Abstract. Colorectal cancer is one of the most common types of cancer worldwide. Previous studies have revealed that certain members of tripartite motif (TRIM) proteins are involved in carcinogenesis regulation, but little is known about the function of TRIM68 in human colorectal cancer. To investigate the role of TRIM68 in colorectal cancer SW1116 and HCT116 cell lines, the present study conducted lentivirus-mediated knockdown against TRIM68 and demonstrated that depletion of TRIM68 notably inhibits colorectal cancer cell proliferation and colony formation ability. Cell cycle arrest in the G0/G1 phase and cycle accumulation in sub-G1 phase provided evidence that TRIM68 may participate in the regulation of colorectal cancer tumorigenesis. The results revealed the significant role of TRIM68 in regulating colorectal cancer cell mitosis and indicated that TRIM68 may be a promising therapeutic target.

Introduction

Malignant colorectal carcinoma (CRC) is one of the most devastating types of cancer worldwide. It is the second leading cause of cancer-associated mortality in Europe and the USA (1,2). CRC causes 500,000 mortalities per year worldwide (3), and it causes mortality in 1/3 of patients (4). Despite improvement in CRC therapy, the disease-specific mortality remains high due to distant metastases and high recurrence rates (5,6). Therefore, novel promising therapeutic molecules are urgently required.

Tripartite motif-containing (TRIM) proteins are recognized as a subfamily of E3 ubiquitin ligases as they contain a really interesting new gene (RING) finger domain (7). It is well known that ubiquitylation is an important type of post-translational modification in eukaryotic cells for selective elimination of short-lived proteins, which also have critical roles in human cancer (8). To date at least 76 known TRIM proteins have been identified and studied (9), and it has been demonstrated that TRIM family proteins are involved in a broad range of biological processes including cell growth, apoptosis, transcriptional regulation and tumorigenesis (10-21). Additionally, previous studies have indicated that TRIM members regulate carcinogenesis, providing positive or negative effects on tumor progression and oncogenesis regulation. Upregulated expression levels of TRIM24 (15,22), TRIM25 (23), TRIM27 (24), TRIM28 (25), TRIM29 (26), TRIM31 (27) and TRIM32 (28) can be observed in multiple types of cancer, including breast, ovarian, lung, bladder, colon and gastric cancer and myeloma (9). Notably gene deletion and reduced expression of TRIM8 (29,30), TRIM13 (31,32), TRIM33 (33,34) and TRIM40 (35) were observed in cancer cells. A number of studies have attempted to elucidate the biological role of TRIM68 in the regulation of cancer; a study by Miyajima et al (36) found that the TRIM68 protein is significantly upregulated in human prostate cancer.

The present study sought to additionally investigate the expression patterns of TRIM68 in colorectal cancer cells by performing the RNA interference (RNAi) technique. To the best of our knowledge, this is the first time evidence has been provided that downregulated expression of TRIM68 suppresses the metastasis properties of colorectal cancer cells and induces cell cycle arrest in the G0/G1 phase. The present study shed light on the promising therapeutic value of TRIM68 against human colorectal carcinoma for future clinical applications.
Materials and methods

Cell culture. Human embryonic kidney HEK293T cells and colorectal cancer HCT116 and SW1116 cell lines were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). HCT116 and SW1116 cells were maintained in RPMI-1640 medium (cat. no. SH30809.01B; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). HEK293T cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; cat. no. SH30243.01B; Hyclone; GE Healthcare Life Sciences). RPMI-1640 and DMEM were supplemented with 10% fetal bovine serum (cat. no. S1810; BioWest, Riverside, MO, USA) and incubated at 37°C, in a humidified atmosphere of 5% CO2.

Lentiviral packaging and virus construction. A short hairpin RNA (shRNA) candidate sequence against the human TRIM68 gene (NCBI reference sequence, NM_018073.6) was screened to be the optimal sequence (5'-CATGGATTTGTATGGAGATTTCTCGAGAAAACCTCCACATTACTGTTTTTT-3'), sequence 1). Concurrently the negative control short hairpin RNA (5'-GCCAGGGTTTGGAGAATATCTCGAGATATTCTTCAACCCCTCGGTTTTT-3') was scrambled. The stem-loop- stem oligos (shRNAs) were synthesized, annealed, and ligated into the pNhel/PacI-linearized shRNA vector pFH-L (Shanghai Hollybio Co., Ltd., Shanghai, China). DNA sequencing was performed to assess that the lentiviral shRNA expressing vectors were constructed without mismatched sequences. The generated plasmids were termed pFH-L-shTRIM68 and pFH-L-shCon.

HEK293T cells (1.0x10⁵) were inoculated into 10 cm dishes and cultured for 24 h at 37°C to reach >80% confluence. The medium was replaced with serum-free DMEM 2 h prior to transfection. The plasmids, consisting of 10 µg of pFH-L-shTRIM68 or pFH-L-shCon, 7.5 µg of pHelper plasmids pSVG-I and 5 µg of pCMVAR8.92 (Shanghai Hollybio Co., Ltd., Shanghai, China), were added to the cells with a mixture of 0.95 ml Opti-MEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and rabbit anti-GAPDH antibody simultaneously (dilution 1:3,000; cat. no. 10494-1-AP; Proteintech Group, Inc., Chicago, IL, USA) overnight at 4°C, followed by 5 washes with PBST. Blots were then incubated with a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (dilution 1:5,000; cat. no. AP1717b; Abgent Biotech Co., Ltd., Suzhou, China) and rabbit anti-GAPDH antibody simultaneously (dilution 1:3,000; cat. no. 10494-1-AP; Proteintech Group, Inc., Chicago, IL, USA) overnight at 4°C, followed by 5 washes with PBST. Blots were then stained with enhanced chemiluminescence detection kit (Applygen Technologies, Inc., Beijing, China) in accordance with the manufacturer's protocol.

Western blot analysis. Prior to applying western blot analysis, SW1116 cells were subcultured and infected at 37°C with recombinant lentiviruses for 4 days. The cells were rinsed with cold PBS twice, and subsequently lysed in cell lysis buffer containing 10 mM EDTA, 4% SDS, 10% glycerin in 100 mM Tris-HCl (pH 6.8) (Shanghai Hollybio Co., Ltd.). Equal amounts of protein samples (60 µg) were subjected to 10% SDS-PAGE in a Tris/HCl buffer (pH 7.4) and electrotransferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) at 300 mA for 1.5 h. The membrane was blocked for 1 h in PBS-T buffer (PBS pH 7.4, 0.05% Triton-100) containing 1% (w/v) bovine serum albumin at room temperature. The membrane was then probed with a rabbit anti-TRIM68 antibody (dilution 1:500; cat. no. API717b; Abgent Biotech Co., Ltd., Suzhou, China) and rabbit anti-GAPDH antibody simultaneously (dilution 1:3,000; cat. no. 10494-1-AP; Proteintech Group, Inc., Chicago, IL, USA) overnight at 4°C, followed by 5 washes with PBST. Blots were then incubated with a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (dilution 1:5,000; cat. no. SC-2054; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature. The blots were developed using an enhanced chemiluminescence detection kit (Applygen Technologies, Inc., Beijing, China) in accordance with the manufacturer's protocol.

Cell proliferation assay and plate colony formation assay. HCT116 cells and SW1116 cells were exponentially cultured and infected with constructed lentiviruses at a MOI of 30 for 48 h. Transfected cells were washed and re-seeded in 96-well plates at a density of 5x10⁴ cells/well for 5 days, respectively. Reverse transcription was performed using quantitative RT-PCR kits (cat. no. KR103; Tiangen Biotech Co., Beijing, China). The mRNA expression level with or without vector transfection was detected, with β-actin acting as a normalizing control. The primers (5′ to 3′) used for gene expression analysis were designed using NCBI Primer-BLAST and presented as follows: TRIM68 forward, 5′-GGAGCCAATCTTGGAGCTTG-3′ and reverse, 5′-TGGACACGTTTCTGTCCCTC-3′; β-actin forward, 5′-GTTGAGATCCTGCGAAAGAC-3′ and reverse, 5′-AAAAGGTTGAA CGAAC-3′. Infected colorectal cells were trypsinized and harvested 5 days subsequent to transduction. Total RNA was isolated using TRIzol reagent (Gibco; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. In total, 1 mg of total RNA was used to synthesize the first strand of cDNA using 200 U/ml SuperScript II RT (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, 2 step RT-qPCR reactions were performed using the BioRad Connect Real-Time PCR platform (Bio-Rad Technologies, Inc., Hercules, CA, USA), which consisted of: Cycle 1 (1x), 95°C, 1 min; and cycle 2 with 40 repeated cycles, 95°C, 5 sec and 60°C, 20 sec. Absorbance data were collected at the end of every extension (60°C) and graphed using ABI 7500 Software v2.0.6 (Applied Biosystems, Inc., Carlsbad, CA, USA). The qPCR data were analyzed using the 2^-ΔΔCT method (S1).
microscopy software (Carl Zeiss AG, Oberkochen, Germany) were performed daily for 5 days.

Colony formation assays were performed by inoculating HCT116 and SW1116 cells into 6-well plates at the end of 96 h of lentivirus infection. In total, 500 cells per well of transfected HCT116 cells and 300 cells per well of SW1116 cells were cultured for 8 days. Colony cells were fixed with methanol and stained with 0.1% crystal violet. The number of colonies containing >50 cells was manually counted by microscopy (LSM710; Carl Zeiss AG).

Cell cycle analysis by flow cytometry. Cell cycle was analyzed by flow cytometry. Prior to cell cycle analysis, HCT116 cells were maintained in RPMI-1640 medium (cat. no. SH30809.01B+; Hyclone; GE Healthcare Life Sciences) and infected with the lentivirus for 5 days. Cells were harvested and washed twice in PBS, then fixed in 75% alcohol overnight at 4°C for permeabilisation. The next day, the cells were washed twice by cold PBS and resuspended in 1 ml PBS with 100 mg RNase A (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) prior to lucifugal incubation at 37°C for 30 min. The cells suspended in 1 ml PBS and 100 mg propidium iodide (cat. no. 81845; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) prior to lucifugal incubation at 37°C for 30 min. Samples were then analyzed on a FACS machine (Beckman, CA, USA) and detected in red fluorescence excitation with 20,000 counted.

Statistical analysis. All statistical analyses were performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). The significant differences between groups were compared using Student's t-test, and data were expressed as the mean ± standard deviation of 3 independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of recombinant lentiviral vectors for TRIM68 by GFP visualization. To investigate the biological role of TRIM68 in colorectal cancers, the present study performed a loss-of-function assay by RNAi: Control lentiviruses (Lv-shCon) and specific TRIM68-targeting lentiviruses (Lv-shTRIM68) were constructed. The most efficient type of shRNA expression cassette was selected and constructed into TRIM68-silencing lentiviral vector, and co-cultured with colorectal cancer cells (shTRIM68). Concurrently, negative blank controls of cells were cultured without lentiviral infection (Con). GFP expression in colorectal cancer cells was observed by fluorescent microscopy 96 h subsequent to infection. As shown in Fig. 1A, >80% of SW1116 or HCT116 cells were GFP positive, indicating infection efficacy. To determine the silencing efficiency, the expression level of TRIM68 mRNA was detected by RT-qPCR. In Fig. 1B and C, the non-silencing lentivirus encoded by the irrelevant sequence had a negligible effect on TRIM68 expression, but the TRIM68-silencing lentivirus significantly downregulated the mRNA expression by 72.3% in the SW1116 group (P=0.000) and by 68.9% in the HCT116 group (P=0.001). In parallel, the protein level was monitored by western blot analysis (Fig. 1D), providing evidence that the protein expression of TRIM68 was noticeably depleted, while control groups (shCon) maintained a similar expression level.

The results of the present study demonstrate that the lentiviruses constructed provide knockdown efficiency and provide an efficient tool to investigate the knockdown effect of TRIM68 in colorectal cancer cells in vitro.

Depletion of TRIM68 represses cell proliferation and colony formation ability of colorectal cancer cells in vitro. Cell proliferation was monitored for 5 days subsequent to colorectal cancer HCT116 or SW1116 cells being infected with the shTRIM68 lentivirus. Solubilized formazan crystals were detected to evaluate cell viability in this assay. It was observed that cells infected with shCon lentivirus exhibited no alteration in cell proliferation and viability (Fig. 2; shCon vs. Con), while a marked inhibition in TRIM68 inhibition group was observed on day 5 compared to non-infected cells (shTRIM68 vs. Con; P=0.000), suggesting that knockdown of TRIM68 led to repressed cancer cell proliferation.

Malignant cancer cells possess a strong ability to form colonies in vitro (37). In the present assay, the colony of HCT116 and SW1116 cells was markedly smaller when infected with TRIM68-silencing lentivirus (Fig. 3A and B) and the respective colony numbers were significantly lower compared with the control cells (Fig. 3C and D, shTRIM68 vs. shCon; P=0.001). The downregulation of TRIM68 significantly suppressed the colony formation capability of colorectal cancer cells.

The present study provided evidence that knockdown of TRIM68 by RNAi markedly suppresses the proliferation and colony formation ability of colorectal cancer cells in vitro.

TRIM68 silencing induces cell cycle arrest. To determine whether the decreased cell proliferation induced by knockdown of TRIM68 was due to cell cycle arrest, PI staining and flow cytometric analysis was performed. Fig. 4 shows that the G0/G1-phase DNA content in TRIM68-silenced cells (61.20%) was significantly increased compared with that in non-silenced cells (53.53%; P=0.001). In addition, the S phase DNA content in the shTRIM68 group (22.85%) was statistically decreased compared with that in the shCon group (29.12%; P=0.000), which indicated that the cell cycle of TRIM68-silenced cells was arrested in the G0/G1 phase. These findings are in agreement with the cell growth inhibition observed in the proliferation assay, which suggests that the TRIM68 gene modulates the growth of human colorectal cancer cell via cell cycle control. The population of sub-G1 infected HCT116 cells was analyzed. Marked accumulation of sub-G1 group cells (Fig. 4C) indicated apoptosis was proceeding when TRIM68 was absent, leading to inhibited cell proliferation and colony formation reduction.

Discussion

The present study has demonstrated that TRIM68 acts as a potential tumor growth promoter in colorectal cancer. TRIM68 expression was observed to be positively associated with cancer cell proliferation and colony formation ability. More significantly, cancer cell cycle arrest by down-regulating endogenous expression of TRIM68 indicated that TRIM68 might be a promising therapeutic target for colorectal cancer.
RNAi has been utilized as an efficient therapeutic tool against human malignant carcinomas in previous decades (38-40), and lentivirus therapy is the most effective type of vehicle for gene transduction and integration (41) with lower immunogenicity compared with the adenovirus. The present study identified and functionally characterized the potential therapeutic value of knocking down the TRIM68 gene in human colorectal cancer cell lines SW1116 and HCT116. It was demonstrated that a downregulated TRIM68 expression level resulted in attenuated cell proliferation and colony formation. Cell cycle arrest and apparent apoptosis were also observed.

TRIM proteins, containing a RING finger, a B-box (B2) and a coiled-coil (CC) motif in the structure (7), may be classified into subfamilies I-XI on the basis of their structural variation (10,42,43). TRIM68, structurally characterized as RING/B2/CC/PRY/Sprouty, has identical domains compared with TRIM22, TRIM27, TRIM39 and TRIM41 (42). The endogenous expression level of TRIM22, which is also termed stimulated transacting factor of 50 kDa, may be upregulated by p53 (44). Additionally, TRIM22 has been implicated in proliferation and differentiation of promyelocytic cells (45). TRIM27, also termed ret finger protein, may serve as a
small ubiquitin-related modifier E3 ligase, which has been implicated in cell cycle regulation, apoptosis and endogenous protein transportation (46). TRIM27 is highly expressed in various cancer cells (47); its expression level correlates with ERBB2 protein expression in breast cancer (24), and studies have demonstrated that TRIM27 triggers apoptosis by the activation of JUN N-terminal kinases (48). TRIM41 is able to associate with protein kinase C to control the function of other proteins by phosphorylation (49). TRIM39 is able to associate and bind to modulator of apoptosis 1 and regulate caspase-dependent apoptosis (50). To date, a number of studies have investigated the molecular role of TRIM68 in cancer cells. Miyajima et al. (36) provided evidence that TRIM68 interacts with androgen receptor (AR) and enhances its transcriptional activity in the presence of dihydrotestosterone. TRIM68 may be implicated as a cofactor for AR-mediated transcription and serve as a promising therapeutic target for prostate cancer. Additional studies will investigate the growth-related signaling
molecules that are associated with aberrant expression levels of endogenous TRIM68.

In conclusion, the present findings indicate that TRIM68 has a role in colorectal cancer cell proliferation. Depletion of TRIM68 triggers cell cycle arrest and apoptosis. Lentivirus-mediated TRIM68 knockdown delivers efficient blocking of cancer cell colony formation, which indicates that TRIM68 is critical for colorectal cancer tumorigenesis. The present study revealed that TRIM68 may be a novel therapeutical target for the treatment of human colorectal cancer and deserves additional study.

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