

Silencing of Btd7 Inhibited Epithelial–Mesenchymal Transition and Chemoresistance in CD133⁺ Lung Carcinoma A549 Cells

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Cancer stem cells (CSCs) are responsible for tumorigenesis and recurrence, so targeting CSCs is an effective method to potentially cure cancer. BTB/POZ domain-containing protein 7 (Btd7) has been found in various cancers, including lung cancer and liver cancer, but the role of Btd7 in non-small cell lung cancer (NSCLC), CSC self-renewal, and chemoresistance is still unknown. Therefore, in this study we found that the ratio of tumor sphere formation and stem cell transcription factors in CD133⁺ cells was dramatically enhanced compared to parental cells, which indicated successful sorting of CD133⁺ cells from A549. Meanwhile, Btd7 and the markers of the epithelial–mesenchymal transition (EMT) process were more highly expressed in CD133⁺ cells than in parental cells. Silencing of Btd7 significantly inhibited the self-renewal and EMT process in CD133⁺ cells. Furthermore, we found that downregulation of Btd7 promoted cell apoptosis and increased the sensitivity to paclitaxel in CD133⁺ and parental cells. In conclusion, our results suggest that Btd7 is a promising agent for the inhibition of survival and chemoresistance of cancer stem-like cells of NSCLC, which may act as an important therapeutic target in NSCLC.

Key words: Btd7; CD133⁺; Epithelial–mesenchymal transition (EMT); Chemoresistance; Non-small cell lung cancer (NSCLC)

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality in the world, with over 1 million deaths worldwide annually^{1,2}. Non-small cell lung cancer (NSCLC)³, one of the two major types of lung cancer, accounts for about 80%–85% of all lung cancer cases. Small cell lung cancer accounts for 10%⁴. Although diagnostic and therapeutic approaches have been developed in recent years, the 5-year survival rate for NSCLC patients (less than 15%) has not increased significantly. Conventional chemotherapeutics can often neither inhibit tumor growth nor prevent its relapse due to tumor resistance to chemotherapy. Therefore, a better understanding of the underlying mechanism in order to find new targets is of great importance in the treatment of patients with NSCLC.

Cancer stem cells (CSCs), a small population of cells in a tumor, have been proposed to be responsible for the initiation and progression of many cancers^{5,6}. Tissue-specific CSCs, characterized by elevated expression of

CSC-related molecules such as SOX2, OCT4, and Nanog, and the potential to self-renew and differentiate into multilineage cancer cell types, have been identified in leukemia and solid tumors^{7,8}. CSCs have been associated with resistance to chemo- and radiotherapy^{9,10}. Epithelial–mesenchymal transition (EMT) is a transdifferentiation program essential for numerous developmental processes that occur during various stages of embryogenesis, tissue fibrosis, and cancer¹¹. When the EMT progress is triggered, epithelial cells lose the expression of intercellular adhesive protein, such as E-cadherin, and gain mesenchymal marker expression, such as vimentin, N-cadherin, MMP-2, and MMP-9¹². Recent studies have revealed a key role for EMT in the potentiation of stem cell features, acquisition of an undifferentiated state, and promotion of tumor progression and metastasis¹³. Furthermore, an important role for EMT in tumor resistance to anti-cancer therapies has increasingly been appreciated¹⁴. Evidence proving that CSCs acquire mesenchymal-like

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characteristics and that EMT cells exhibit stem cell-like traits suggests that EMT and CSCs are mechanistically linked to chemoresistance¹⁵.

Btbd7 (BTB/POZ domain-containing 7), a BTB/POZ domain-containing protein, was identified as a regulatory gene that promotes epithelial tissue remodeling and formation of branched organs by regulating E-cadherin¹⁶. Microarray analysis showed a different expression pattern in cancer¹⁷. It was reported that Btbd7 promotes dispersion of the cells and downregulates E-cadherin in pulmonary adenocarcinoma¹⁸. Increased expression in Btbd7 was detected in NSCLC tissues compared to the adjacent nontumorous lung tissues. Btbd7 expression was associated with reduced membrane expression of E-cadherin and abnormal cytoplasmic N-cadherin expression¹⁹. Pathological analysis showed that overexpression of Btbd7 in NSCLC was associated with lymph node metastasis and advanced TNM stages. Btbd7 contributes to the metastasis of NSCLC, so Btbd7⁺ NSCLC may have a high potential for metastasis and thereby a poor prognosis²⁰. The role of Btbd7 in controlling the EMT process has been suggested in NSCLC, but its role in regulating the growth of CSCs and chemoresistance has never been addressed.

In the context of understanding the effects of Btbd7 on CSC growth and chemoresistance, our study led to findings that the protein is overexpressed in CSCs and involved in chemoresistance.

MATERIALS AND METHODS

Human Non-Small Cell Lung Cancer Stem Cell Line A549 CD133⁺ Sorting

Cell suspension was centrifuged at 1,300 r/min for 5 min, and A549 cell precipitation was resuspended in 300 μ l of buffer per 10⁸ total cells after aspirating supernatant completely. FCR Blocking Reagent (Miltenyi Technic, Boston, MA, USA) and CD133/CD133 MicroBeads (Miltenyi Technic) were successively added at 100 μ l per 10⁸ total cells. Cells were mixed and incubated for 30 min at 2°C–8°C. Buffer was then added at 1–2 ml per 10⁸ cells to wash cells, which were then centrifuged at 1,300 r/min for 5 min. The cells were resuspended up to 10⁸ cells in 500 μ l of buffer. An appropriate MACS Column and MACS Separator were chosen according to the number of total cells and the number of CD133⁺ cells. CD133⁺ cells were cultured for further experiments.

Cell Lines and Plasmids

A549 cell lines were maintained in RPMI-1640 (Thermo Scientific, Waltham, MA, USA) with 10% (FBS; Thermo Scientific), which were provided by Cell Bank, Chinese Academy of Sciences (Shanghai, P.R. China). The NSCLC CSC line was maintained in a DMEM/F12

medium supplemented with ITS, 20 ng/ml EFG, and 20 ng/ml FGF. All cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. Btbd7-siRNA (sc-92326) and NC (sc-37007) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA)

Real-Time PCR

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) and RNase-free DNase (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was prepared using mRNA retrovirus kits (Fermentans; Thermo Scientific, Waltham, MA, USA), and the sequences of primers are shown in Table 1. mRNA was tested by quantitative real-time PCR using an ABI 7300 Sequence Detector (Applied Biosystems, Foster City, CA, USA) with the SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA). PCR parameters were as follows: 95°C for 3 min, 40 cycles of 95°C for 10 s, and 58°C for 30 s. The relative expression level was calculated using the Ct method. Human β -actin was used as an internal control.

Western Blotting

The protein concentration of samples was detected by the BCA kit (Auragene, Changsha, P.R. China). Proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Invitrogen). PVDF membranes were blocked with 5% dry milk-TBST for 30 min at 37°C. The blots were then incubated with an antibody [anti-Btbd7 antibody (sc-241937; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-E-cadherin antibody (ab15148; Abcam, Cambridge, MA, USA), anti-N-cadherin antibody (ab12221; Abcam), anti-vimentin antibody (ab8978; Abcam), anti-CD45 antibody (ab10558; Abcam), anti-CD44 antibody (ab157107; Abcam), CD133 polyclonal antibody (18470-1-AP; Proteintech, Chicago, IL, USA), OCT4 polyclonal antibody (11263-1-AP; Proteintech), SOX2 polyclonal antibody (11064-1-AP; Proteintech), anti-ALDH1A1 antibody (ab52492; Abcam), and anti-Nanog antibody (ab109250, Abcam)] overnight at 4°C. Following three washes, the membranes were then incubated with a secondary antibody for 40 min at 37°C in TBST. Signals were visualized by ECL chemiluminescence detection kit (Auragene).

Flow Cytometry

Aliquots of CD133⁺ cells were evaluated for purity by flow cytometry with a FACSCalibur machine (BD Biosciences, San Jose, CA, USA) using CD133/2 (293C3)-PE antibody (Miltenyi Biotec, Germany).

Cells were transfected with Btbd7 siRNA and treated with paclitaxel at 1 μ M for 48 h, and then cells were collected for apoptosis detection and stained with annexin

Table 1. The Primers of Stemness and EMT Genes

Gene		Sequence
CD133	Sense	TCAAGGACTTGCGAACTCTC
	Antisense	GTCTCCTTGATCGCTGTTG
SOX2	Sense	CCCTGTGGTTACCTTTTCTC
	Antisense	AGTGCTGGGACATGTGAAG
CD45	Sense	TGAAAGAGTGAGAGTGGACGAT
	Antisense	ATTCTGGTGTCTGCCTGCTT
ALDH1	Sense	TTACCTGTCTACTCACCGATT
	Antisense	CAACATCCTCCTTATCTCCTTC
CD44	Sense	AGCAGCACTTCAGGAGGTAC
	Antisense	CCATGTGAGTGTCTGGTAGCA
Nanog	Sense	CTCTCCTCTTCCTTCCTCCAT
	Antisense	TTGCGACACTCTTCTCTGC
OCT4	Sense	TTCAGCCAAACGACCATCT
	Antisense	GCTTTGCATATCTCCTGAAGA
Btbd7	Sense	TAAGTGATGCAATGAAAAGA
	Antisense	TGACCATGTAGAGCGTGT
E-cadherin	Sense	TCTGGCACCACACCTTCTAC
	Antisense	GGAAGGAAGGCTGTAAGAGT
N-cadherin	Sense	GTGCCATTAGCCAAGGGAATTCAGC
	Antisense	GCGTTCCTGTTCCACTCATAGGAGG
Vimentin	Sense	CTCCGCGCCTACGCCA
	Antisense	GCCCAGGCGGAGGTACTCC
β-Actin	Sense	AGGGGCCGGACTCGTCATACT
	Antisense	GGCGGCACCACCATGTACCCT

V-FITC and propidium iodide using the TACS Annexin V kit (Kaigen, Nanjing, P.R. China) according to the manufacturer's protocol. All samples were analyzed using a BD FACSCanto II cytometer (BD Biosciences) with BD FACSDiva software. All experiments were performed in triplicate.

Sphere Formation Assay

Suspensions of CD133⁺ and parent cells were plated at 100 cells/well in 96-well plates and grown for 7 days. The numbers of spheres in each well were then counted. The experiment was repeated in triplicate. The rate of sphere formation was calculated based on the formula: rate of sphere formation % = (number of spheres/number of plated cells) × 100%.

Cell Proliferation Assay

Cells at 5,000 cells/well were seeded in 96-well plates and treated with paclitaxel at 0, 0.01, 0.1, 1, 5, 10, and 20 μM for 24, 48, and 72 h after culture. Cell proliferation was detected by MTT assay using a microplate reader at 570-nm wavelength.

Statistics

The values given are mean ± SD. The *p* values were determined for experimental versus control treatments

by two-tailed Student's *t*-test (*p* < 0.05, *p* < 0.01, and *p* < 0.001).

RESULTS

Isolation and Characterization of CD133⁺ Cells From NSCLC Cell Line A549

Previous studies have shown that Btbd7 contributes to lung cancer cell invasion and metastasis through regulating the expression of EMT-associated proteins and correlates with a poor prognosis. Here we assessed whether Btbd7 regulates the function of NSCLC CSCs. In NSCLC, several studies have reported the isolation of CSCs based on various markers including ALDH1, side population phenotype, and CD133 positivity²¹. Considering that the CD133 molecule is the most widely used surface marker for the NSCLC CSCs²², we isolated CD133⁺ in the CSC-like cell population of the A549 cell line by immunomagnetic separation using CD133 antibody-conjugated microbeads. The percentage of CD133⁺ cells was analyzed by flow cytometry, which reached 89.5% after CD133 magnetic sorting (Fig. 1A). To examine whether the isolated CD133⁺ cells had CSC characteristics, we analyzed the expression of CSC markers Nanog, CD44, ALDH1, OCT4, SOX2, and CD45 in parental versus CD133⁺ NSCLC cells on the mRNA level (Fig. 1B–I). Higher expression levels

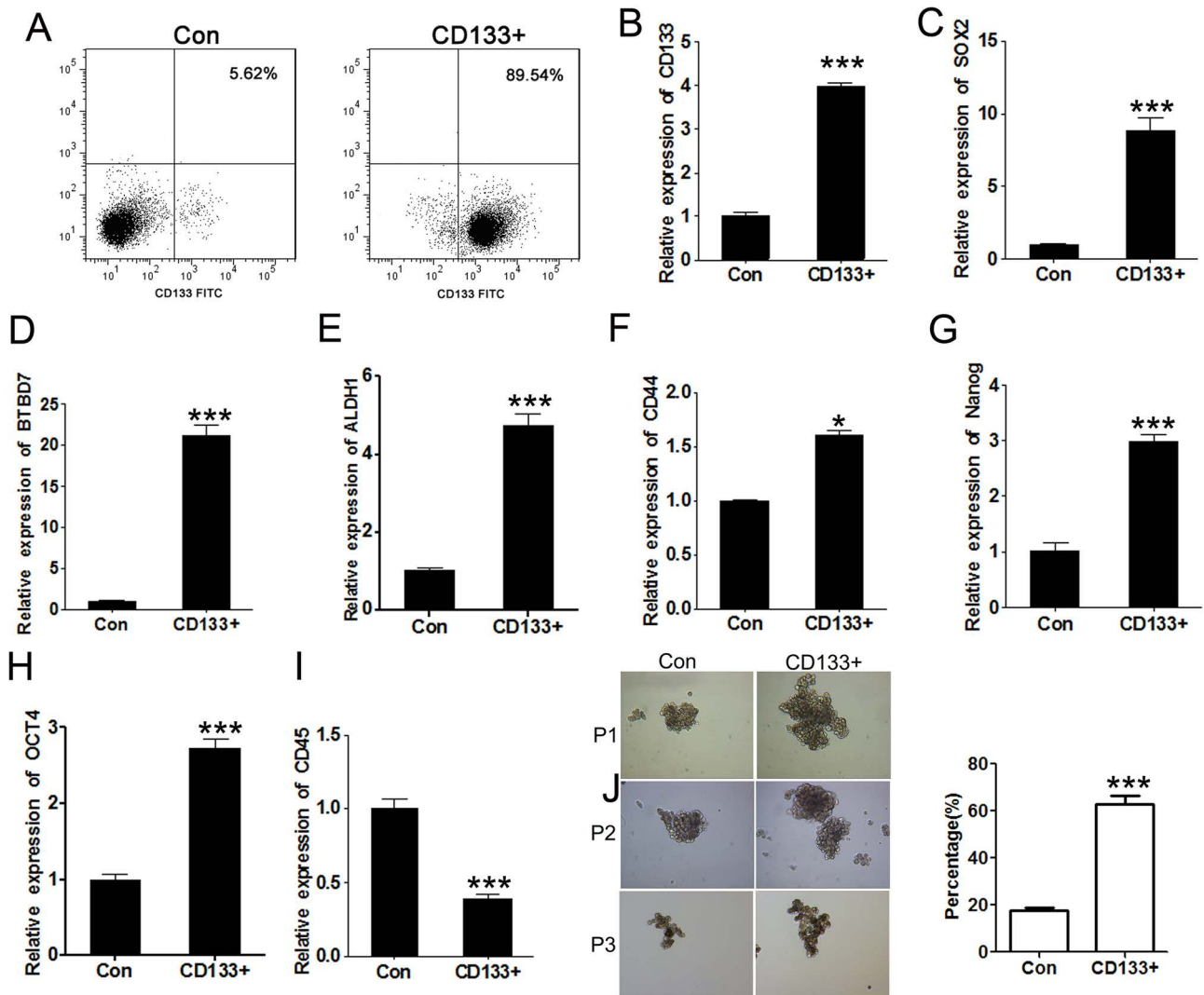


Figure 1. Isolation of CD133⁺ CSC-like cells. (A) Flow cytometry analysis of the proportion of CD133 in CD133⁺ and parental cells of A549. (B–I) Real-time PCR results of the expression of pluripotency-associated genes in CD133⁺ and parental cells of A549. (J) Sphere formation capacity of CD133⁺ and parental cells of A549. Bars represent mean values \pm SD. $n=3$. * $p<0.05$, *** $p<0.001$.

of these CSC markers were consistently detected in isolated CD133⁺ cells when compared with parental cells, except CD45, which was downregulated in CD133⁺ cells. Sphere formation assays were also performed. The capacity of sphere formation in CD133⁺ cells (62.85%) is significantly increased when compared with parental cells (17.71%) (Fig. 1H). Based on these results, we applied the enriched CD133⁺ cells as a source of putative CSCs in subsequent experiments.

Expression of *Btbd7* and EMT-Associated Molecules in CD133⁻ and CD133⁺ Cells

Accumulating evidence indicates that the induction of an EMT phenotype by different factors also induces

CSC-like cells, and CSCs display EMT phenotypes. So the expression of EMT-associated molecules in A549 cells was detected, including epithelial protein E-cadherin, mesenchymal marker vimentin, and N-cadherin. Both mRNA and protein results showed that CD133⁺ cells expressed higher levels of N-cadherin and vimentin. Conversely, E-cadherin expression in CD133⁺ cells was significantly lower, indicating that CD133⁺ cells have EMT-associated characteristics, which is consistent with other studies²³.

Btbd7 was suggested to be involved in regulating the EMT process, so we tested the expression of *Btbd7* in parental and CD133⁺ cells using real-time PCR and Western blotting analysis (Fig. 2A and B). Real-time

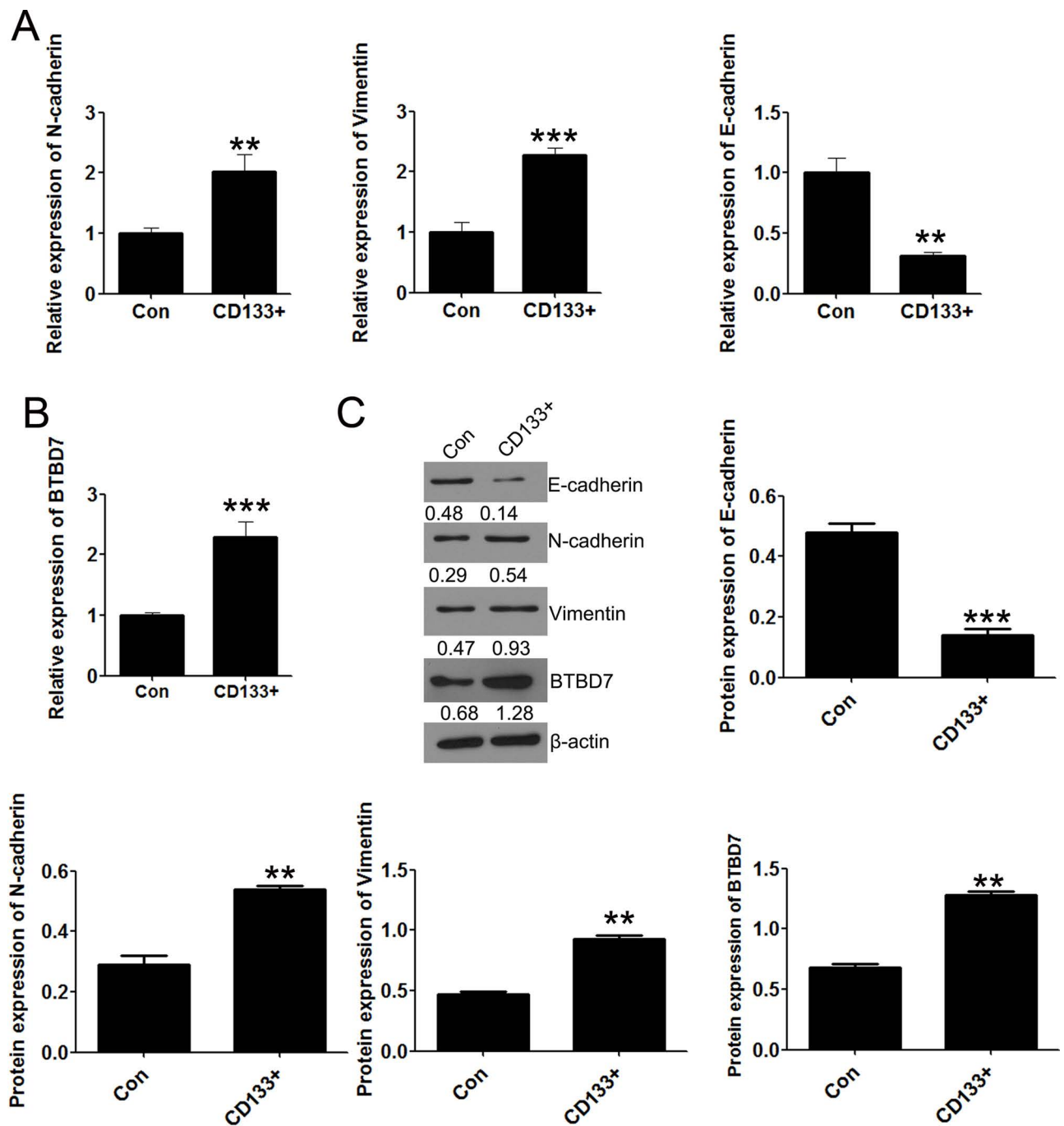


Figure 2. Btd7 and EMT-associated molecules in parental and CD133⁺ cells. (A) Analysis of the mRNA expression of the epithelial–mesenchymal transition marker in A549 CD133⁺ and parental cells by real-time PCR. (B) Quantitation of mRNA expression of Btd7 in CD133⁺ and parental cells of A549. (C) Protein expression of epithelial–mesenchymal transition markers and Btd7 was analyzed by Western blot. Bars represent mean values \pm SD. $n=3$. ** $p<0.01$, *** $p<0.001$.

PCR analysis showed that the Btd7 mRNA expression (2.3-fold) was higher in CD133⁺ cells compared with the parental cells. The Btd7 protein level shown by Western blot analysis was also higher in CD133⁺ cells than in parental cells.

Silencing Btd7 Inhibited EMT Features in CD133⁺ Cells

To elucidate the effects of Btd7 on CD133⁺ cells, Btd7 was knocked down in CD133⁺ cells by transfecting with Btd7 siRNAs, which was confirmed by real-time

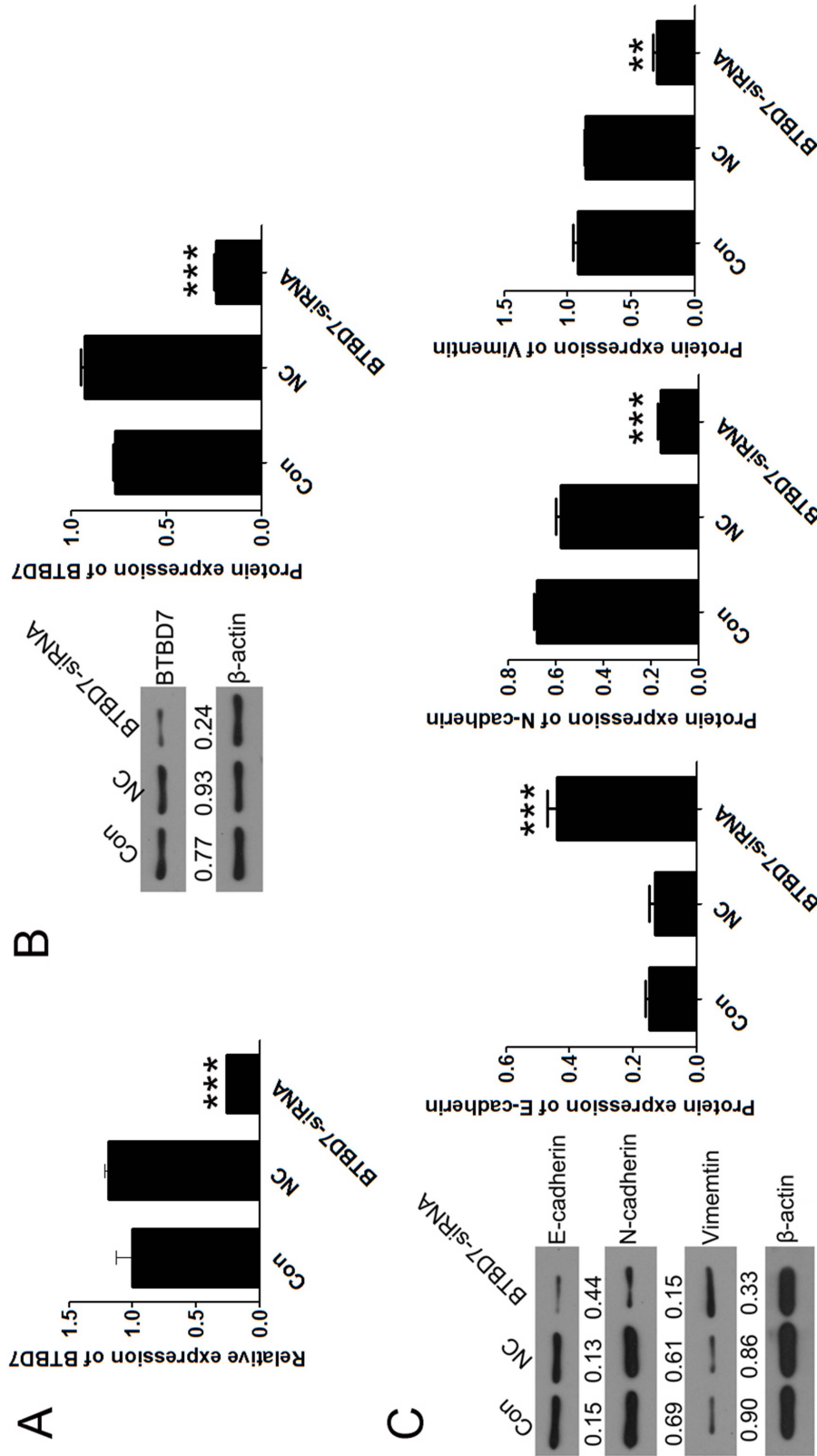


Figure 3. Silencing Btbd7-inhibited EMT features in CD133⁺ cells. (A) Real-time PCR assay detected the Btbd7 level in A549 cells upon Btbd7 downregulation. (B) Protein expression of Btbd7 was analyzed by Western blot. (C) The expression of epithelial-mesenchymal transition markers in CD133⁺ cells was analyzed by Western blot after Btbd7 silencing. Bars represent mean values ± SD. *n* = 3. ***p* < 0.01, ****p* < 0.001.

PCR and Western blotting (Fig. 3A and B). The expression levels of EMT-related genes were examined first. E-cadherin was upregulated in Btbd7 knockdown cells, which is consistent with the negative regulatory effect of Btbd7 on E-cadherin (Fig. 3C). Downregulation of the expression of vimentin and N-cadherin was observed in Btbd7 knockdown CD133⁺ cells (Fig. 3C), indicating the reversal of the EMT phenotype of CSCs caused by Btbd7 knockdown in CD133⁺ cells.

Silencing Btbd7 Decreased Self-Renewal and Chemotherapy Resistance in CD133⁺ Cells

Two important signatures of CSCs are their self-renewal capacity and resistance to chemotherapy. To investigate the effects of Btbd7 on the regulation of the self-renewal of CD133⁺ cells, a sphere formation assay was performed. Depletion of Btbd7 by siRNAs reduced the ability of CD133⁺ cells to form spheres by 50%, compared with cells transfected with a nontargeting control siRNA (Fig. 4A). Also, the size of the spheres that formed was significantly smaller when compared with the siRNA-transfected cells. Next, whether depletion of Btbd7 affects stem-like functions of CD133⁺ cells by modulating the expression of core embryonic stem cell transcription factors SOX2, OCT4, and Nanog was examined. Both real-time PCR and Western blotting results showed that all three transcription factors were reduced upon Btbd7 depletion (Fig. 5A and B). In addition, the expression of CSC markers including CD133, CD44, and ALDH1 was downregulated in Btbd7 knockdown CD133⁺ cells, and the expression of CD45 showed the contrary. These results demonstrated that Btbd7 is important for CD133⁺ self-renewal.

CSCs are believed to utilize a variety of mechanisms to evade eradication by conventional therapy such as chemotherapy and radiation. We assessed the effect of Btbd7 inhibition on chemoresistance of the stem cells. We first evaluated the sensitivity of parental cells and CSCs under different concentrations of paclitaxel treatment. Exposure of A549, CD133⁻, and CD133⁺ cells to a range of paclitaxel (0–20 μ M) concentrations resulted in IC₅₀ values of 2.502 and 6.996 μ M (Fig. 6A), respectively. CD133⁺ cells have a higher drug resistance to paclitaxel treatment than CD133⁻ cells. We then mainly used 1 μ M paclitaxel in the following assays. We next assessed the Btbd7 inhibition on chemoresistance of stem cells. MTT assays showed that cell proliferation was decreased in Btbd7 knockdown CD133⁺ cells in the presence of paclitaxel compared to control cells (Fig. 6B). Flow cytometry assays showed that the apoptotic percentage of CD133⁺ cells was obviously increased by silencing Btbd7 expression when compared with control cells under paclitaxel treatment (Fig. 6C). These

results demonstrate that Btbd7 mediates the cells' resistance to chemotherapy.

DISCUSSION

In this study, we first enriched CSCs by cell sorting based on CD133 from the A549 cell line. CD133 is the most commonly used among the CSC markers^{24,25}. CD133⁺ cells had CSC-like characteristics by showing the capacity to form spheres and express higher levels of the stem cell markers OCT4, SOX2, and Nanog, which is in accordance with previous studies²⁶. Moreover, these cells manifested an EMT gene expression profile, which has been considered to be an important peculiarity linked to CSCs²⁷.

Btbd7 contains 1,130 amino acids with two putative BTB/POZ domains. The BTB domain is a protein–protein interaction motif that was first identified as a sequence motif in genes of a DNA virus²⁸. The function of BTB-containing proteins is now well known to be mainly transcriptional regulation and protein degradation. Btbd7 was found to act as a dynamic regulator that promotes epithelial tissue remodeling and formation of branched organs through downregulating E-cadherin¹⁶. The absence of E-cadherin or its decreased expression is recognized as a hallmark of EMT²⁹. A recent study using MDCK cells showed that Btbd7 promotes dispersion of the cells and downregulates E-cadherin¹⁸. Knockdown of Btbd7 induces E-cadherin expression and restrains fibronectin and TWIST1 expression in HCC cells, resulting in metastasis and recurrent suppression in HCC³⁰. Lung cancer metastasis was prone to occur in patients with positive Btbd7 expression, which may contribute to patients' poor clinical outcome in NSCLC¹⁹. We found a significantly higher expression of Btbd7 in CD133⁺ A549 cells, indicating that Btbd7 may induce the production of CSCs through EMT by regulating the expression of E-cadherin. Downregulation of Btbd7 using siRNA reversed the EMT phenotype in CD133⁺ cells and decreased the self-renewal of CD133⁺ cells.

Despite great efforts that have been made in the management of lung cancer, such as improved diagnostic abilities and the use of targeted therapies, relapse of lung cancer due to the resistance of tumors to conventional chemotherapeutics remains a serious clinical problem³¹. Many studies have shown that lung CSCs, characterized by expressing certain specific molecules and enriched by different methods, appear to mediate resistance to chemotherapy³². EMT, a complex process by which epithelial cells become invasive, migratory mesenchymal cells, could give rise to CSCs. Increasing studies demonstrate an important role for EMT on conferring drug resistance characteristics to cancer cells³³. We found that CD133⁺ cells showed a greater potential for drug tolerance. Downregulation of Btbd7

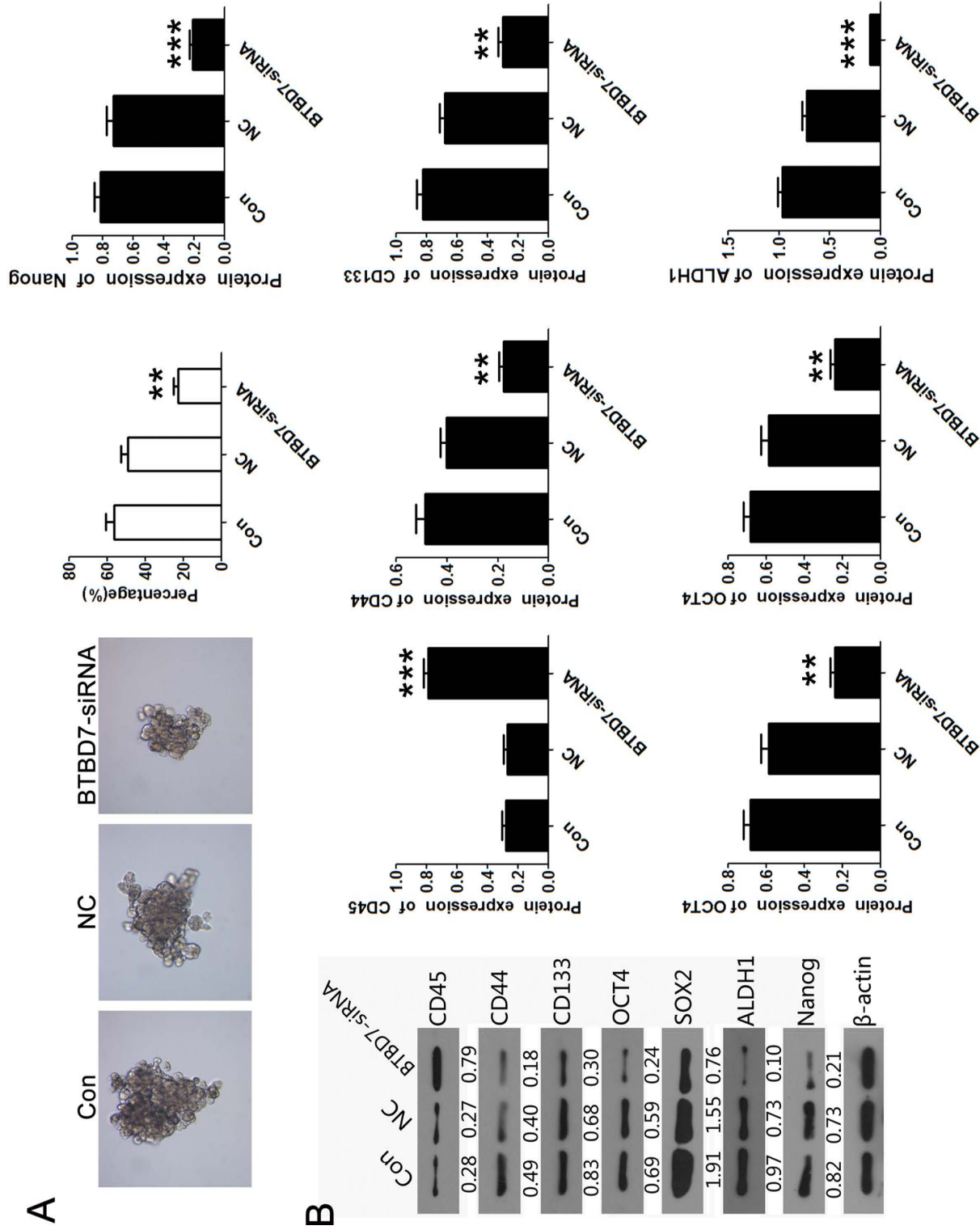


Figure 4. Silencing Btbid7 decreased self-renewal in CD133⁺ cells. (A) The rate of the sphere formation capability of CD133⁺ in A549 cells after Btbid7 silencing. (B) Protein expression of stemness markers was analyzed in A549 CD133⁺ cells by Western blot after Btbid7 was silenced. Bars represent mean values \pm SD. *n* = 3. *******p* < 0.01, ********p* < 0.001.

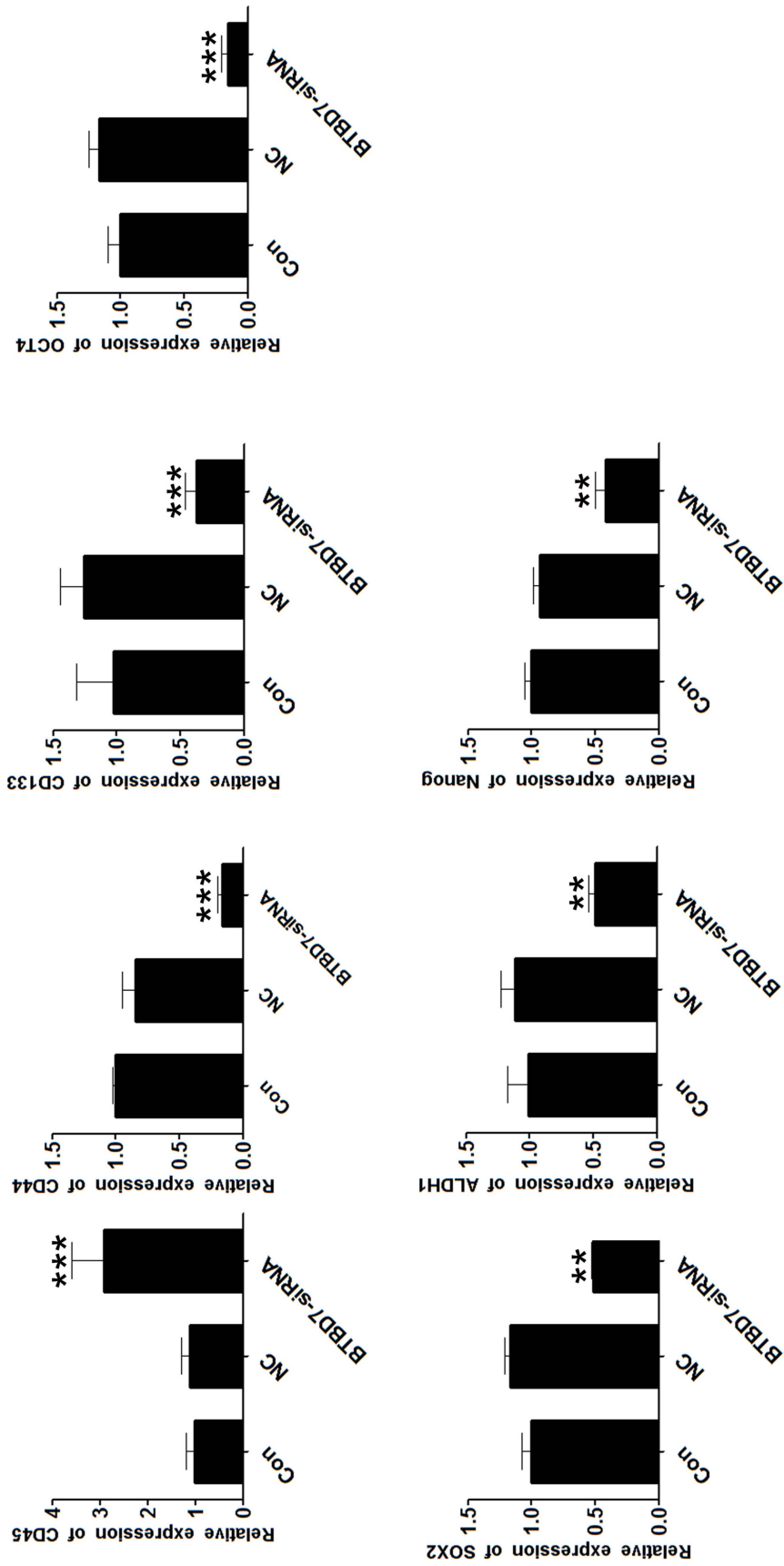


Figure 5. Expression of stemness marker in A549 CD133⁺ cells when Btbd7 was downregulated. The mRNA expression of stemness markers was analyzed by real-time PCR after Btbd7 silencing. Bars represent mean values \pm SD. *n* = 3. ***p* < 0.01, ****p* < 0.001.

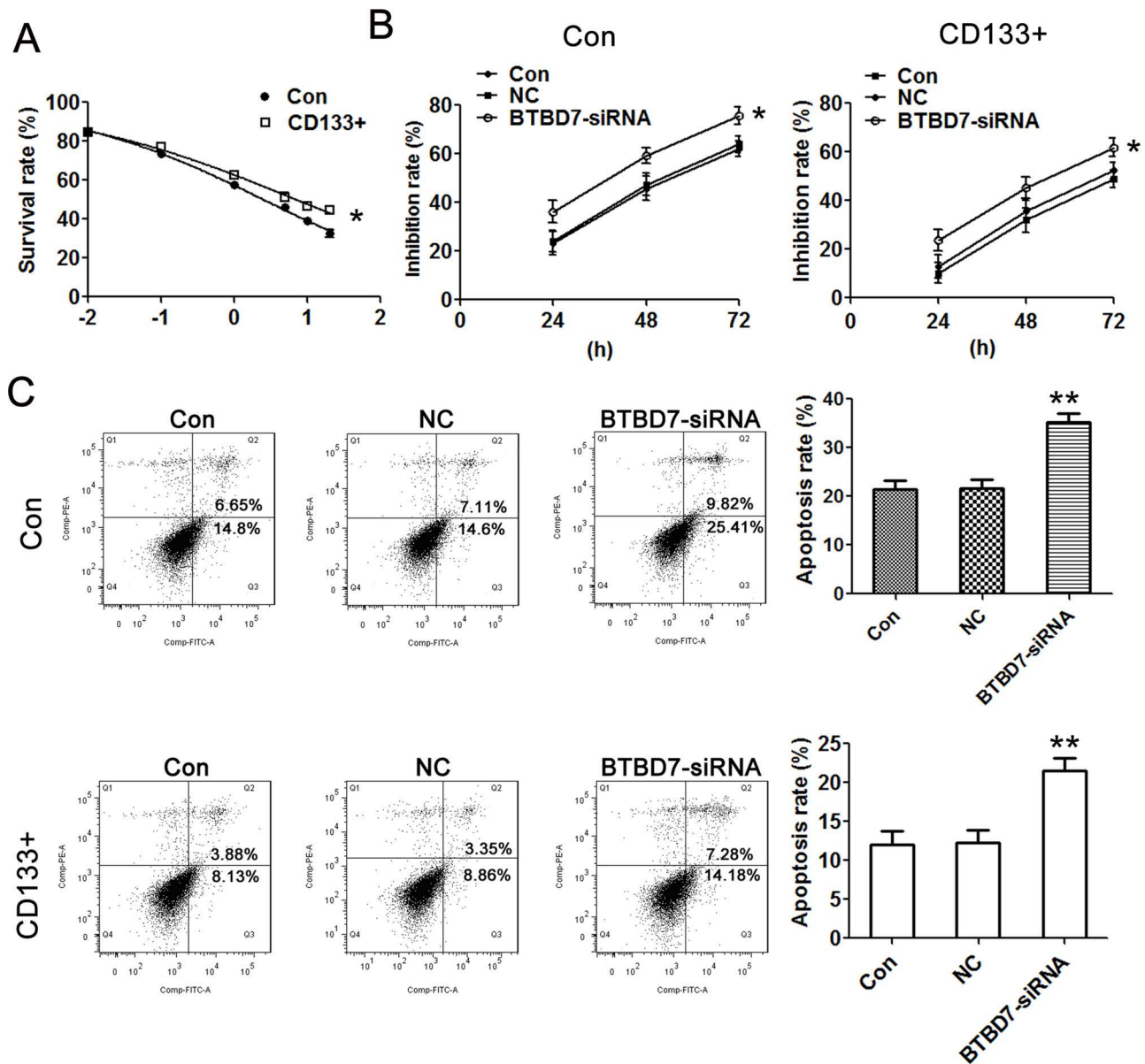


Figure 6. Btbd7 downregulation sensitized cells to chemotherapy in CD133⁺ cells. (A) Cell proliferation ability was used to detect cell proliferation in CD133⁺ and parental cells exposed to paclitaxel. (B) Cell proliferation ability was used to detect cell proliferation in CD133⁺ and parental cells of A549 exposed to paclitaxel. (C) Flow cytometry analysis showed the rates of cell apoptosis in CD133⁺ and parental cells exposed to paclitaxel. Bars represent mean values \pm SD. $n=3$. * $p<0.05$, ** $p<0.01$.

using siRNA restored the sensitivity of CD133⁺ cells to chemotherapy, suggesting an important role for Btbd7 in chemoresistance, particularly in CSCs.

Taken together, we found that Btbd7 is overexpressed in CSCs and induces EMT by regulating the expression of EMT markers such as E-cadherin, N-cadherin, and vimentin. Downregulation of Btbd7 using siRNA reduces the self-renewal capacity of CSCs and restores sensitivity of CSCs to chemotherapy. Btbd7 is associated with

the expression of stem cell marker genes. These results demonstrate the potential of Btbd7 as an important therapeutic target, which gives a combinatorial therapeutic approach to blocking EMT induction of epithelial cells and preventing CSC survival and chemoresistance and could be a better approach than strategies targeting tumor cells or CSCs alone.

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