

## Original Article

# Knockdown of tetraspanin 13 inhibits proliferation of colorectal cancer cells

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**Abstract:** Colorectal cancer (CRC) is one of the most common malignant tumors with higher morbidity and mortality. Studies have demonstrated the tetraspanin 13 (TSPAN13) is overexpressed in various cancers. However, the possible role of TSPAN13 in CRC remains unclear. The aim of this study is to investigate the role of TSPAN13 in CRC and explore its underlying mechanism. In our study, RNA interference lentivirus system was firstly used to deplete TSPAN13 expression in CRC cell lines, HCT116 and HT29, as confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis. Then a series of functional assays, including MTT, colony formation and flow cytometry analysis were conducted on CRC cells after knockdown of TSPAN13. Our results indicated that knockdown of TSPAN13 significantly inhibited CRC cell proliferation and colony formation through inducing cell cycle arrest and apoptosis. Furthermore, knockdown of TSPAN13 enhanced the expression of p27, cleaved caspase-3 and cleaved PARP and decreased CDK4 expression. Our findings suggest that TSPAN13 might be an oncogene and serve as a potential therapeutic target for the treatment of CRC.

**Keywords:** Colorectal cancer, RNA interference, tetraspanin 13, proliferation

## Introduction

Colorectal cancer (CRC) represents one of the major causes of cancer-related death in the world [1]. Despite huge advances in medical and surgical treatment in cancer therapy, the diagnosis and prognosis of CRC patients in advanced stage is still poor, mainly due to local recurrence and metastasis [2]. Currently, clinicopathological parameters are applied to manage CRC, but not considered as reliable predictors of prognosis [3]. Therefore, it is necessary to identify novel biomarkers associated with the prevention, diagnosis and treatment of CRC.

Tetraspanins are integral transmembrane proteins with 33 family members identified in mice and humans [4], which contain short N-terminal and C-terminal cytoplasmic tails, small and large extracellular loops and four transmembrane domains [5]. The predominant view has pointed tetraspanins play important roles in cell adhesion, migration, proliferation and apoptosis [6-8]. To date, several tetraspanins have

been investigated in cancer progression. Tetraspanin 1 has been reported to be overexpressed in CRC and its suppression inhibits proliferation of cancer cells [9]. Similar results have been also observed in human skin squamous carcinoma [10]. Tetraspanin 8 (TSPAN8), as a cell surface protein, has been implicated as human tumor-associated antigen in certain cancers, such as colon carcinoma [11] and hepatocellular carcinoma [12]. Moreover, TSPAN8 could promote gastric cancer cell proliferation and invasion reported by Wei et al [13]. Tetraspanin 13 (TSPAN13), also as a member of transmembrane tetraspanins, is overexpressed in various cancer samples by microarray and Oncomine analysis [14-17]. However, the basic mechanisms underlying the effect of TSPAN13 on CRC cell growth are still unclear and deserve investigation.

In the present study, we aimed to investigate the potential role of TSPAN13 in CRC. Using lentivirus-mediated RNA interference, the expression of TSPAN13 was specifically knocked down in CRC cells. Then loss-of-function assays

were performed in CRC cells. In addition, we also investigated the underlying mechanisms underlying TSPAN13's function on CRC cells.

### Materials and methods

#### Cell culture

Human CRC cell lines (HCT116 and HT29) and human embryonic kidney 293T (HEK293T) cell line were all provided from the Cell Bank of the Chinese Academy of Sciences. HCT116 cells were cultured in RPMI-1640 (Hyclone, Biowest) supplemented with 10% fetal bovine serum (FBS). HT29 cells were grown in MCCOYS'5A (SIGMA) medium containing 10% FBS. HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) plus 10% FBS. All the above cells were maintained in a humidified incubator containing 5% CO<sub>2</sub> at 37°C.

#### TSPAN13 knockdown in CRC cell lines

According to the sequence of TSPAN13 gene (NM\_014399.3), shRNA target sequence (5'-GTTCCGAAGTGTTAACCCAACTCGAGTTTGGGTAACTTTCGGAAGTTT-3') and the non-silencing sequence (5'-TTCTCCGAACGTGTCACGT-3') were designed and cloned into the pFH-L-green fluorescent protein (GFP) lentiviral vector (Shanghai Hollybio, China) between restriction enzyme sites NheI and PacI. Then the recombinant pFH-L-GFP vector was transfected into the HEK293 cells together with two-helper vector (pVSVG-I and pCMVΔR8.92) (Shanghai Hollybio, China) using Lipofectamine 2000 (Invitrogen, United States) according to the manufacturer's instructions. For lentivirus infection, both HCT116 and HT29 cells were cultured in 6-well plates and transduced with TSPAN13 shRNA-expressing lentivirus (shTSPAN13) or non-targeting shRNA-expressing lentivirus (sh-Con), with a multiplicity of infection (MOI) of 30 and 40, respectively. After 3 days of infection, cells were observed under fluorescence microscope (DMI4000B; Leica Microsystems, Germany).

#### Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

To confirm the TSPAN13 was knocked down at transcriptional level, primers (forward: 5'-CC-TCTAGCCAGCCAACCTTCC-3' and reverse: 5'-GACCCTCCACCTCCCTCAG-3') were designed

to detect TSPAN13 mRNA expression. Actin (forward: 5'-GTGGACATCCGCAAAGAC-3' and reverse: 5'-AAAGGGTGTAAACGCAACTA-3') was used as endogenous control. Total RNAs were extracted from HCT116 and HT29 cells using TRIzol reagent (Invitrogen) after 4 days lentiviral infection. Single strand cDNA was synthesized from 2 µg total RNA using Superscript II Reverse Transcriptase (Invitrogen). Subsequently, the expression of TSPAN13 mRNA was determined on BioRad Connet Real-Time PCR platform containing 20 µl PCR reaction mixture (10 µl 2×SYBR premix ex taq, 0.5 µl primers (2.5 µM), 5 µl cDNA and 4.5 µl cDNA) based on the following PCR procedure: initially denatured at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 5 s, and extension at 60°C for 20 s. The expression of TSPAN13 was calculated and normalized to Actin using the 2<sup>-ΔΔCt</sup> method.

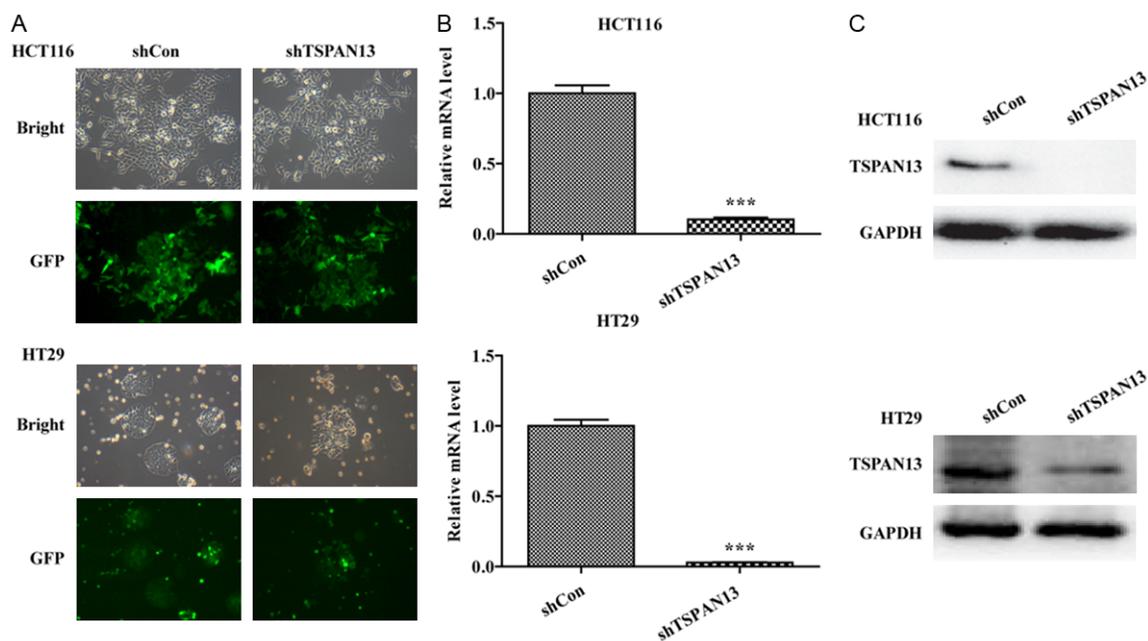
#### Western blot analysis

Total protein was extracted from cells using 2 mL ice-cold RIPA buffer (Beyotime, Shanghai, China). Equal amount of lysates (30 µg) were separated on 10% polyacrylamide-sodium dodecyl sulfate (SDS-PAGE) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking by 1% bovine serum albumin in TBST for 1 h, the membranes were incubated with primary antibodies (anti-TSPAN13, 13570-1-AP, Proteintech; anti-PARP, #9542, Cell signaling; anti-caspase-3, #9661, Cell signaling; anti-CDK4, 11026-2-AP; Proteintech; anti-p27, #3686, Cell signaling; anti-GAPDH, 10497-1-AP, Proteintech) overnight at 4°C. After washed by TBST, membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit (SC-2054, Santa Cruz) for 2 h at room temperature. The signals were detected with enhanced chemiluminescence kit (Pierce Biotechnology, USA).

#### Cell viability analysis using MTT assay

MTT assay was used to evaluate cell viability in CRC cells after TSPAN13 knockdown. After 72 h of infection, HCT116 and HT29 cells were seeded in 96-well plates at a density of 3000 cells/well and 4000 cells/well, respectively, and incubated for 1, 2, 3, 4, or 5 days. Briefly, 20 µl MTT solution (5 mg/mL) was added into each well at each time point and incubated for another 4 h. Then 100 µl acidic isopropanol (10% SDS, 0.01 mol/L HCl and 5% isopropanol) was

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**Figure 1.** Knockdown efficiency of TSPAN13 by lentivirus infection in the colon cancer cells, HCT116 and HT29 (A) Microscopic images of HCT116 and HT29 cells infected with lentivirus. (B) qRT-PCR analysis of TSPAN13 knock-down efficiency in HCT116 and HT29 cells. (C) Western blot analysis validated the knockdown efficiency of TSPAN13 in HCT116 and HT29 cells. \*\*\* $P < 0.001$ , versus control cells GAPDH and actin were used as the internal controls for the qRT-PCR or Western blot assay.

added to stop the reaction. The OD value in each well at a wavelength of 595 nm was measured with an ELISA reader (Bio-Rad, USA).

### Colony formation analysis

A colony formation assay was performed to further evaluate cell proliferation capacity in CRC cells. Briefly, HCT116 cells (600 cells per well) were seeded in 6-well plates and cultured for 8 days. Then the cells were washed and fixed by paraformaldehyde, stained with crystals purple for 10 min. Cell colonies (more than 50 cells per colony) were imaged using micropublisher 3.3 RTV (Olympus) and counted under a microscope.

### Flow cytometric analysis of cell cycle

For cell cycle analysis, HCT116 cells (80,000 cells/well) were cultured in 6-cm dishes and incubated for 6 days. Then cells were trypsinized and centrifuged, washed with cold PBS, and fixed in pre-cold 70% ethanol. After centrifugation, cells were resuspended in PI/RNase/PBS buffer for incubation in dark. The percentages of cells in different phases of cell cycle were measured with flow cytometer (Cell Lab Quanta, Beckman Coulter).

### Flow cytometric analysis of apoptosis

For apoptosis analysis, HCT116 cells (50,000 cells/dish) seeded in 6 cm dishes were infected with lentivirus-mediated shTSPAN13 or shCon for 6 days. Then cells were collected and subjected to Annexin V-APC/7-AAD double staining according to the manufacturer's instruction. Data acquisition and analysis were performed by flow cytometer (Cell Lab Quanta, Beckman Coulter).

### Statistical analysis

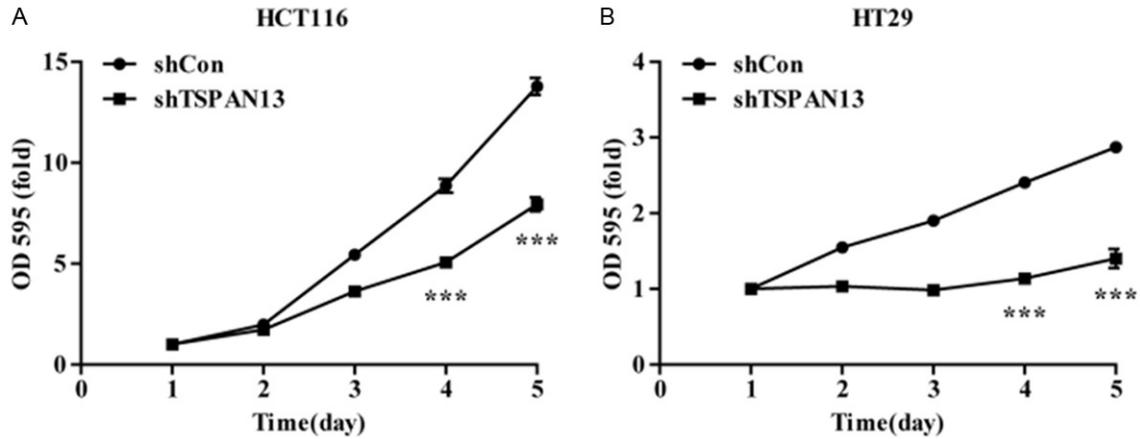
All statistical data were analyzed using SPSS 13.0 software and expressed as mean  $\pm$  SD. The statistical difference was evaluated using Student's t test and accepted at  $P < 0.05$ .

## Results

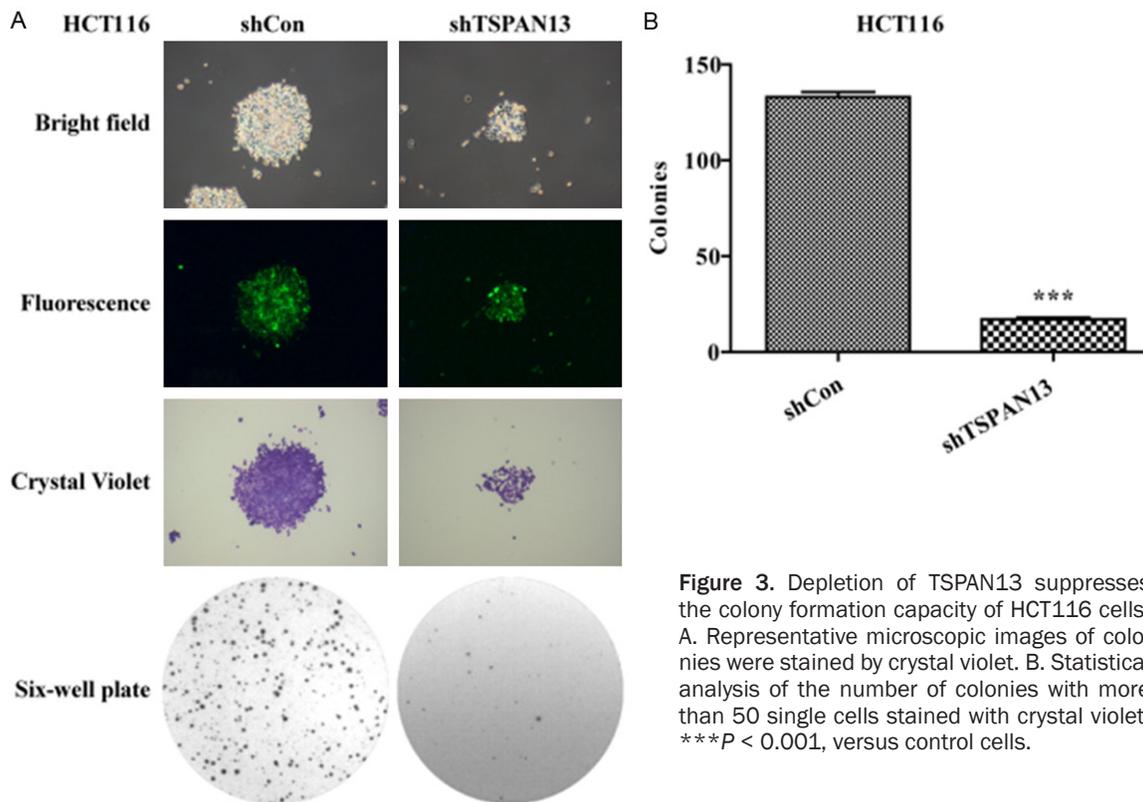
### Efficient knockdown of TSPAN13 in human CRC cell lines

To better evaluate the functions of TSPAN13 in human CRC cells, the expression TSPAN13 was specifically knocked down in HCT116 and HT29 cells using lentivirus-mediated RNAi strategy. As shown in **Figure 1A**, the infection efficiency

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**Figure 2.** Down regulation of TSPAN13 inhibits the proliferation of HCT116 and HT29 cells as verified by MTT assay. \*\*\* $P < 0.001$ , versus control cells.



**Figure 3.** Depletion of TSPAN13 suppresses the colony formation capacity of HCT116 cells. A. Representative microscopic images of colonies were stained by crystal violet. B. Statistical analysis of the number of colonies with more than 50 single cells stained with crystal violet. \*\*\* $P < 0.001$ , versus control cells.

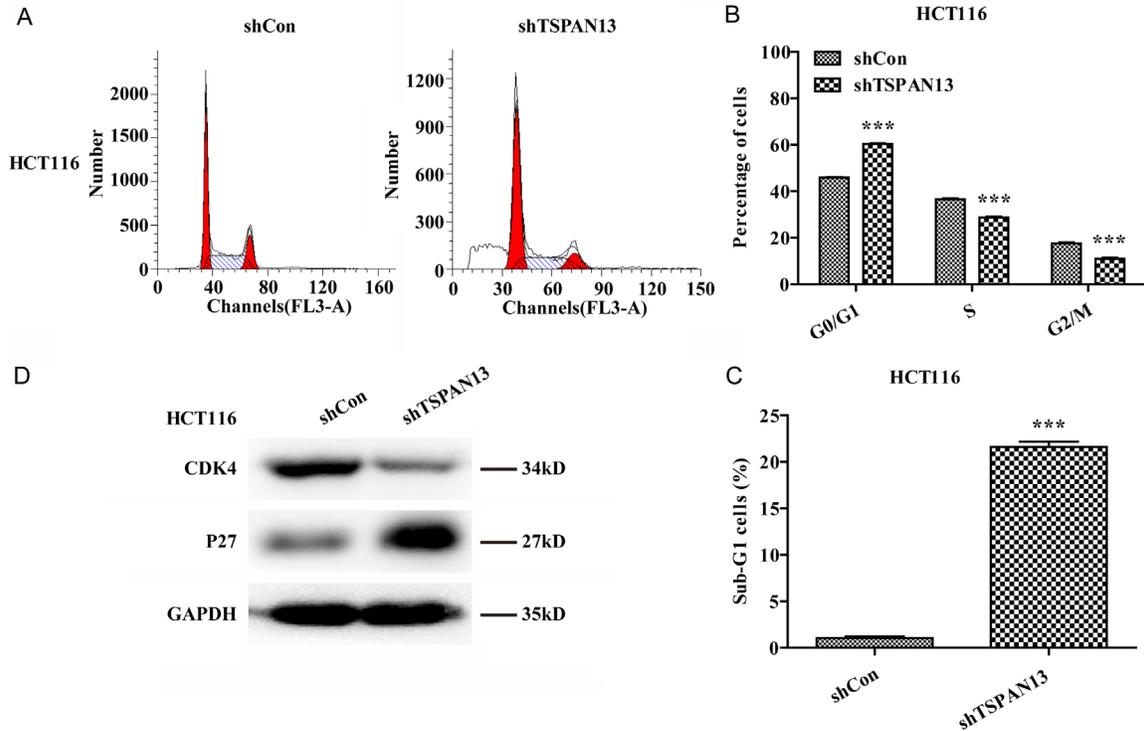
of recombinant lentiviruses was more than 80% in HCT116 and HT29 cells, as revealed by GFP expression. Then we evaluate the knockdown efficiency at mRNA and protein levels, respectively. As shown in **Figure 1B**, lentivirus-mediated shTSPAN13 infection caused about 89.8% reduction in TSPAN13 mRNA in HCT116 cells and 97.2% in HT29 cells compared to shCon infection ( $P < 0.001$ ). Consistently, Western blot analysis (**Figure 1C**) showed that knockdown

of TSPAN13 strongly suppressed the protein level of TSPAN13 in both HCT116 and HT29 cells.

### *Downregulation of TSPAN13 inhibited cell viability in CRC*

After confirming the knockdown efficiency of the shRNA targeting TSPAN13, MTT was used to determine the effect of TSPAN13 depletion

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**Figure 4.** Knockdown of TSPAN13 induced G0/G1 phase cell cycle arrest in HCT116 cells. A. Representative images of HCT116 cells in different cell cycle phases analyzed by flow cytometry. B. Statistical analysis of cells in different phase in HCT116 cells infected with TSPAN13-shRNA. C. Statistical analysis of cells in sub-G1 phase in HCT116 cells infected with TSPAN13 knockdown. D. The effect of TSPAN13 knockdown on the expression of cell cycle associated molecules in HCT116 cells, including CDK4 and p27, as determined by Western blotting. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , versus control cell; GAPDH was used as the internal controls for Western blot assay.

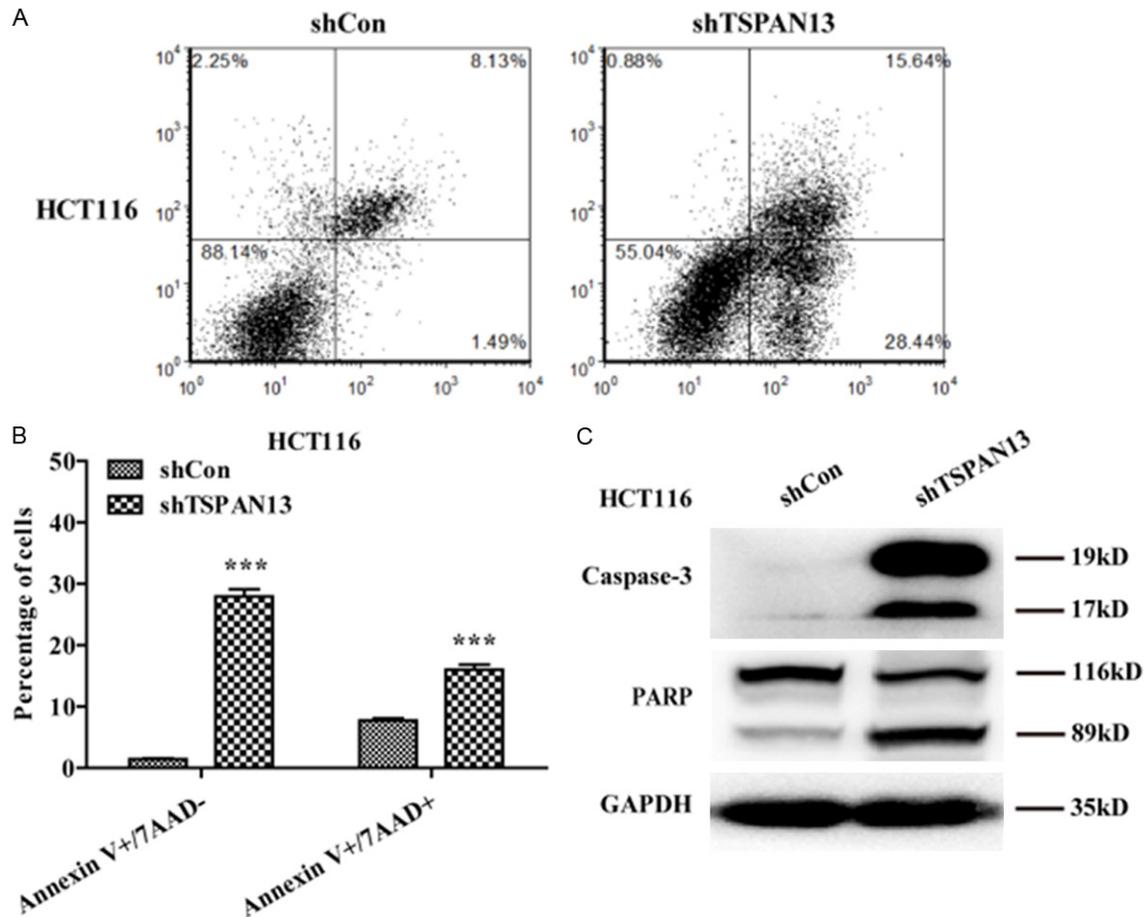
on cell growth on HCT116 and HT29 cells. As shown in **Figure 2**, there was a significant decrease in the cell viability of HCT116 infected with shTSPAN13, compared to shCon infection in a time-dependent manner ( $P < 0.001$ ). The similar results were clearly observed in HT29 cells. Based on these evidences, we could infer TSPAN13 played a positive role on the cell viability of CRC cells.

### *Downregulation of TSPAN13 impaired colony formation ability in CRC cells*

Furthermore, we carried out colony formation assay in HCT116 cells to evaluate the impact of TSPAN13 depletion on the clonogenic capacity of the CRC cells. As shown in **Figure 3A** and **3B**, both the size of single colony and the number of colonies formed in HCT116 cells were obviously decreased after shTSPAN13 infection, compared to that in shCon infection ( $P < 0.001$ ). Thus these results strongly support TSPAN13 is essential for the colony formation in CRC cells.

### *Downregulation of TSPAN13 induced cell cycle arrest in CRC cells*

To explore the underlying mechanisms of cell proliferation impairment by TSPAN13 knockdown, the cell cycle distribution was examined in HCT116 cells infected with shTSPAN13 (**Figure 4A**). According to the analysis of flow cytometry, the percentage of cells in G0/G1 phase was significantly increased, but that in S and G2/M phase were remarkably decreased in HCT116 cells infected with shTSPAN13 (**Figure 4B**,  $P < 0.001$ ). In addition, TSPAN13 knockdown led to an obvious accumulation in the sub-G1 phase (**Figure 4C**,  $P < 0.001$ ), which represents apoptotic cells. Moreover, the expression of cell cycle regulators, including Cyclin dependent kinase 4 (CDK4) and p27 have been determined in HCT116 cells infected with shTSPAN13. The result of western blot analysis (**Figure 4D**) showed the expression of CDK4 was downregulated and p27 was upregulated in shTSPAN13 groups compared with shCon group in HCT116 cells. Collectively, these re-



**Figure 5.** Knockdown of TSPAN13 promoted cell apoptosis in HCT116 cells. A. Representative images of HCT116 cells apoptosis after Annexin-V-7AADD staining. B. Statistical analysis of cells in early-stage (AV+/ADD-) and late-stage (AV+/ADD+) apoptosis. C. The effect of TSPAN13 knockdown on the expression of apoptosis associated molecules in HCT116 cells, including caspase-3, cleaved caspase-3 and PARP, as confirmed by Western blot analysis. \*\*\* $P < 0.001$ , versus control cells; GAPDH was used as the internal controls for Western blot assay.

sults indicated TSPAN13 knockdown might impair cell cycle progression via altering the cell cycle regulators levels, thus suppressing cell proliferation in CRC cells.

*TSPAN13 knockdown induces cell apoptosis associated with caspase-3 and PARP signaling*

To further verify the role of TSPAN13 knockdown in cell growth inhibition, Annexin V/7-AAD double staining was applied in HCT116 cells following lentivirus infection. As shown in **Figure 5A**, there was a remarkable difference in the population distribution of viable (Annexin V-/7-AAD-), necrotic (Annexin V-/7-AAD+), early (Annexin V+/7-AAD-) and late apoptotic (Annexin V+/7-AAD+) cells between shTSPAN13 and shCon groups in HCT116 cells. Statistical analysis (**Figure 5B**) demonstrated apoptotic cells,

including early apoptosis and late apoptosis, were significantly increased in the shTSPAN13 group, as compared to the shCon ( $P < 0.001$ ). In addition, we analyzed the expression of apoptotic markers, as shown in **Figure 5C**. The expression of caspase-3 and cleavage of PARP were upregulated in shTSPAN13 group compared with shCon group in HCT116 cells.

**Discussion**

The present study focused on the possible roles of TSPAN13 involved in the tumorigenic process of CRC. Previous studies reported that TSPAN13 was significantly overexpressed in cancer tissues compared to normal tissues in 75% of the samples analyzed [14]. To further investigate the role of TSPAN13 in CRC progression, we employed lentiviral-mediated shRNA to

inhibit TSPAN13 expression in HCT116 and HT29 CRC cells. The TSPAN13 shRNA infection effectively reduced the expression of TSPAN13 in HCT116 and HT29 cells, as determined by qRT-PCR and Western blot analysis.

It is well established that TSPAN13 gene codes a membrane protein that belongs to the tetraspanin family proteins. Tetraspanins have been reported to be involved in fundamental cellular processes, including cell proliferation, differentiation and tumor metastatic [18-20]. Based on gene sequence similarities, we evaluated the effect of TSPAN13 knockdown on cell growth and proliferation of CRC cells. The results showed downregulation of TSPAN13 inhibited the proliferation of HCT116 cells by inhibiting cell cycle progression at G0/G1 phase and promoting cell apoptosis, which was supported by earlier reports in different cancer cells [9, 13, 21]. Furthermore, Western blot analysis showed TSPAN13 shRNA significantly enhanced the expression levels of p27, cleaved caspase-3, cleaved PARP and suppressed the expression levels of CDK4 in HCT116 cells.

Number studies suggest that cell cycle deregulation is closely related with carcinogenesis [22], which consists of four distinct sequential phases (G0/G1, S, G2, and M) [23]. In eukaryotes, cell cycle could be regulated by cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs) [24]. Deregulation of CDKs activity has been observed in a variety of human tumors and affects normal cell proliferation [25]. In different phases, cell cycle arrest is mostly due to the loss of CDK activity [26]. By using flow cytometry analysis we found the inhibitory effect of shTSPAN13 on HCT116 cell proliferation was associated with the blockage of G1 to S progression. G1/S transition, as main checkpoints of cell cycle, has been demonstrated to be responsible for initiation and completion of DNA replication, which is strongly regulated by the combined activity of the cyclin D/CDK4 [27]. In addition, p27, as a proliferation inhibitor, can inhibit the activity of cyclin/CDK complexes to suppress G1/S progression [28]. Based on these evidences, we could infer knockdown of TSPAN13 arrests cell cycle at G0/G1 phase might through upregulating the expression of p27 and downregulating the expression of CDK4 in HCT116 cells.

Cell apoptosis plays a crucial role in maintaining cellular homeostasis between cell division and cell death [29]. Caspase-caspase is a central part of cell apoptosis and plays an important role in process of programmed cell death. Caspase-3, as a member of the cysteine-aspartic acid protease family [30], is a crucial executor required in the caspase-caspase activation [31]. Together with its specificity substrate PARP, cell apoptosis will be induced [32]. The present study found knockdown of TSPAN13 promoted the apoptosis rates of HCT116 cells. Further western blot analysis revealed that caspase-3, cleaved caspase-3 and cleaved PARP were activated by TSPAN13 knockdown. Thus, we could infer activating caspase cascade is the mechanism of TSPAN13 knockdown inducing cell apoptosis.

In summary, our data demonstrated that TSPAN13 might play an important role in cell proliferation in CRC cells by regulating cell cycle progression and apoptosis. These findings provide an experiment basis for TSPAN13 as a potential therapeutic target for the treatment of CRC.

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### Disclosure of conflict of interest

None.

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