Knockdown of reticulon 4C by lentivirus inhibits human colorectal cancer cell growth

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Received May 13, 2014; Accepted January 21, 2015

DOI: 10.3892/mmr.2015.3569

Abstract. Colorectal cancer is the third most common type of cancer worldwide with high cell motility and metastatic potential. Reticulon 4C (RTN4-C) is the shortest isoform of the reticulon family protein RTN4, which may act to induce cell apoptosis and suppress tumor development. The aim of the present study was to determine the role of RTN4-C in colorectal cancer, and potentially identify a novel target for anti-tumor therapy. To investigate the biological role of *RTN4-C* in colorectal cancer, the expression levels of *RTN4-C* were initially analyzed in six colorectal cancer cell lines by reverse transcription-quantitative polymerase chain reaction and western blot analysis. In addition, lentivirus-based RNA interference was utilized to knock down *RTN4-C* expression in RKO and DLD-1 cells with low and high levels of RTN4-C, respectively. The rate of proliferation decreased in *RTN4-C* silenced RKO and DLD-1 cells compared with the control, as determined using MTT and colony formation assays. Flow cytometric analysis revealed that *RTN4-C* knockdown in RKO cells led to cell cycle arrest at the G0/G1 phase, particularly at the sub-G1 phase representing apoptotic cells. These results indicate that *RTN4-C* has an important role in colorectal cancer cell growth, which may provide a potential therapeutic approach for human colorectal cancer.

Introduction

Colorectal cancer is a cancer of the cells lining the colorectal epithelium and has been identified as the third most common type of cancer worldwide (1,2). It is caused by a combination of a variety of factors, including diet, genetic mutations in several different loci, and reproductive or exogenous hormone use (3). Colorectal cancer is characterized by high cell motility and metastatic potential. Metastases from the cancer, rather than the primary tumors, were observed to be the main causes of mortality (4). To prevent further deterioration, current therapies for colorectal cancer at different stages concentrate on fluorouracil plus leucovorin based conventional chemotherapeutics; however, these are accompanied by negative side effects, high recurrence rates of >50% and high costs (5). At present, the underlying molecular mechanisms to suppress human colorectal cancer development are poorly understood. Therefore, developing novel genetic therapeutic targets for the potential treatment of colorectal cancer is urgently required.

Reticulons (RTNs) have been identified as endoplasmic reticulum integral membrane proteins involved in multiple apoptotic signaling pathways (6). The family comprises four paralogs termed RTN1, RTN2, RTN3 and RTN4 (7). Previous studies have revealed that overexpression of RTN3 may induce cell apoptosis in normal HeLa cells via ectopic overexpression of Bcl-2 (8). A transcript variant 3 of RTN4 termed RTN4-C is highly expressed in the HEK293 human embryonic kidney cell line and was observed to induce cell apoptosis through the c-Jun N-terminal kinase-c-Jun signaling pathway (9). Additionally, *RTN4-C* inhibited the growth of SMMC7721 hepatocellular carcinoma cells via inducing apoptosis (10). However, the functional role of *RTN4-C* in colorectal cancer remains to be elucidated and requires further investigation.

To determine the role of *RTN4-C* in colorectal cancer, and potentially identify a novel target for anti-tumor therapy, the expression levels of *RTN4-C* were detected in multiple colorectal cancer cell lines: SW480, SW620, RKO, DLD-1, HCT116 and HT-29. Subsequently, lentivirus-mediated short hairpin RNA (shRNA) was adopted to target *RTN4-C* in colorectal cancer cells. Proliferation, colony formation and cell cycle assays were also conducted.

Materials and methods

Cell culture. SW480, SW620, RKO, DLD-1, HCT116, HT-29 human colorectal cancer cell lines and the HEK293T human embryonic kidney cell line were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SW480, SW620, RKO and DLD-1 cells were cultured in RPMI-1640 (Gibco-BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA).

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Key words: colorectal cancer, reticulon 4C, lentivirus, knockdown

HCT116 and HT-29 cells were cultured in McCoys 5A medium (Sigma-Aldrich, Poole, UK) supplemented with 10% FBS. HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% FBS. All cells were maintained in a humidified incubator with 5% CO_2.

Construction of RTN4-C shRNA-expressing lentivirus. The cDNA sequence of *RTN4-C* was obtained from NCBI (GenBank, NM_007008.2). Sequence-specific knockdown of *RTN4-C* was induced with an shRNA with the following sequence: 5'-CCGG GCTATATCTGAGGAGTTGGTTCTC-GAGAACCAACTCC TCAGATATAGCTTTTTTG-3'. The non-silencing shRNA had the following sequence: 5'-CCGGCCAAGGAAGTGCAATTGCATACTCGAGTATG-CAATTGCACTTCCTTGGTTTTTTG -3' and was used as a control. The shRNAs were purchased from Shanghai Hollybio (Shanghai, China). The two synthesized shRNAs were ligated into the pFH-L vector (Shanghai Hollybio, Shanghai, China) containing a green fluorescent protein (GFP) reporter driven by the cauliflower mosaic virus 35S promoter. The generated plasmids were transfected into HEK293T cells with the packaging vectors pVSVG-I and pCMVΔR8.92 (Shanghai Hollybio) using Lipofectamine 2000® (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. For lentivirus transfection, RKO and DLD-1 cells were cultured in 6-well plates at a density of $5x10^4$ cells/well and transfected with lentiviruses (shRTN4 or shControl) at a multiplicity of transfection of 20, respectively. Transfection efficiency was determined by counting GFP-expressing cells under a fluorescence microscope (Olympus Corporation, Tokyo, Japan) 96 h after transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The RT-qPCR experiment was conducted to elucidate *RTN4-C* gene expression in the SW480, SW620, RKO, DLD-1, HCT116 and HT-29 colorectal cancer cell lines. In addition, it was performed to detect the knockdown efficiency of the *RTN4-C* gene in cells transfected with shControl or shRTN4, RT-qPCR was performed following transfection for 5 days. TRIzol reagent (Invitrogen Life Technologies) was used to extract the total RNA of the cultured cells. The primers sequences used were as follows: Forward 5'-CTCCTCTGGTCTCGTCCTC-3' and reverse 5'-GTCCTCGTCCTCCTCTTCC-3' for *RTN4-C*; and forward 5'-GTGGACATCCGCAAAGAC-3' and reverse 5'-AAAGGGTGTAACGCAACTA-3' for β-actin. Fold changes in expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Following transfection for 5 days, RKO and DLD-1 cells were lysed in 2X SDS sample buffer (10 mM EDTA, 4% SDS and 10% Glycine in 100 mM Tris-HCl buffer, pH 6.8) for 1 h at 4˚C. Equal quantities of proteins (30 μg) were loaded and separated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Following blocking with 5% skimmed milk, the membranes were exposed to the primary antibodies RTN4-C (ab47085, 1:500 dilution; Abcam, Cambridge, MA, USA) and mouse monoclonal GAPDH (sc-32233, 1:3,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4˚C. Following incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (sc-2054, 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature, the expression of the target proteins were visualized with chemiluminescence reagents (ECL kit; Amersham Pharmacia Biotech, Amersham, UK). Bands were analyzed using the Imagequant densitometric scanner (Molecular Dynamics, Sunnyvale, CA, USA).

MTT proliferation assay. To detect the effect on proliferation of RKO and DLD-1 cells transfected by shRTN4 or shControl, an MTT assay was conducted. Lentivirus-transduced RKO and DLD-1 cells were reseeded in 96-well plates at a density of $2x10^3$ cells/well, respectively. Viable cell numbers were determined following seeding for 1, 2, 3, 4 and 5 days. A total of 10 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was added to each well. Following incubation for 3 h, 100 μl acidic isopropanol containing 10% SDS, 5% isopropanol and 0.01 mol/l HCl was added into each well to dissolve the formazan crystals. Finally, the absorbance at 595 nm was recorded using the Shimadzu UV-1603 spectrophotometer (Shimadzu, Kyoto, Japan).

Plate colony formation assay. Lentivirus-transduced RKO and DLD-1 cells were cultured in 6-well plates at a density of 400 cells/well. The medium was changed regularly. After 8 days of culture, the adherent cells were washed twice with phosphate-buffered saline and fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed cells were then stained with crystal violet (Beyotime Institute of Biotechnology, Haimen, China). The number of colonies (>50 cells/colony) were observed and counted using a fluorescence microscope (Olympus BX50; Olympus, Tokyo, Japan).

Cell cycle analysis. The RKO cells transfected with shRTN4 or shControl were seeded at $5x10^4$ cells/dish in 6-cm dishes and incubated for 72 h. Cell cycle progression was subsequently monitored using a flow cytometer (Navios; Beckman Coulter, Miami, FL, USA) and cell cycle analysis kit (C1052; Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Statistical analysis. All data are expressed as the mean ± standard deviation from three independent experiments. Student's t-test was performed for statistical analysis. Statistical analyses were conducted using SPSS version 19.0 (IBM, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Differential transcription and translation of RTN4-C in six colorectal cancer cell lines. Firstly, RT-qPCR was used to analyze *RTN4-C* expression at the transcriptional level in six colorectal cell lines SW480, SW620, RKO, DLD-1, HCT116, and HT-29 (Fig. 1A). As a result, the SW620 cells exhibited the highest expression level of *RTN4-C* among these cells, while RKO cells exhibited the lowest. The SW480, DLD-1, HCT116 and HT-29 cell lines exhibited relatively high expression patterns of *RTN4-C* compared with RKO cells. The translational levels of RTN4-C in these six cell lines were detected using western blot analysis. As shown in Fig. 1B, SW480, DLD-1, HCT-116

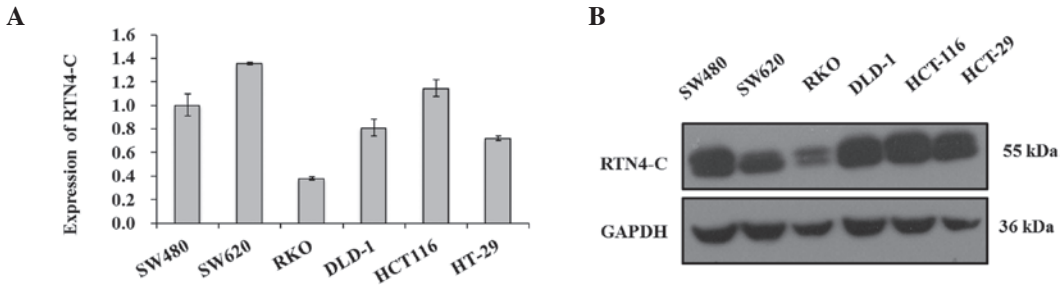

Figure 1. Expression of *RTN4-C* in six human colorectal cancer cell lines. Expression patterns of *RTN4-C* in colorectal cell lines of SW480, SW620, RKO, DLD-1, HCT-116, and HCT-29 were evaluated by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis. RTN4-C, Reticulon 4C.

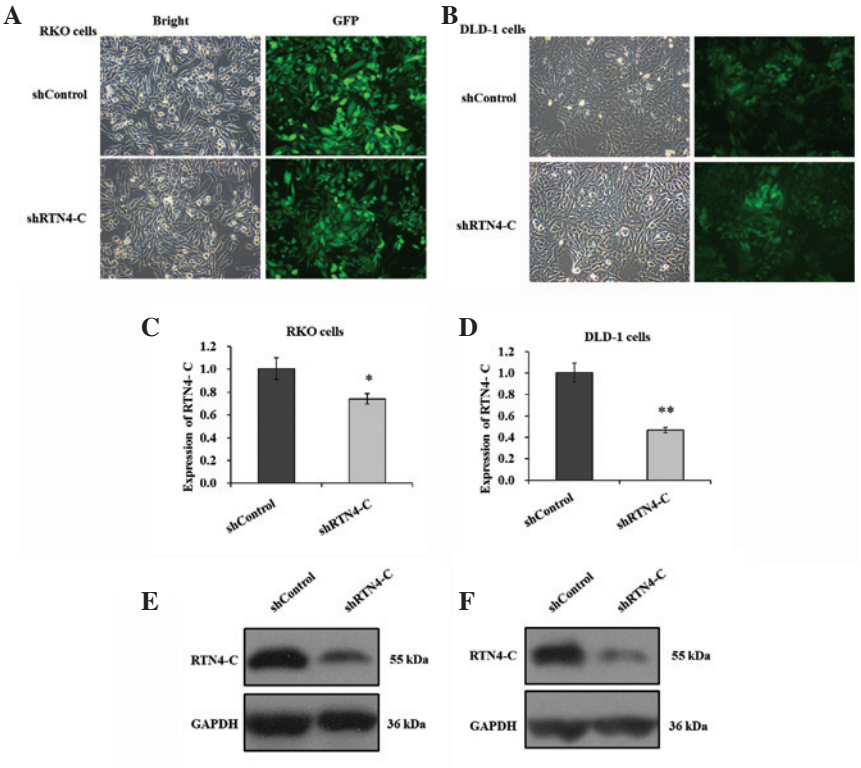

Figure 2. Validation of *RTN4-C* knockdown efficiency in shRTN4-transfected colorectal cancer cells. GFP expression in (A) RKO and (B) DLD-1 cells indicated successful transfection using lentiviral-delivered shRNA against *RTN4-C*. Knockdown efficiency of *RTN4-C* was confirmed by reverse transcription-quantitative polymerase chain reaction in (C) in RKO and (D) DLD-1 cells and western blot analysis in (E) RKO and (F) DLD-1 cells. *P<0.05 and **P<0.01, compared with control. RTN4-C, Reticulon 4C; GFP, green fluorescent protein.

and HT-29 cells exhibited high expression levels of RTN4-C protein. The RKO cells, which exhibited the lowest expression levels of *RTN4-C* mRNA expression also revealed the lowest translational pattern. However, SW620 cells with the highest transcriptional level of *RTN4-C* exhibited a relatively low protein expression. To investigate the biological function of *RTN4-C* in colorectal cancer, the DLD-1 and RKO cell lines with relatively high and low RTN4-C expression patterns were selected.

Successful depletion of RTN4-C in DLD-1 and RKO cells by lentivirus-derived RNA interference (RNAi). RKO and DLD-1 cells transfected with recombinant lentiviruses harboring shRTN4 or shControl were used to assess the effects of RTN4-C knockdown on colorectal cancer growth. As shown in Fig. 2A and B, the transfection rates in RKO and DLD-1 cells were >80%, as detected by GFP-fluorescence. The mRNA levels of *RTN4-C* were significantly decreased in the two cell lines following shRTN4 transfection compared with shControl, as measured by RT-qPCR (Fig. 2C and D). The knockdown efficiency of *RTN4-C* was 26.2 and 53.2% in RKO and DLD-1 cells, respectively. The endogenous RTN4-C protein level was estimated in the two cell lines using western blot analysis. This revealed that the expression levels of RTN4-C were reduced markedly in shRTN4-transfected RKO and DLD-1 cells in comparison with shControl (Fig. 2E and F). In conclusion, it was inferred that RTN4-C depletion was successfully introduced in DLD-1 and RKO cells via shRTN4 transfection.

Cell proliferation and colony formation of DLD-1 and RKO cells are inhibited by RTN4-C downregulation. To evaluate whether the proliferation of RKO and DLD-1 cells

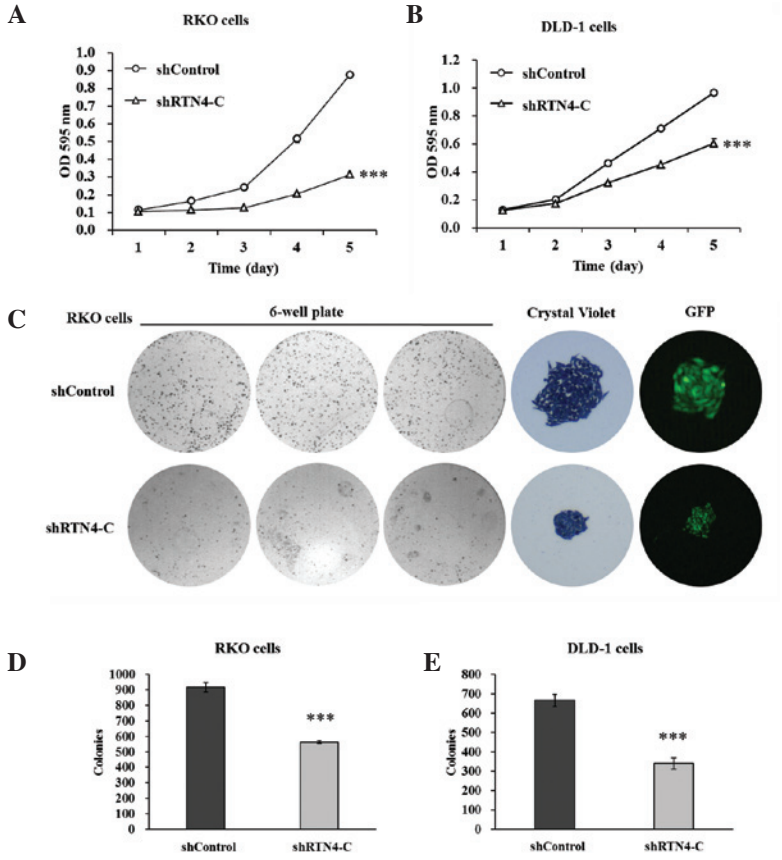

Figure 3. *RTN4-C* knockdown inhibited the growth of colorectal cancer cells. Cell proliferation was detected using an MTT assay in (A) RKO and (B) DLD-1 cells following shRTN4 transfection. (C) Representative images of colony formation in RKO cells. Colony numbers in (D) RKO and (E) DLD-1 cells following shRTN4 transfection. ***P<0.001, compared with control. RTN4-C, Reticulon 4C; GFP, green fluorescent protein.

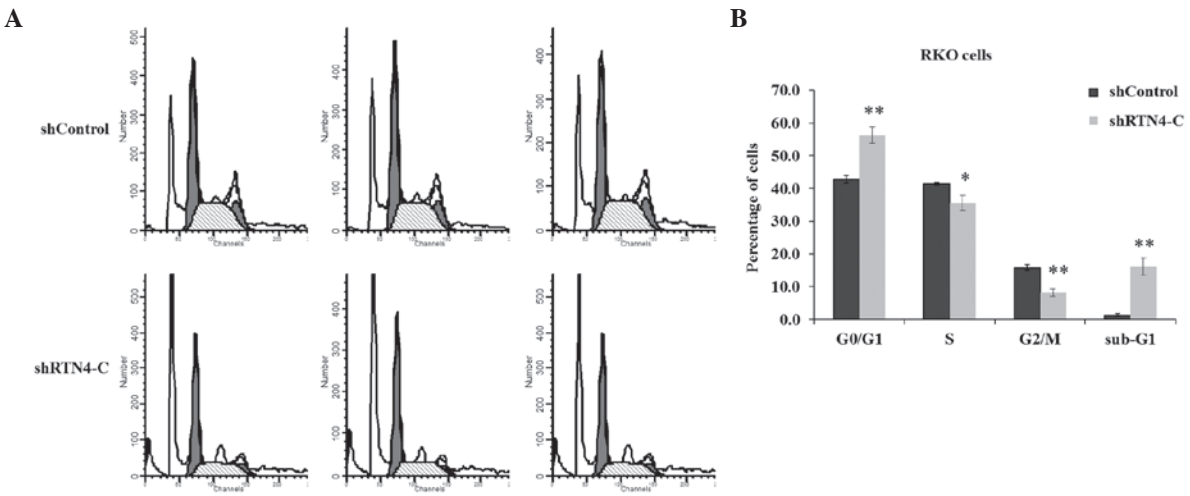

Figure 4. Effect of *RTN4-C* knockdown on cell cycle progression in RKO cells. (A) Flow cytometric histograms of RKO cell numbers under each phase. (B) RKO cells were blocked in G0/G1 and sub-G1 phases in shRTN4 group compared with control. *P<0.05 and **P<0.01, compared with control. RTN4-C, Reticulon 4C.

was affected by *RTN4-C* downregulation, an MTT assay was performed. Figure 3A and B show that the proliferation rates were decreased markedly in shRTN4-transfected RKO and DLD-1 cells relative to the shControl. The proliferation of shRTN4-transfected RKO cells was suppressed by 60 and 64% at days 4 and 5 compared with the shControl. The proliferation of DLD-1 cells was reduced by 36 and 37% at days 4 and 5, respectively, in the shRTN4 group compared with the shControl group.

In addition, a colony formation assay was conducted to determine the effect of *RTN4-C* on the *in vitro* tumorigenicity of colorectal cancer cells. The number of colonies was fewer and the size of each colony was markedly smaller in the shRTN4 groups compared with the shControl groups (Fig. 3C).

Total colony numbers were reduced significantly as indicated in Fig. 3D and E. There were 564±28 colonies formed of shRTN4-transfected RKO cells, while 917±10 colonies of the shControl cells formed. There were 340±18 colonies formed of shRTN4-transfected DLD-1 cells, while there were 666±30 colonies in the shControl groups. These results demonstrated that *RTN4-C* knockdown may inhibit colorectal cancer cell proliferation and colony formation.

RTN4-C knockdown blocks cell cycle progression in RKO cells. To examine the mechanisms of cell growth inhibition by lentivirus-mediated *RTN4-C* knockdown, the cell cycle progression of RKO cells was determined using flow cytometry (Fig. 4A). As shown in Fig. 4B, cell numbers in shRTN4 groups were significantly increased in the G0/G1 phase and the sub-G1 phase representing apoptotic cells, compared with those in the shControl groups. The percentage of cells was increased by ~24 or ~92% in the G0/G1 phase or the sub-G1 phase, respectively, following shRTN4 transfection. Whereas the percentage of cells was decreased by 14 or 48% in the S phase or the G2/M phase following shRTN4 transfection. Knockdown of *RTN4-C* may inhibit colorectal cancer cell growth possibly via induction of cell cycle arrest and apoptosis.

Discussion

Colorectal cancer is regarded as one of the most common malignancies and it is difficult to treat (11,12). Previous studies have indicated that >55,000 mortalities occur due to metastatic colorectal cancer annually in the United States alone (13). To gain insight into the biological function of *RTN4-C* in colorectal cancer, lentivirus-based *RTN4-C* knockdown in RKO and DLD-1 colorectal cancer cells were constructed and further investigated. The results revealed that the cell proliferation and colony formation were restrained in shRTN4 transfected RKO and DLD-1 cells.

Several previous studies have reported that changes in cell cycle distribution contribute to cell proliferation inhibition. Notably, microRNA-125b arrested the cell cycle at the G1 to S transition to prevent cell proliferation and metastasis in human liver cancer (14). Nemo-like kinase expression knockdown blocks the G0/G1 phase to S phase transition to inhibit human adenosquamous carcinoma cells CAL-27 proliferation and colony formation (15). During the present study, knockdown of RTN4 caused a decrease in the percentage of cells in S phase and G2/M phase, but resulted in an increase of cells in G0/G1 phase, in particular there was an increased number of apoptotic cells, as indicated by an increase of cells in sub-G1 phase, which suggests that *RTN4-C* may regulate G0/G1 to S phase transition and inhibit cell apoptosis. Nevertheless, cell apoptosis was promoted in SMMC7721 hepatocellular carcinoma cells with an increased level of *RTN4-C* (10). Additionally, Chen *et al* (9) reported that *RTN4-C* induced HEK293 cell apoptosis through its involvement in the c-Jun N-terminal kinase-c-Jun pathway. These studies suggest that *RTN4-C* may function differentially in cancer cell growth and apoptosis in various human organs. In the present study, *RTN4-C* knockdown may have altered gene expression to directly or indirectly activate the cell apoptosis pathway in colorectal cancer cells. In conclusion, *RTN4-C* knockdown may induce cell apoptosis and block cell cycle progression at the G0/G1 phase to suppress cell proliferation and colony formation in colorectal cancer cells. However, the altered gene expression and the activated apoptosis signaling pathway remain to be elucidated and require further study.

It is well-established that tumor metastasis is facilitated by cell proliferative activity (16). Therefore, controlling cell proliferation may contribute to the prevention of tumor development. The present study demonstrated that knockdown of *RTN4-C* by RNAi resulted in a significant inhibition of colorectal cancer cell growth via induction of G0/G1 phase cell cycle arrest and apoptosis. The present study improves the understanding of *RTN4-C* function in colorectal cancer and may aid in the development of a novel therapeutic approach.

Acknowledgements

The present study was supported by the Research Fund of the Science and Technology Commission of Shanghai Municipality (grant no. 12ZR1418000).

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