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Suppression of TSPAN1 by RNA interference inhibits proliferation and invasion of colon cancer cells in vitro

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ABSTRACT

Aims and background. To investigate effect of TSPAN1 downregulation by RNA interference (RNAi) on proliferation and invasion of human colon cancer cells *in vitro*.

Methods and study design. RNAi was performed using the vector (pU6H1-GFP)-based small-interfering RNA (siRNA) plasmid gene silencing system to specifically knock down TSPAN1 expression in a colon cancer cell line, HCT-8. The expression of TSPAN1 mRNA was detected by reverse-transcription polymerase chain reaction. TSPAN1 protein expression was observed using Western blots and immunofluorescent microscopy. Cell proliferation and cell cycle assay were measured using methyl thiazolyl tetrazolium (MTT) and flow cytometry, respectively. The invasive ability of HCT-8 cells was examined using a duel culture chamber separated by polycarbonate membranes coated with Matrigel (8.0-μm pore size).

Results. After transfection with the TSPAN1 siRNA plasmid, TSPAN1 mRNA and protein expression was significantly decreased. The decrease in mRNA and protein was associated with a significant decrease in TSPAN1 fluorescent staining and a decrease in cell proliferation due to cell cycle arrest in the G1/G0 phase. A significant decrease in the number of invading HCT-8 cells was associated with these changes.

Conclusion. RNAi-mediated downregulation of TSPAN1 expression significantly inhibits the proliferation and invasion of colon cancer cells *in vitro*. This finding suggests that TSPAN1 plays an important role in colon cancer progression, and RNAi targeting of TSPAN1 may be a potential therapeutic strategy for the treatment of colon cancer. Free full text available at www.tumorionline.it

Key words: TSPAN1, colorectal cancer, invasion, MTT, RT-PCR, immunofluorescence, cell cycle.

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Introduction

Human colon cancer is the second most common cause of cancer mortality worldwide[1]. Although treatments such as surgery, adjuvant chemotherapy and radiotherapy have achieved great progress, the reported survival rate of colon cancer patients after 5 years is not encouraging[2]. There is an urgent need for new treatment strategies to improve the survival rate of these patients. Targeted gene therapy may be a promising way to achieve this goal.

Tetraspanins constitute a large family of ubiquitously expressed membrane proteins. Several tetraspanin molecules such as CD9, CD82, CD63 and CD151 have been identified and implicated in the regulation of cell development, differentiation, proliferation, motility and tumor cell invasion[3-7]. TSPAN1 (formerly referred to as NET-1) is a new member of the tetraspanin family. Sequence analysis of TSPAN1 reveals a structure typical for tetraspanins, with the presence of 4 transmembrane domains delimiting 2 extracellular regions as well as conserved amino acid residues[8]. Overexpression of TSPAN1 has been observed in some tumors such as gastric, hepatocellu-

lar, ovarian and cervical cancer[9-13], as well as in colon cancer tissues[14,15]. Studies have demonstrated that TSPAN1 protein not only stimulates cell proliferation[9] but also enhances invasion and migration of gastric carcinoma cells[16]. These reports suggested that TSPAN1 may play an important role in tumor progression in many of these cancers, including colon cancer.

Small-interfering RNAs (siRNAs) (21 nucleotides), normally generated from long double-stranded RNAs during RNA interference (RNAi), are now frequently used to suppress or inhibit specific gene targets to gain insight into their functions[14]. SiRNAs can be introduced into cells by using either chemically synthesized siRNA oligonucleotides, or vector-based siRNA (shRNA), which allows long-lasting and more stable gene silencing[17-19]. To investigate the role of TSPAN1 siRNA in colon cancer progression, we transfected colon cancer cells with the recombinant TSPAN1 siRNA plasmid and observed the suppression of TSPAN1 expression and the inhibition of proliferation and invasion of colon cancer cells *in vitro*.

Material and methods

Cell culture

The human colon cancer cell line, HCT-8, was purchased from the Shanghai Cellular Institute of the Chinese Scientific Academy, and cultured at 37 °C in a humidified incubator (5% CO_2) in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Plasmid construction

The siRNA sequences were designed on the basis of the published TSPAN1 sequence from GenBank (accession number AF065388) using Qiagen siRNA software (QIAGEN, Shanghai, China). The siRNA sense sequence for TSPAN1 was 5'-TGTGGTCTTTGCTCTTGGTTTCC-3', and the antisense sequence was 5'-GGAAACCAAGAGCAAAGACCACA-3'. The selected sequences were submitted to BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/) to ensure that the selected gene was specifically targeted. The target sequence of the negative control group had no homology to any human gene sequence. The recombinant plasmids (pU6H1-GFP-siRNA TSPAN1 and pU6H1-GFP-siRNA, a negative control) were constructed and sequenced by Biomics Biotechnologies (Nantong, JS, China). The green fluorescent protein construct was used to assay the function of the transfected siRNA.

Cell transfection

HCT-8 cells (1.5×10^5 cells per well) were plated in 6-well plates (Nunc, Rochester, NY, USA). When the cells reached 70% confluence, they were transfected with the plasmids using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. At 48 hours post-transfection, images were analyzed by direct fluorescence microscopy (Olympus, Beijing, China). The cells were divided into 3 groups: HCT-8/PU6H1-GFP-SiRNA TSPAN1 as HCT-8/silence(+), HCT-8/PU6H1-GFP-SiRNA as the negative control HCT-8/silence(-), and HCT-8/no-plasmid as a blank control.

Reverse-transcription polymerase chain reaction (RT-PCR)

HCT-8 cells were collected and total RNA was extracted 48 hours after transfection using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of the total RNA was assessed using an ultraviolet spectrophotometer. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. The primer sequences for the genes and expected product sizes were as follows:

5'-CCAATAAGCTTATGCAGTGCTTCAGCTTCATTAAGA-3' (forward), 5'-CCAATGAATTCTTGTAGATTGCAGTACAGATACATG-3' (reverse) for TSPAN1 (300bp); 5'-CGAAGTCAACGGTGGTCGTAT-3' (forward), 5'-AGCCTTCTCGGTGGTGAAGAC-3' (reverse) for GAPDH (240bp).

RNA samples were subjected to reverse transcription into cDNAs using a reverse-transcription kit (Invitrogen) according to the manufacturer's instructions. PCR amplification was performed under the following reaction conditions: 30 seconds at 94 °C, 30 seconds at 62 °C, and 40 seconds at 72 °C for 40 cycles. The PCR products were electrophoresed on 1% agarose gel, visualized by ethidium bromide staining under ultraviolet light, and analyzed using the Gel-Pro Analyzer 4.0 software. The relative quantity of TSPAN1 expression was calculated based on the TSPAN1/GAPDH ratio.

Western blot assay

HCT-8 cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA) 48 hours after transfection and equal amounts of protein were separated by 10% SDS-PAGE and then transferred to a PVDF membrane. Nonspecific binding was blocked for 2 hours with 5% nonfat milk in TBST (Tris-buffered saline containing 0.1% Tween-20). After incubation with the primary antibodies overnight at 4 °C (a rabbit anti-TSPAN1 polyclonal antibody at 1:200 dilution; the antibody was made by the authors in cooperation with Genemed Biotechnologies Inc, South San Francisco, CA, USA; a rabbit anti-β-actin antibody, at 1:2000 dilution [Sigma-Aldrich]), membranes were washed 3 times in TBST for 5 minutes and subsequently incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody (1:2500 dilution, [Sigma-Aldrich]) for 1 hours at room temperature, and developed using a chemiluminescence system (Pierce, Rockford, IL, USA). The film was scanned and the density of

the bands measured using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA), and then expressed as the percentage of the density of the β-actin band.

Immunofluorescence microscopy

HCT-8 cells were cultured on coverslips in 24-well plates, transfected, and 48 hours later the coverslips were washed twice with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde for 1 hour. The cells were permeabilized with 0.2% Triton X-100 for 5 minutes then blocked with 1% BSA in PBS for 1 hour at room temperature. Cells were incubated overnight at 4 ºC with the TSPAN1 primary antibody (1:200 dilution) and washed extensively, followed by labeling with a secondary TRITC-labeled antibody (1:100 dilution [Sigma-Aldrich]) for 2 hours at 37 ºC. The nuclei were counterstained by Hoechst 33258 (5 µg/mL; Invitrogen) for 30 minutes and the stained cells observed using immunofluorescent microscopy.

Thiazolyl blue tetrazolium bromide assay

Cells (3×10^4 per well) were cultured in 96-well plates each containing 200 µL of RPMI-1640 complete medium. After 24, 48 and 72 hours post-transfection, 20 µL of 5 mg/mL thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich) was added to each well and wells were cultured for a further 4 hours, after which 200 µL of DMSO was added to the wells and absorbance was measured on a spectrophotometer (490 nm).

Cell cycle analysis by flow cytometry

After 48 hours in culture, transfected HCT-8 cells (1×10^6) were washed twice with ice-cold PBS, harvested by trypsinization, and then fixed in 70% cold ethanol at 4 °C overnight. The cell pellets were resuspended in a staining solution of 0.1% triton-X, DNase-free RNase and propidium iodide (Sigma-Aldrich) for 30 minutes at room temperature in the dark. Flow-activated cell sorter analysis was carried out using a Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and CELLQUEST software.

In vitro invasion assay

The invasive potential of HCT-8 cells was determined by a invasion assay using polycarbonate membranes (8.0-µm pore size) in the upper half of 24-well Transwell culture chambers coated with Matrigel (Costar, Corning, NY, USA). After transfection for 24 hours, HCT-8 cells (1×10^5) were suspended with 100 µL serum-free RPMI-1640 medium and placed in the upper chamber. The lower compartment of the chamber was filled with 500 µL RPMI-1640 medium containing serum. After 24 hours' incubation in 5% CO_2 at 37 °C, nonmigratory cells on top of the filters were gently removed with cotton swabs. The invading cells on the underside of the filter were fixed for 10 minutes in 10% formaldehyde, stained with 1% crystal violet for 5 minutes, and washed with PBS. The number of cells was quantified by counting the cells in at least 5 random fields per filter (magnification, 20 × 10).

Statistical analysis

All experiments were performed a minimum of 3 separate times with at least 3 replicates per experiment. Data are presented as means ± SD analyzed by SPSS 13.0 (SPSS Inc., Chicago, IL, USA). A Mann-Whitney U test was used to measure statistical significance between experimental groups. $P < 0.05$ was considered statistically significant.

Results

Suppression of TSPAN1 mRNA levels in HCT-8 cells by siRNA

The recombinant plasmids (pU6H1-GFP-siRNA TSPAN1 and pU6H1-GFP-siRNA as negative control) were constructed using the pU6H1-GFP vectors. Forty-eight hours after transfection, we observed significant inhibition of TSPAN1 mRNA expression in the HCT-8/silence(+) group compared with the other 2 groups ($P < 0.05$). There was no significant difference between the blank control group and the HCT-8/silence(-) group ($P > 0.05$) (Figure 1).

Downregulation of TSPAN1 protein expression in HCT-8 cells by siRNA

The levels of TSPAN1 protein in cells were detected using Western blotting. Analysis showed a significant decrease in protein expression in the HCT-8/silence(+) group compared with the blank control and HCT-8/silence(-) groups ($P < 0.05$). There was no significant difference between the blank control group and the HCT-8/silence(-) group ($P > 0.05$) (Figure 2). We also observed the distribution of TSPAN1 protein in the 3 groups using immunofluorescence microscopy. TSPAN1 protein was located as a small polar concentration in the cytoplasm near the nucleus of HCT-8 cells. The level was decreased in the HCT-8/silence(+) group compared with the other 2 groups (data not described) (Figure 3).

Effect of TSPAN1 downregulation on proliferation of HCT-8 cells

We used the MTT assay to investigate the effect of TSPAN1 siRNA on proliferation in HCT-8 cells. After 48 and 72 hours in culture, the mean proliferation rate of the HCT-8/silence(+) group was significantly lower than that of the other groups ($P < 0.05$); however, there was no significant difference between the blank control group

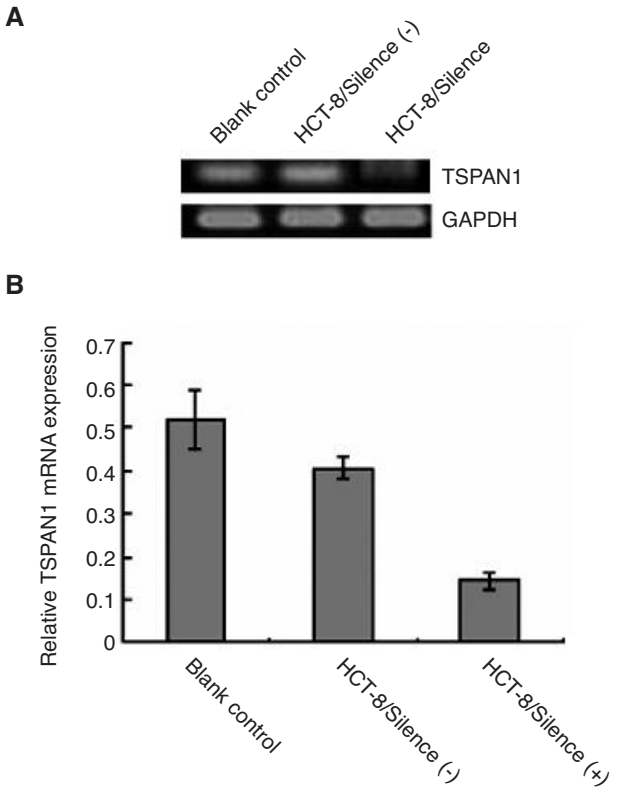

Figure 1 - A) HCT-8 cells were transfected with recombinant plasmids (pU6H1-GFP-siRNA TSPAN1 and pU6H1-GFP-siRNA as negative control) and after 48 h in culture the expression of TSPAN1 mRNA was assessed by RT-PCR. GAPDH was used as internal control. B). The PCR products were electrophoresed on 1% agarose gel, visualized by ethidium bromide staining under ultraviolet light, and analyzed using the Gel-Pro Analyzer 4.0 software. TSPAN1 mRNA expression was quantified relative to GAPDH levels in the HCT-8 cells. Values represent the mean ± SD. TSPAN1 mRNA levels in the HCT-8 /silence(+) group, HCT-8/silence(-) group, and blank control group were 0.14 ± 0.02, 0.40 ± 0.03, and 0.52 ± 0.07, respectively. Statistical analysis showed that the expression of TSPAN1 mRNA in HCT-8 cells was significantly downregulated after transfection with TSPAN1 siRNA plasmid (P <0.05).

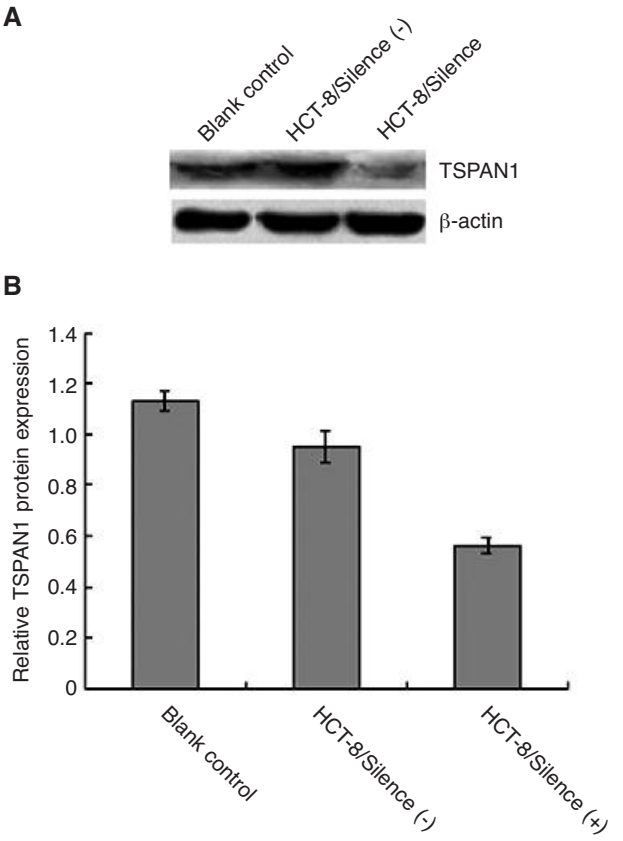

Figure 2 - A) The transfected HCT-8 cells were analyzed for protein expression of TSPAN1 using Western blot analysis. Beta-actin was used as internal control. B) The density of the TSPAN1 protein bands was measured using ImageQuant software and expressed as the percentage of the density of β-actin in HCT-8 cells. Values represent the mean ± SD. TSPAN1 protein levels in the HCT-8/silence(+) group, HCT-8/silence(-) group, and blank control group were 0.56 ± 0.03, 0.95 ± 0.06, and 1.13 ± 0.04, respectively. Statistical analysis showed that the expression of TSPAN1 protein in HCT-8 cells was significantly downregulated after transfection with TSPAN1 siRNA plasmid (P <0.05).

and the HCT-8/silence(-) group at any time (P >0.05) (Figure 4).

Effect of TSPAN1 downregulation on cell cycle of HCT-8 cells

We used flow cytometry to determine whether the inhibitory effect of TSPAN1 siRNA on cell proliferation was mediated through cell cycle progression. We found that 71.8 ± 8.6% of HCT-8/silence(+) cells were in the G0/G1 phase of the cell cycle, which was significantly higher than in the HCT-8/silence(-) group (51.5 ± 5.7%) and the blank control group (53.9 ± 5.5%) (P<0.05), whereas there was no obvious difference between the HCT-8/silence(-) group and the blank control group (P >0.05). These data indicate that the inhibition of cell growth by TSPAN1 siRNA was associated with the arrest of a significant number of cells at the G0/G1 phase of the cell cycle (Figure 5).

Effect of TSPAN1 downregulation on invasion of HCT-8 cells

We used an invasion assay to detect any inhibition by TSPAN1 siRNA of the ability of colon cancer cells to invade through the chamber membrane. The average numbers of migrating cells in the HCT-8/silence(+), blank control, and HCT-8/silence(-) groups were 25.8 ± 8.5, 45.8 ± 5.7, and 42.0 ± 4.6, respectively. This shows that the ability of HCT-8/silence(+) cells to invade was significantly decreased compared with the other 2 groups (P <0.05).

Discussion

Cancer is a disease of genes, whether based on aberrant changes in sequence or expression (epigenomics).

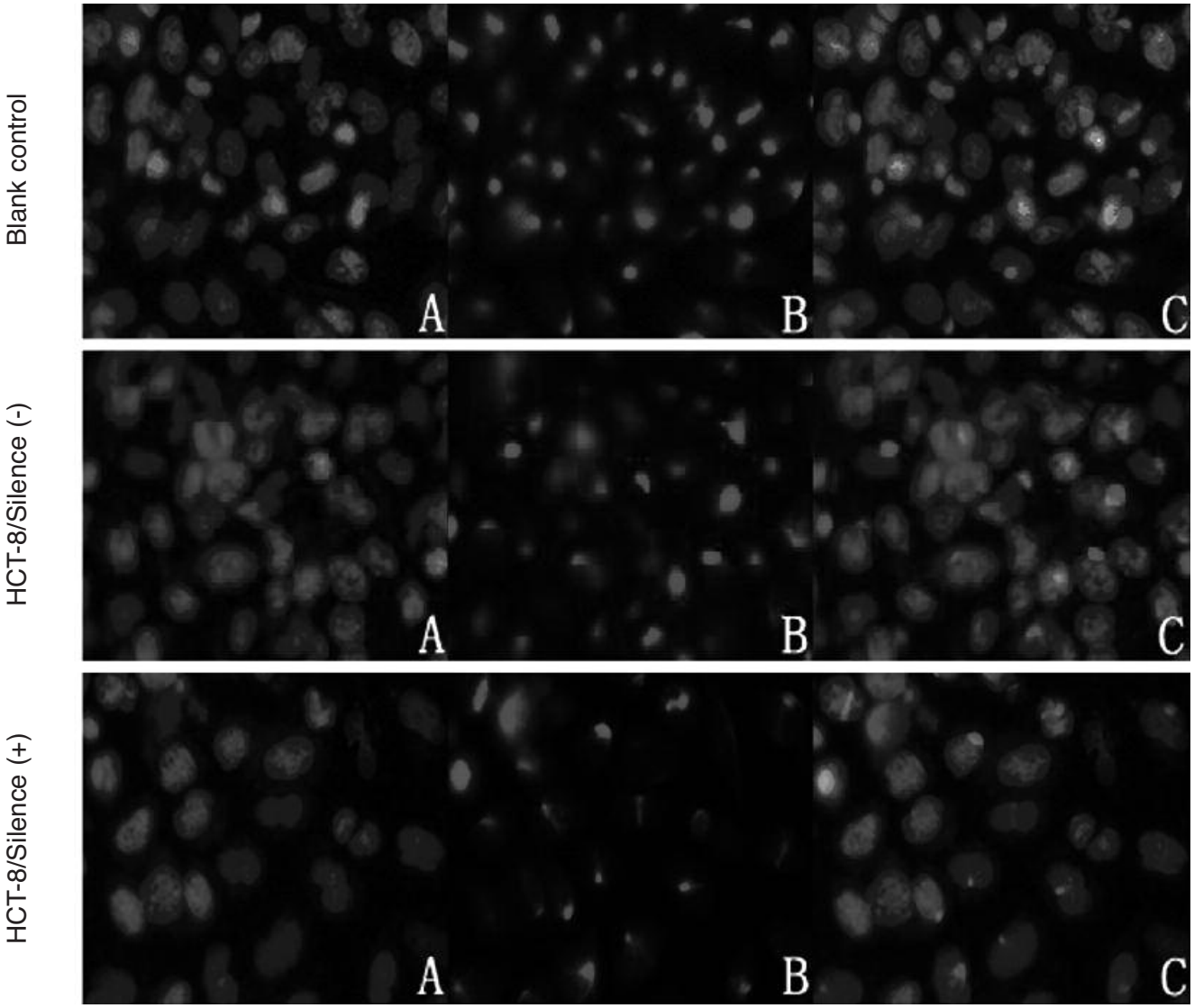

Figure 3 - TSPAN1 protein expression in the cytoplasm of HCT-8 cells using immunofluorescent microscopy. HCT-8 cells were cultured on coverslips in 24-well plates, transfected, and 48 h later stained with a TSPAN1 primary antibody followed by labeling with a secondary TRITC-labeled antibody. A) HCT-8 cell nuclei were homogeneously stained using Hoechst 33258. B) HCT-8 cells stained for TSPAN1 protein. C) Merged micrographs. TSPAN1 protein was located as a small polar concentration in the cytoplasm near the nuclei of the HCT-8 cells. The level decreased in the HCT-8/silence(+) group compared with the other 2 groups.

The constellation of genetic and epigenetic abnormalities characterizing cancer cells presents new and more specific targets for cancer treatment and prevention[20]. TSPAN1, a new member of the tetraspanin group, is a recently discovered tumor-related gene. In our previous study, we found that TSPAN1 was frequently expressed in human hepatocellular carcinoma (HCC) and colon cancer tissues at a higher level compared to the levels in peritumoral tissue[11,14]. In addition, there is a strong correlation between the level of TSPAN1 expression and pathological grading and clinical stages of HCC and colon cancer[11,14]. Shen *et al.*[21] also suggested that the expression of TSPAN1 may be related to proliferation, metastasis and clinical stage of HCC. Taken together, these reports suggest that TSPAN1 may play a critical role in the progression of tumor growth and metastasis in numerous human tumors including colon cancer. Targeting the specific downregulation of TSPAN1 may thus be a potential therapeutic strategy against human cancers, colon cancer included.

RNA interference uses double-stranded RNA to target specific mRNAs for degradation, thereby specifically silencing their expression. Theoretically, mRNA encoding any protein that is associated with a disease can be

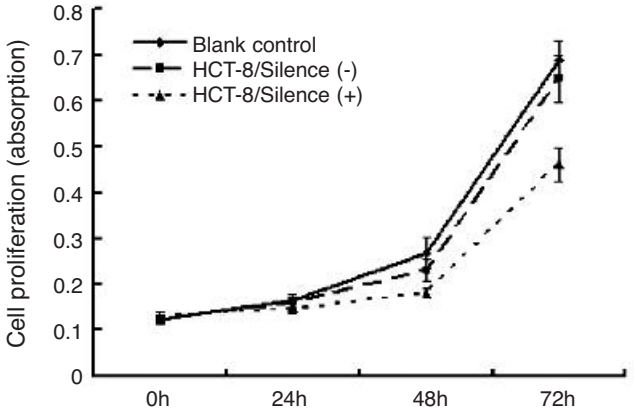

Figure 4 - Proliferation of HCT-8 cells *in vitro*. Cells (3 × 10^4 per well) were transfected and cultured for 24, 48 and 72 hours. Proliferation was determined by MTT assay and optical density measurements using a spectrophotometer (490 nm). Values represent the mean ± SD. Twenty-four, 48 and 72 h after transfection, the optical density was 0.15 ± 0.01, 0.18 ± 0.01, and 0.46 ± 0.04, respectively, in the HCT-8/silence(+) group, 0.16 ± 0.02, 0.23 ± 0.02, and 0.65 ± 0.05 in the HCT-8/silence(-) group, and 0.17 ± 0.01, 0.27 ± 0.03, and 0.69 ± 0.04 in the blank control group. Statistical analysis showed that after 48 and 72 hours' transfection, the proliferation of cells transfected with TSPAN1 siRNA plasmid was significantly notably. (P <0.05).

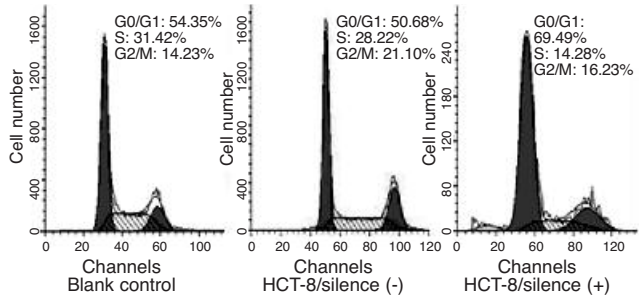

Figure 5 - HCT-8 cells (1 × 10^6) were transfected and cultured for 48 h then trypsinized and fixed in 70% cold ethanol at 4 °C overnight. The cells were resuspended in DNase-free RNase and propidium iodide and cell cycle numbers were determined using a flow-activated cell sorter and CELLQUEST software. The graphs show an example following cell sorting. The result showed that 71.8 ± 8.6% of HCT-8/silence(+) cells were in G0/G1 phase, which was significantly higher than the fraction of HCT-8/silence(-) cells (51.5 ± 5.7%) or blank control cells (53.9 ± 5.5%) (P <0.05).

cleaved selectively by siRNA. Accordingly, we hypothesized that RNAi has the potential to silence TSPAN1 expression in colon cancer cells. Our results clearly showed that the level of TSPAN1 mRNA and protein significantly decreased in the HCT-8/silence(+) group, and suggested that the transfection plasmid led to suppression of TSPAN1 expression because of post-transcriptional mechanisms in which double-stranded RNA effectively silenced TSPAN1 in HCT-8 cells.

We observed that transfection caused the arrest of the cell cycle at the G0/G1 phase and a concomitant reduction of cell proliferation for the HCT-8/silence(+) group, the mechanism of which may be through delaying the progress of the cell cycle from G1 to S phase.

A characteristic feature of any malignant tumor is the ability of tumor cells to migrate and invade into surrounding and/or distal tissue. TSPAN1 siRNA plasmid transfection clearly reduced the ability of HCT-8 cells to invade across the chamber barrier. Similar to the present findings, Leyden *et al.*[9] reported that siRNA-mediated downregulation of TSPAN1 expression resulted in decreased proliferation and invasion of gastric cancer cells *in vitro*.

In comparison to TSPAN1, some members of this family, in particular CD9, CD63 and CD82, are known as metastasis suppressor genes[22,23], while others, like CD151 and Co-029, are supposed to promote metastasis formation[7,24]. For example, the transfection of CD9 or CD63 in melanoma cells reduces the metastatic potential of these cells[25].

The mechanism of tetraspanins' affecting tumor cell behavior has been studied in recent years. Tetraspanins form complexes by interacting with other tetraspanins and with a variety of transmembrane and cytosolic proteins that are required for their functioning[26,27]. They also associate with other molecules that play a critical role in cell signaling and apoptosis[28]. For example, CD151 regulates cell migration, mostly through its association with α3β1, α6β4, and MMPs[24]. Berditchevski *et al.*[29] reported that in MDA-MB-231 cells, CD9 and CD151 were distributed evenly in a dot-like manner in the cytoplasm and cell membrane. In the present study, we observed that TSPAN1 protein was located as a small polar concentration in the cytoplasm near the nucleus of HCT-8 cells, the area where the Golgi apparatus is frequently located. So we guessed that TSPAN1 may regulate tumor progression by interacting with other transmembrane and cytosolic proteins when located on the the membrane of secretion organelles, and carry out functions in the cytoplasm, like other tetraspanins. We are currently pursuing the question which other tetraspanins and molecules may be affected by TSPAN1.

Taken together, these data indicate that RNAi-mediated downregulation of TSPAN1 expression significantly inhibited the proliferation and invasion of HCT-8 cells *in vitro*. Consistent with several reports, we conclude that TSPAN1 may play an essential role in the progression of some malignant tumors including colon cancer, and is important for colon cancer growth and metastasis. RNAi-directed targeting of TSPAN1 may be used as a potent and specific tool for the treatment of colon cancer, especially in inhibiting and/or preventing cancer cell progression.

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