 h. The optical density at 450 nm was determined and the inhibition rate was calculated. The proper concentration of CuB was chosen for further studies.

For cell proliferation assay, the cells were transfected with siNC or siBTG3 (GenePharm, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction and then the siRNA-transfected cells were seeded in 96-well plates 24 h post-transfection. After being cultured for 0-72 h, the cells were incubated with CCK-8 solutions and then the optical density was measured at 450 nm.

**Cell transfection.** Cells were seeded at $4\times10^5$ cells/plates in 6-well plates and cultured in a 5% $CO_2$ incubator. After 1 h of serum starvation, the cells were transfected with 100 pm siNC or siBTG3 (GenePharm) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours post-transfection, the cells were harvested and then treated with 5 μM CuB (Selleck Chemicals) for different time (0-72 h) prior to further studies.

**Western blot.** Total proteins were extracted from tissue samples or CRC cells using RIPA lysis buffer (Beyotime, Haimen, China). Bicinchoninic acid (BCA) Assay Kit (Beyotime) was used to measure protein concentration and an equal amount of proteins (40 μg) were separated by SDS-PAGE prior to transfer to PVDF membranes (Millipore, Bedford, USA). Then, the membranes were blocked with nonfat milk (Yili Group, Hohhot, China), and then incubated with primary antibody against BTG3 (1:500 dilution) (Sangon, Shanghai, China), DNMT1 (1:1000 dilution), DNMT3a (1:1000 dilution), DNMT3b (1:500 dilution) (BIOSS, Beijing, China), Cyclin D1 (1:1000 dilution), Cyclin E1 (1:1000 dilution), cleaved caspase-3 (1:1000 dilution), and cleaved PARP (1:1000 dilution) (Cell Signaling Technology, Beverly, USA) at 4 °C overnight. Afterwards, the membranes were incubated with horseradish peroxidase (HRP)-labeled IgG (1:5000 dilution) (Beyotime) at 37 °C for 45 min. Protein bands were detected with ECL reagent (Beyotime) and then quantified by Gel-Pro Analyzer (Media Cybernetics, Bethesda, USA).

**Real-time PCR.** Total RNAs were extracted from CRC cells using TRIpure reagent (BioTeke, Beijing, China), followed by determination of RNA concentration. Then, RNAs were reverse-transcribed into cDNAs and real-time PCR was performed using Exicycler$^{TM}$ 96 Real-Time Quantitative Thermal Block (Bioneer, Daejeon, Korea). The primer sequences are as follows: BTG3-forward, 5'-ATGAAATTGCTGCCGTTGTCT-3' and BTG3-reverse, 5'-GCCTGTCCTTTCGATGGTTTT-3'; β-actin-forward, 5'-CTTAGTTGCGTTACACCCTTTCTTG-3' and β-actin-reverse, 5'-CTGTCACCTTCACCGTTCCAGTTT-3'.

**Bisulfite-sequencing PCR (BSP).** Genomic DNA was isolated from CRC cells using Genomic DNA Extraction Kit (BioTeke Corporation, Beijing, China) and then subjected to bisulfite conversion using EZ DNA Methylation-Gold Kit (ZYMO Research, Tustin, USA) according to the manufacturer's instructions. The bisulfite-converted genomic DNA was subjected to PCR, and then the PCR products were separated by 1.5% agarose gel electrophoresis and purified using DNA Gel Extraction Kit (BioTeke Corporation). The sequences of primers used are as follows: BTG3-forward, 5'-GTTTAAAATATAGTAGGG-3' and BTG3-reverse, 5'-CCCTACCCTAAACCTAAC-3'. The purified PCR products were cloned into pGEM-T Easy Vector (Promega Corporation, Madison, USA) and five clones were randomly chosen for bisulfite sequencing.

**Immunofluorescence.** The slides were fixed in 4% paraformaldehyde, washed three times with PBS, and treated with 0.1% Triton X-100 for 30 min. After being washed, the slides were blocked with goat serum, and then incubated with primary antibody against Ki67 (1:100 dilution) (Proteintech, Wuhan, China) at 4 °C overnight. Afterwards, the slides were incubated with Cy3-labelled secondary antibody (1:200 dilution) (Beyotime) at room temperature for 1 h. Nuclei were counterstained with DAPI (Beyotime) and subsequently the slides were photographed under a fluorescent microscope (Olympus, Tokyo, Japan).

**Cell cycle distribution.** Cells were harvested by centrifugation, washed twice with PBS, and fixed in 70% ethanol at 4 °C for 12 h. The fixed cells were resuspended, and then stained with 25 μl Propidium Iodide (PI) (Beyotime) and 10 μl RNase A (Beyotime) at 37 °C for 30 min in the dark. Cell cycle distribution was analyzed by flow cytometry.

**Cell apoptosis.** Cell apoptosis was examined using Annexin V-FITC Apoptosis Detection Kit (Beyotime) according to the manufacturer's instructions. Briefly, the cells were washed with PBS

and resuspended in binding buffer. Then, 5 μl Annexin V-FITC and 10 μl PI were added to stain the cells for 15 min in the dark before flow cytomeric analysis.

**Statistical analysis.** Data are expressed as mean ± SD. One-way ANOVA followed by Tukey *post-hoc* test or Student's *t* test was used for statistical analysis. $P<0.05$ was considered to be statistically significant.

## Results

**BTG3 is downregulated in human CRC tissues.** To determine BTG3 expression in 20 paired of human CRC tissues and adjacent normal colorectal tissues, western blot was performed. The results showed that BTG3 is markedly downregulated in CRC tissues compared with adjacent normal tissues (Figure 1A).

**Selection of cell lines and CuB concentration.** BTG3 expression levels in four CRC cell lines were determined by western blot. We found that SW480 and Caco-2 cells expressed lower levels of BTG3 than HCT116 and Colo205 cells (Figure 1B). Thus, SW480 and Caco-2 cells were chosen for further studies.

CCK-8 assay was performed to choose the proper concentration of CuB. The results showed that CuB significantly reduced cell viability in a dose-dependent manner in both SW480 and Caco-2 cells (Figure 2). In the present study, 5 μM of CuB was chosen.

**CuB reactivates BTG3 by promoter demethylation of BTG3 in CRC cells.** To investigate the effect of CuB on the expression levels of BTG3, CRC cells were incubated with CuB for 48 h and then subjected to real-time PCR and western blot. 5-Aza-dC was used as a positive control. The results showed that CuB and 5-Aza-dC significantly elevated the mRNA and protein levels of BTG3 in both SW480 and Caco-2 cells compared with the vehicle control (Figure 3A). Next, we investigated whether this elevation of BTG3 expression is due to the promoter demethylation in CRC cells. The results showed that BTG3 promoter was hypermethylated in the vehicle (DMSO)-treated cells (Figure 3B), as determined by bisulfite-sequencing PCR (BSP). CuB or 5-Aza-dC treatment significantly induced promoter demethylation of BTG3 in both SW480 and Caco-2 cells. We then determined the levels of three key DNA methyltransferases by western blot. The results showed that the levels of DNMT1, DNMT3a, and DNMT3b were markedly reduced in CuB- or 5-Aza-dC-treated cells compared with those in the vehicle control cells (Figure 4).

**CuB inhibits cell proliferation in CRC cells in vitro**. To explore whether BTG3 is involved in CuB-induced modulation of CRC progression, BTG3 was knocked down in CRC cells and then incubated with 5 μM CuB. We found that CuB treatment led to elevation of BTG3 expression at both mRNA and protein levels. However, siBTG3 transfection attenuated CuB-induced increases in BTG3 levels (Figure 5A). Cell proliferation was examined by CCK-8 assay. The results showed that CuB significantly inhibited cell proliferation in both SW480 and Caco-2 cells *in vitro*, which was reversed by BTG3 knockdown (Figure 5B). Ki67 expression was measured by immunofluorescence. We found that CuB treatment decreased Ki67 expression in both SW480 and Caco-2 cells; whereas, BTG3 knockdown blocked the effects of CuB (Figure 5C).

**CuB arrests the cell cycle at G1 phase in CRC cells in vitro.** Cell cycle distribution was analyzed by flow cytometry. The results showed that CuB caused cell cycle arrest at G1 phase in both SW480 and Caco-2 cells, which was reversed by siBTG3 transfection (Figure 6A). Cyclin D1 and Cyclin E1 levels were determined by western blot. The results showed that CuB incubation significantly decreased Cyclin D1 and Cyclin E1 levels in both SW480 and Caco-2 cells. Knockdown of BTG3 restored the levels of Cyclin D1 and Cyclin E1 even in the present of CuB (Figure 6B).

**CuB induces cell apoptosis in CRC cells in vitro.** Cell apoptosis was analyzed by flow cytometry. The results showed that CuB significantly induced cell apoptosis in both SW480 and Caco-2 cells *in vitro*. BTG3 knockdown inhibited the promoting effect of CuB on cell apoptosis (Figure 7A). Next, the levels of cleaved capase-3 and cleaved PARP were determined by western blot. We found that SW480 and Caco-2 cells treated with CuB expressed higher levels of cleaved capase-3 and cleaved PARP compared with the vehicle control cells. BTG3 knockdown reversed the elevated levels of cleaved capase-3 and cleaved PARP induced by CuB treatment (Figure 7B).

## Discussion

DNA methylation is an important mechanism for gene silencing, including tumor suppressor genes, inhibitors of oncogenes, DNA repair genes, and cell cycle regulators [17, 18]. A previous study has revealed that CuB suppresses cell proliferation and induces cell apoptosis in NSCLC by epigenetically regulating the expression of tumor-related genes [16]. This is the first report to investigate whether CuB reactivates BTG3 through DNA demethylation and then affects cell proliferation, cell cycle distribution, and cell apoptosis in CRC.

In the present study, we demonstrated that BTG was significantly downregulated in 20 paired of human CRC tissues compared with adjacent normal tissues, which are consistent with previous observations [5, 9]. In addition, aberrant hypermethylation status of BTG3 promoter has been reported in several human cancers [6, 11, 19]. Therefore, we hypothesized that promoter methylation in the CpG island of BTG3 may contribute to BTG3 silencing in CRC tissues. Although there are other mechanisms that mediate BTG3 suppression, *eg.,* miR-20a-5p and miR-106b-5p [7, 8], promoter methylation is still the frequent mechanism of tumor suppressor gene (TSG) silencing in cancers. 5-Aza-dC, an inhibitor of DNA methyltransferase [20], was used as a positive control in our study. We found that both CuB and 5-Aza-dC increased BTG3 expression in both CRC cells, as evidenced by real-time PCR and western blot. The methylation status of BTG3 promoter region was then determined by bisulfite-sequencing PCR (BSP). The results showed that CuB and 5-Aza-dC induced demethylation of BTG3 promoter in both SW480 and Caco-2 cells. In mammals, DNA methylation is catalyzed by three key enzymes, namely DNMT1, DNMT3a, and DNMT3b [17]. Decreased levels of DNMT1, DNMT3a, and DNMT3b were observed in CuB- or 5-Aza-dC-treated cells compared with the vehicle control. The results suggest that CuB may reactivate BTG3 expression by promoter demethylation.

Cell proliferation plays an important role in cancer development and progression [21]. Increasing evidences have reported that CuB treatment suppresses cell proliferation in various cancers, including lung cancer, breast cancer, glioblastoma, and cutaneous squamous cell carcinoma [14, 22-24]. Consistently, treatment with CuB significantly inhibited proliferation of SW480 and Caco-2 cells *in vitro*, as determined by CCK-8 assay. Ki67, one of the widely used markers of cell proliferation, is strongly expressed in proliferating cells, but not in nondividing cells [25, 26]. Ki67 expression was analyzed by immunofluorescence to assess cell proliferation. The results showed that CuB treatment significantly decreased Ki67 expression levels in both SW480 and Caco-2 cells compared with the vehicle control. The data indicated that CuB has an anti-proliferative effect on CRC cells *in vitro*.

Cancer is characterized by uncontrolled cell proliferation caused by abnormal activation of cell cycle-related proteins [27]. Cyclin D1 regulates G1/S-phase transition through forming complex with Cdk4 and Cdk6 [28, 29]. Cyclin E1, expressed in late G1 and S phase of the cell cycle, also promotes cell cycle progression from G1 phase into S phase [30]. Then, we evaluated whether cell proliferation inhibition induced by CuB is due to aberrant expression of cell cycle-related proteins.

The results showed that treatment with CuB led to decreases in Cyclin D1 and Cyclin E1 levels in CRC cells, suggesting that CuB may inhibit cell proliferation via regulation of cell cycle progression.

Apoptosis (also called programmed cell death), an important form of cell death, has been reported to be dysregulated in human cancers [31]. In the present study, flow cytometric analysis revealed that CuB greatly induced SW480 and Caco-2 cell apoptosis *in vitro* and the pro-apoptotic effect of CuB was previously observed in other cancer cells [13, 32, 33]. Caspases are a family of cysteine proteases functioning in cell apoptosis [34]. Both extrinsic and intrinsic apoptosis pathways can activate the executioner caspase-3, followed by cleavage of downstream substrates to amplify the apoptotic signal [35]. Poly(ADP-ribose) polymerase (PARP) is a major substrate of caspase-3 and can be cleaved into 89- and 24-kDa fragments by activated caspase-3 during apoptosis [36]. We found that CuB significantly increased cleaved caspase-3 and cleaved PARP in both SW480 and Caco-2 cells, suggesting that CuB may induce CRC cell apoptosis through regulating the activation of caspase-3 and PARP.

Our previous study and other reports have demonstrated that BTG3 overexpression suppresses cell proliferation and promotes apoptosis in CRC, epithelial ovarian cancer, and esophageal adenocarcinoma [37-40]. These evidences demonstrate that BTG3 exerts anti-proliferative and pro-apoptotic effects in CRC and other human cancers. To further verify the involvement of BTG3 in CuB-induced proliferation inhibition and apoptosis induction, both cell lines were transfected with siBTG3 or siNC and then treated with CuB prior to analyses. The results showed that BTG3 knockdown rescued the effects of CuB on cell proliferation, cell cycle distribution, and cell apoptosis, suggesting the involvement of BTG3.

In conclusion, promoter methylation might contribute to epigenetic silencing of BTG3 in CRC. Furthermore, CuB, which possessed similar effects to 5-Aza-dC, inhibited cell proliferation, arrested the cell cycle at G1 phase, and induced cell apoptosis through promoter demethylation of BTG3 in CRC.

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## Figure Legends

**Figure 1.** BTG3 expression in colorectal cancer (CRC) tissues and four CRC cell lines. A) The levels of BTG3 in 20 pairs of CRC tissues (T) and adjacent normal tissues (N) were determined by western blot. B) BTG3 expression levels in four CRC cell lines (SW480, Caco-2, HCT116 and Colo205) were examined by western blot. β-actin was used as internal control. Results are expressed as Mean ± SD. ** indicates $P<0.01$.

**Figure 2.** Selection of proper CuB concentration. SW480 and Caco-2 cells were incubated with increasing doses of CuB (0.1, 0.5, 1, 5, 10, 25, and 50 μM) for 48 h. Inhibition rate was measured by CCK-8 assay. Results are expressed as Mean ± SD. * indicates $P<0.05$ and ** indicates $P<0.01$ compared with the DMSO group.

**Figure 3.** Effect of CuB and 5-Aza-dC on the expression and methylation status of BTG3. The cells were treated with CuB (5 μM) or 5-Aza-dC (5 μM) and then subjected to further analyses. A) BTG3 expression was determined by real-time PCR and western blot. β-actin was used as internal control. B) Bisulfite-sequencing PCR (BSP) analysis of BTG3 promoter in SW480 and Caco-2 cells. Results are expressed as Mean ± SD. * indicates $P<0.05$ and ** indicates $P<0.01$ compared with the DMSO group.

**Figure 4.** Effect of CuB and 5-Aza-dC on the levels of DNA methyltransferases. After incubation with CuB or 5-Aza-dC, the levels of DNMT1, DNMT3a and DNMT3b were determined by western blot. β-actin was used as internal control. Results are expressed as Mean ± SD. * indicates $P<0.05$ and ** indicates $P<0.01$ compared with the DMSO group.

**Figure 5.** Effect of CuB on cell proliferation in CRC cells *in vitro*. SW480 and Caco-2 cells were transfected with siBTG3 or siNC and then incubated with 5 μM CuB for different time 24 h post-transfection. A) After 48 h of incubation with CuB, real-time PCR and western blot were performed to determine BTG3 expression. β-actin was used as internal control. B) Cell proliferation was determined by CCK-8 assay (0, 24, 48, and 72 h). C) Ki67 expression was analyzed by immunofluorescence. Scale bar represents 50 μm. Results are expressed as Mean ± SD. * indicates

$P<0.05$ and ** indicates $P<0.01$ compared to the DMSO group. # indicates $P<0.05$ and ## indicates $P<0.01$ compared with the CuB+siNC group.

**Figure 6.** Effect of CuB on cell cycle distribution in CRC cells *in vitro*. SW480 and Caco-2 cells transfected with siBTG3 or siNC were treated with 5 μM CuB for 48 h prior to further analyses. A) Cell cycle distribution was analyzed by flow cytometry. B) The levels of Cyclin D1 and Cyclin E1 were determined by western blot. β-actin was used as internal control. Results are expressed as Mean ± SD. * indicates $P<0.05$ and ** indicates $P<0.01$ compared to the DMSO group. # indicates $P<0.05$ and ## indicates $P<0.01$ compared with the CuB+siNC group.

**Figure 7.** Effect of CuB on cell apoptosis in CRC cells *in vitro*. The siRNA-transfected cells were incubated with CuB for 24 h and then subjected to further analyses. A) Flow cytometric analysis of cell apoptosis. B) The levels of cleaved caspase-3 and cleaved PARP were determined by western blot. β-actin was used as internal control. Results are expressed as Mean ± SD. * indicates $P<0.05$ and ** indicates $P<0.01$ compared to the DMSO group. # indicates $P<0.05$ and ## indicates $P<0.01$ compared with the CuB+siNC group.

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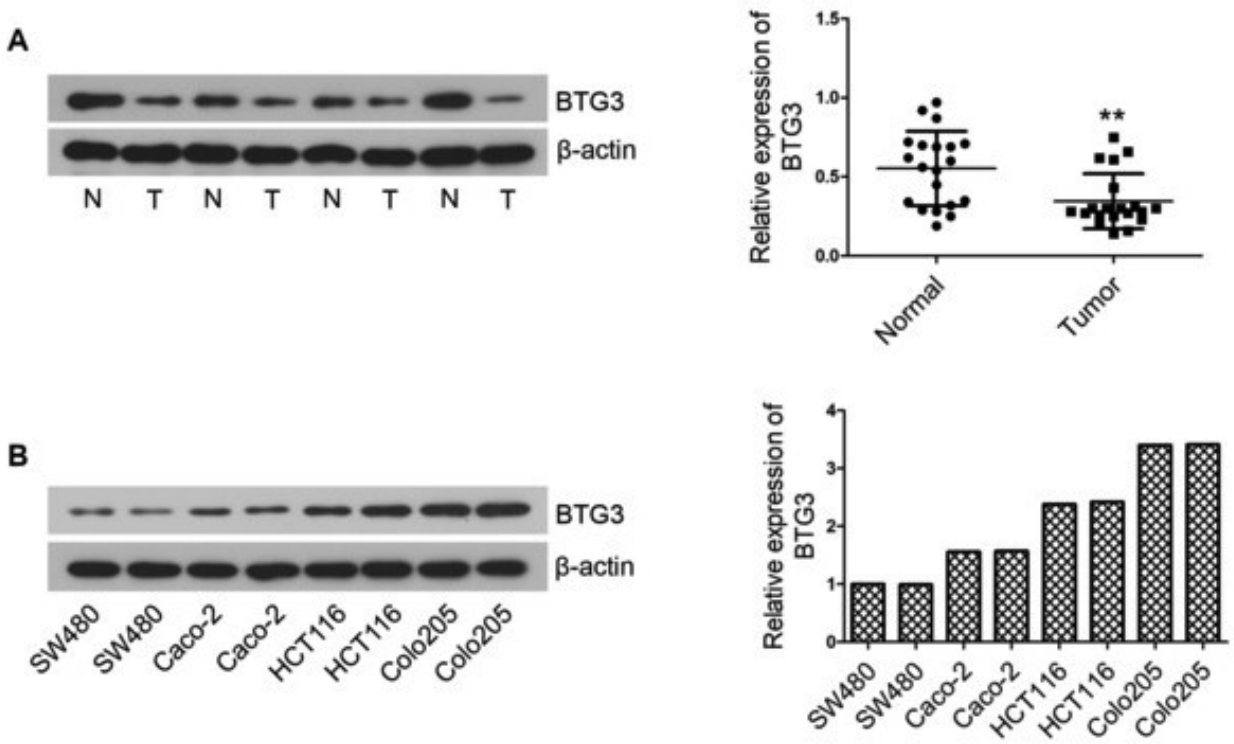

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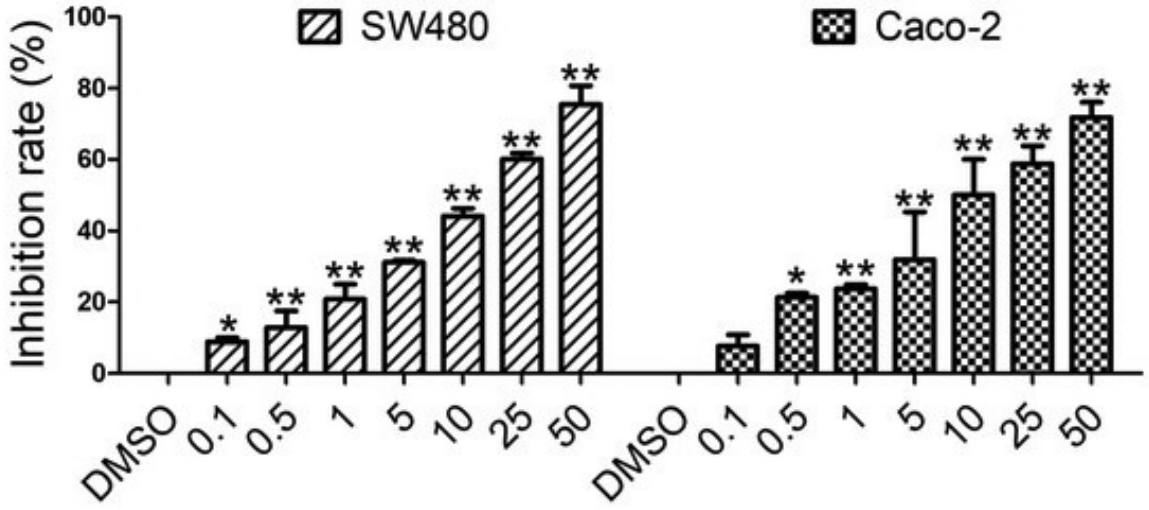

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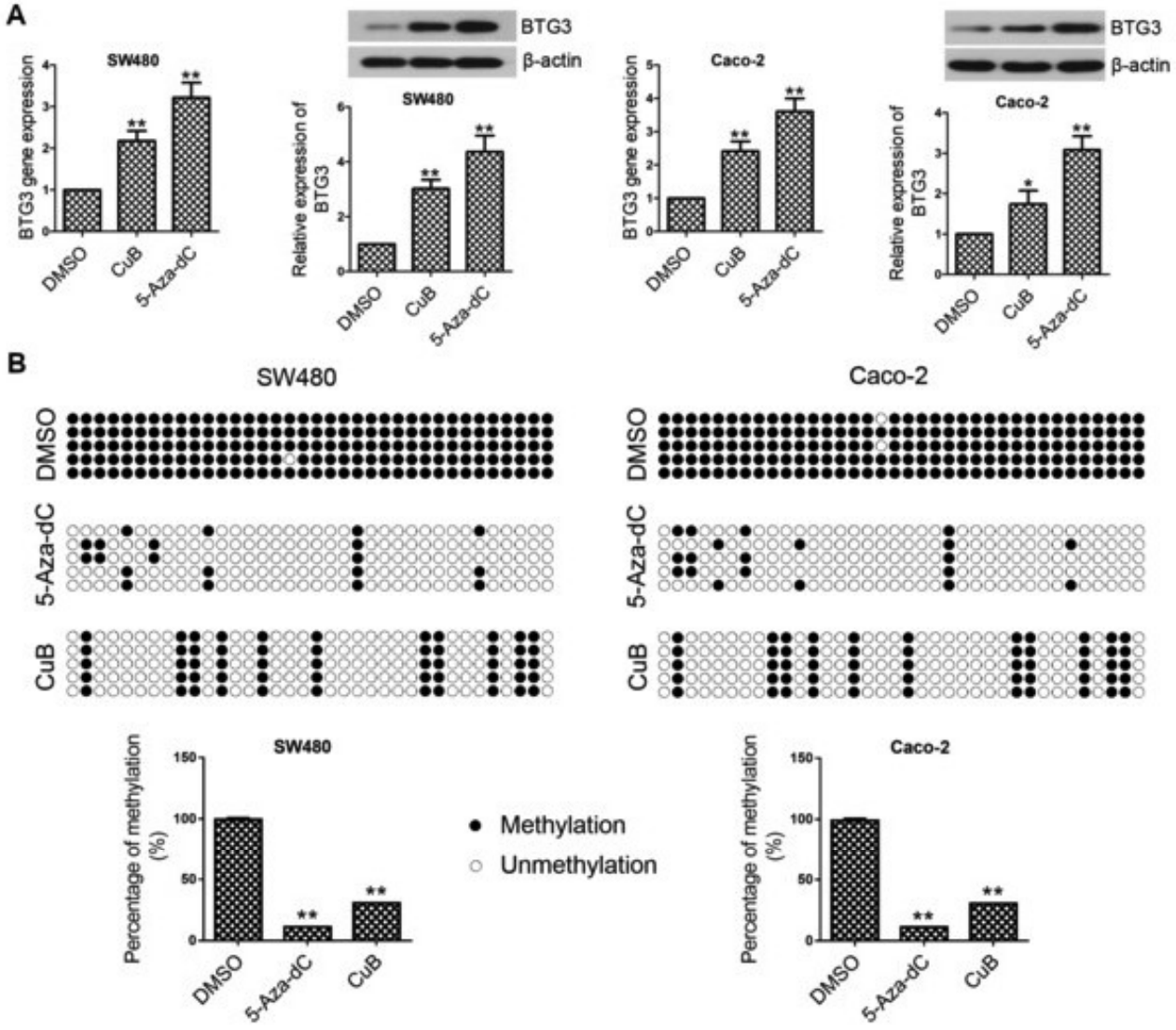

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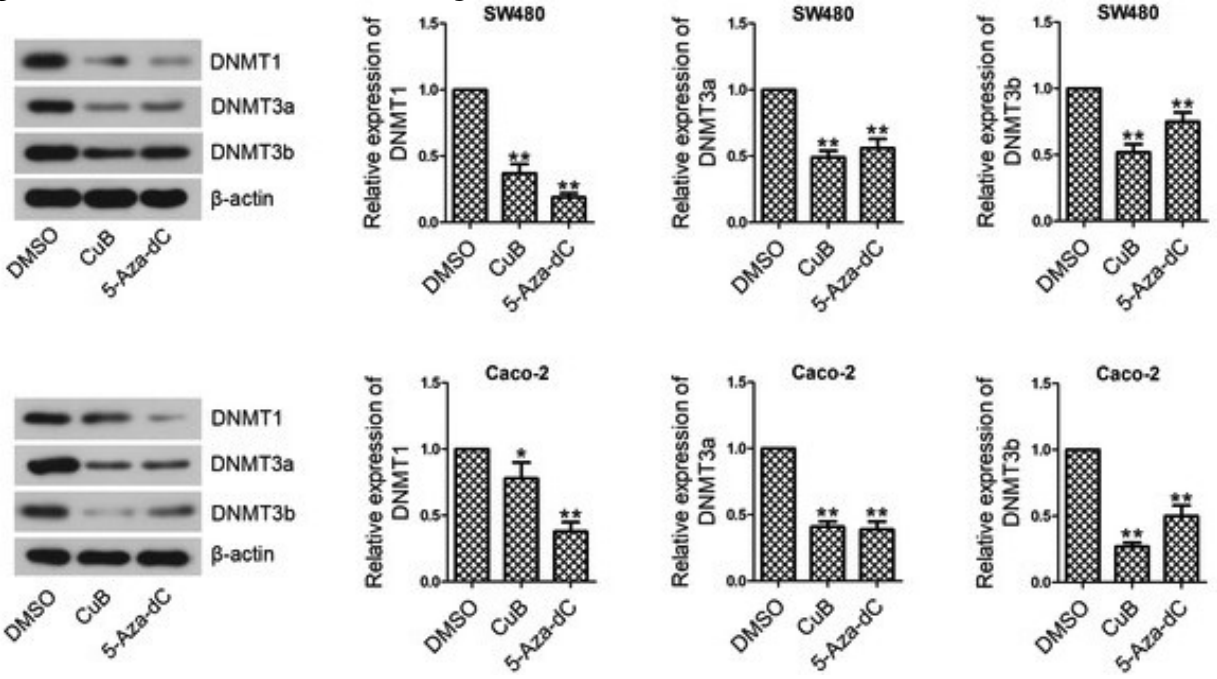

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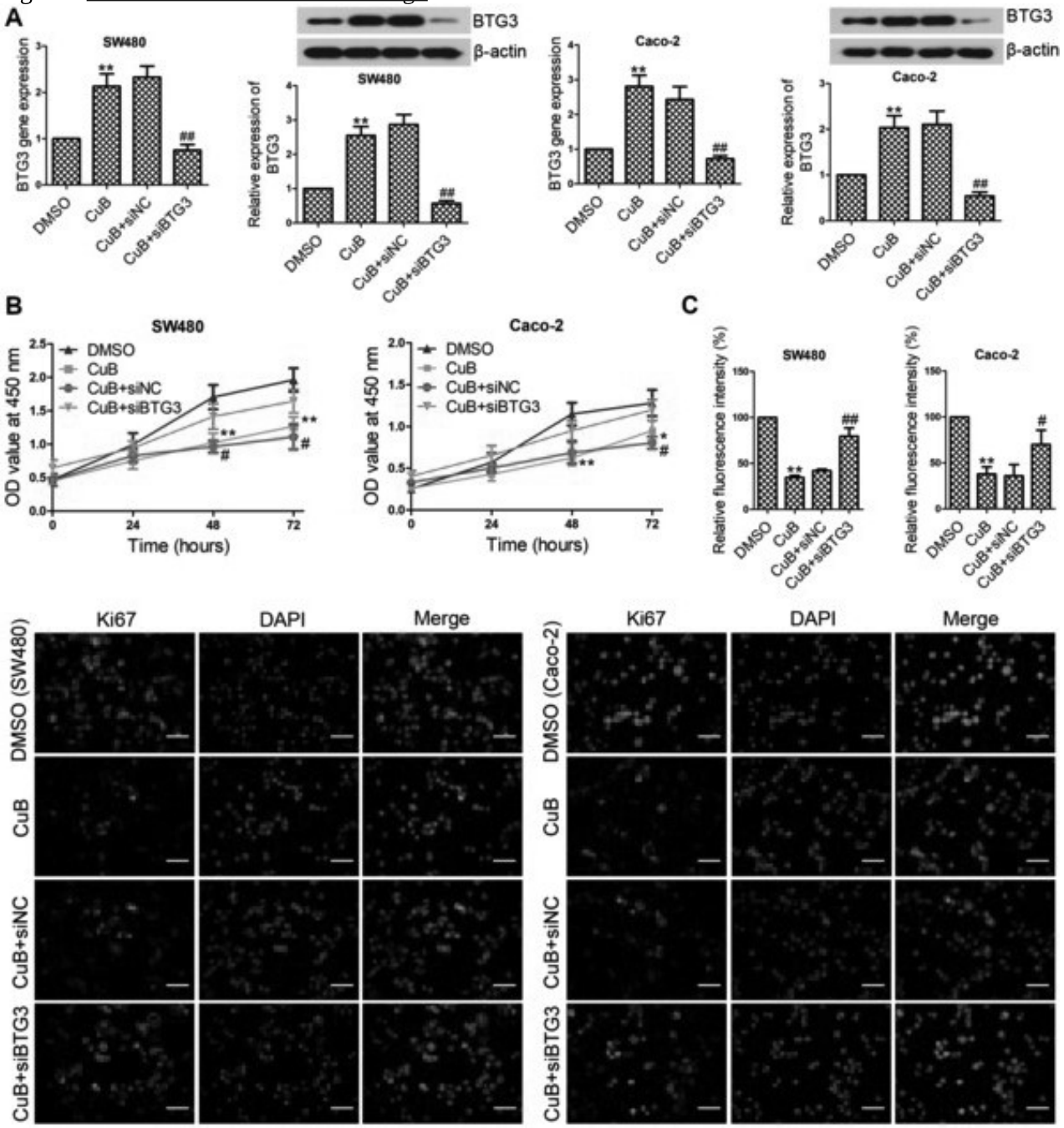

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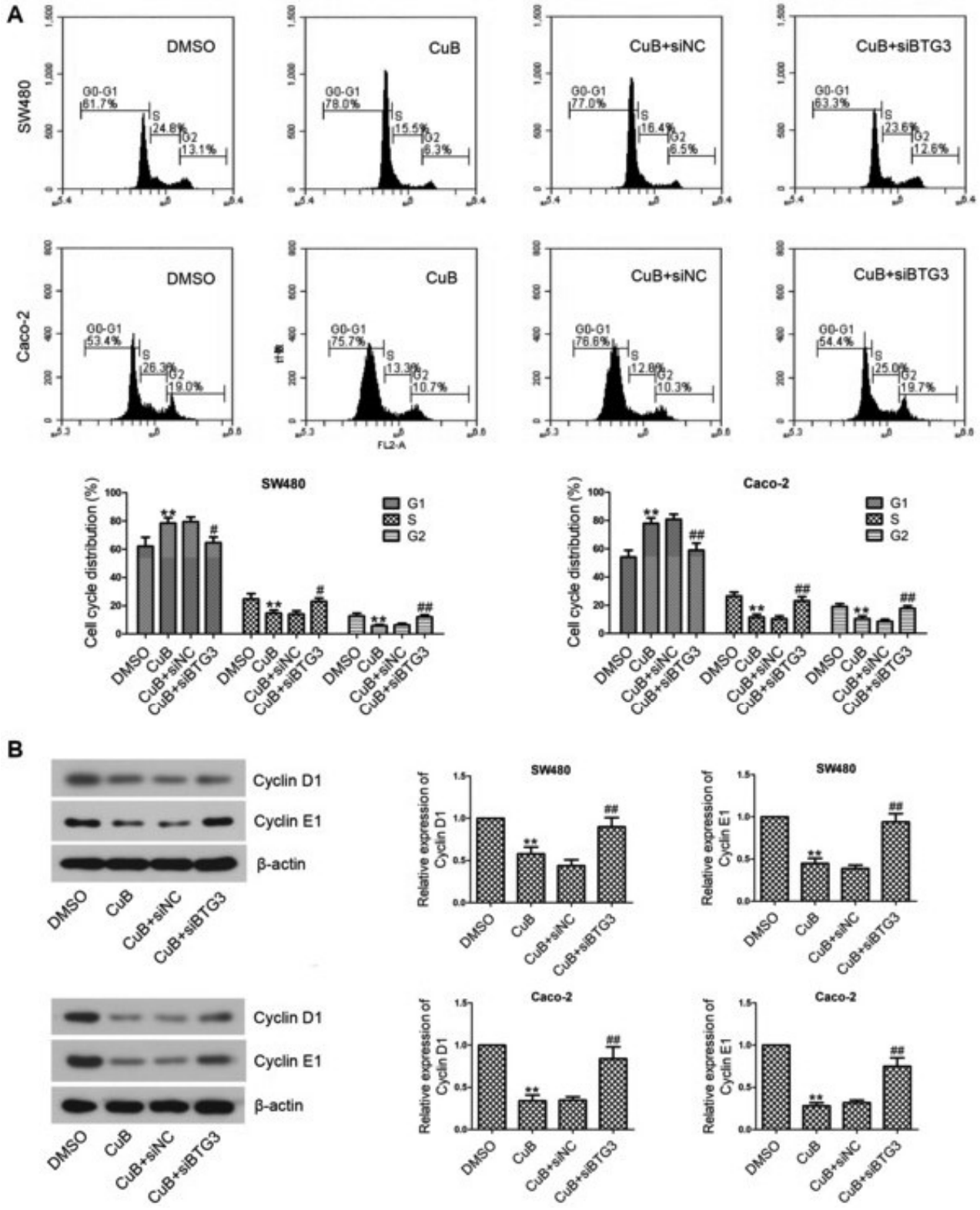

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