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Effect of siRNA-mediated knockdown of *eIF3c* gene on survival of colon cancer cells

Ning SONG[§], Yan WANG[§], Xiao-dong GU, Zong-you CHEN, Liu-bin SHI[†‡]

(*Department of General Surgery, Huashan Hospital, Fudan University, Shanghai 200040, China*)

[†]E-mail: linbinshi@163.com

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Abstract: Eukaryotic initiation factor subunit c (*eIF3c*) has been identified as an oncogene that is over-expressed in tumor cells and, therefore, is a potential therapeutic target for gene-based cancer treatment. This study was focused on investigating the effect of small interfering RNA (siRNA)-mediated *eIF3c* gene knockdown on colon cancer cell survival. The *eIF3c* gene was observed to be highly expressed in colon cancer cell models. The expression levels of the gene in *eIF3c* siRNA infected and control siRNA infected cells were compared via real-time polymerase chain reaction (PCR) and western blotting analysis. Cell proliferation levels were analyzed employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and colony formation assays. Furthermore, the effects of *eIF3c* gene knockdown on the cell cycle and apoptosis were analyzed using flow cytometry. The results showed that suppression of *eIF3c* expression significantly (P<0.001) reduced cell proliferation and colony formation of RKO colon cancer cells. The cell cycle was arrested by decreasing the number of cells entering S phase. Further, apoptosis was induced as a result of *eIF3c* knockdown. Collectively, *eIF3c* deletion effectively reduced the survival of colon cancer cells and could be used as a therapeutic tool for colon cancer therapy.

Key words: Eukaryotic initiation factor subunit c (*eIF3c*), Colon cancer, RKO cells, Small interfering RNA (siRNA)
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1 Introduction

Colon cancer is the second major cause of malignancy-related death worldwide after lung cancer (Mclornan *et al.*, 2010). Although much research has been carried out on the development of chemotherapeutics, because of inherent or acquired drug resistance, optimal chemotherapeutics for the treatment of colon cancer have not yet been found (Longley *et al.*, 2006). Due to this resistance and the high risk factors associated with chemotherapy, other means of cancer treatment are constantly being researched. Gene expression profiling provides insights into understanding and identifying different subsets of genes with differential expression during cancer progression (van de Veer *et al.*, 2002). This understanding has led to gene-targeted therapies to control cancer cell survival. Among those technologies, the use of interfering RNA (RNAi) to manipulate identified genes related to cancer progression has been gaining more attention in cancer therapy research. This method has been used in studies related to colon cancer on the inhibition of the β-catenin gene by small interfering RNA (siRNA). Successful silencing resulted in down-regulation of β-catenin-dependent gene expression and inhibited cellular proliferation in both in vitro and in vivo models (Verma *et al.*, 2003). Therefore, the identification of critical genes related to colon cancer cell progression and their silencing may be a promising approach in cancer gene targeting therapy.

Translation is a fundamental process which can be divided into initiation, elongation, termination, and ribosome recycling. During the initiation phase of translation, the eukaryotic initiation factor 3 (eIF3) complex is essential as it is required for the interaction between the 40S ribosomal subunit which binds

‡ Corresponding author
§ The two authors contributed equally to this work

to the ternary complex (eIF2-GTP-methionine) and the 5′-end of the mRNA (Sonenberg and Hinnebusch, 2009). The expression of eIF subunits in cancers has not been well characterized. However, it has been found recently that altered expression of eIF subunits in tumors such as the over-expression of *eIF4e*, *eIF2a*, *eIF3c*, or *eIF3h* and the under-expression of *eIF3e* can induce cellular proliferation by initiating protein translation (Scoles *et al.*, 2006). Scoles *et al.* (2006) showed that the *eIF3c* gene has oncogenic properties and that its over-expression induces the proliferations of gliomas, meningiomas, and ovarian carcinomas. *eIF3c* is the p110 subunit of *eIF3*. In the initiation of protein translation, *eIF3c* has a significant role in binding to two AUG recognition factors, *eIF1* and *eIF5*. The *eIF3c* gene *eIF3S8* is located on chromosome 16p11.2, which is an unstable region of the genome, and therefore duplication of the entire *eIF3S8* gene is observed regularly (Loftus *et al.*, 1999). This high duplication rate of the *eIF3c* gene may contribute to higher expression levels of *eIF3c* in various tumors (Scoles, 2008). Furthermore, it has been found that *eIF3c* is associated with neurofibromatosis 2 (NF2) tumor suppressor proteins. High expression levels of *eIF3c* have adverse effects on NF2 expression and thereby induce cell proliferation (Scoles *et al.*, 2006) via the hyperactivation of translation initiation machinery (Zhang *et al.*, 2007).

Based on these literature and clinical findings, the current study was focused on the use of *eIF3c* knockdown as a therapeutic tool for colon cancer therapy. Lentivirus-mediated *eIF3c* siRNA delivery was used as the mode of knockdown, and the effects of knockdown on colon cancer cell proliferation were observed in RKO colon cancer cell models.

2 Materials and methods

2.1 Cell culture

Colon cancer cells (RKO, HCT116, SW480, SW620, and LoVo) and human embryonic kidney cells (HEK293) were obtained from the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 5% CO_2.

2.2 Construction of *eIF3c* short hairpin RNA (shRNA) lentivirus

The sequences of the siRNA for *eIF3c* and control siRNA were synthesized as 5′-GAC CAT CCG TAA TGC CAT GAA-3′ and 5′-TTC TCC GAA CGT GTC ACG T-3′, respectively. These nucleotide sequences were inserted into the plasmid using the shRNA expressing vector pFUGW (Hollybio, Shanghai, China) and lentiviral packing vectors pVSVG-1 and pCMVΔR8.92 (Hollybio, Shanghai, China). The identities of the generated lentiviral based shRNA expressing vectors were confirmed by DNA sequencing. For the transfection, HEK293T cells (1×10^7) were seeded in 10-cm dishes and cultured for 24 h to reach 70%–80% confluence. Two hours before transfection, the medium was replaced with serum-free DMEM and the three plasmids, including 20 μg of silencing sequence or control sequence, 15 μg of packaging vector pCMVΔR8.92, and 10 μg of VSVG-1 plasmid, were added to 200 μl of opti-MEM and 15 μl of Lipofectamine 2000. The mixture was added to the cells and incubated for 8 h prior to replacement with 10 ml of DMEM medium containing 10% FBS. The supernatant was collected after 48 h of transfection and lentiviral particles were harvested by ultra-centrifugation (4000×*g*) at 4 °C for 10 min (Soneoka *et al.*, 1995).

2.3 Infection of RKO cells with *eIF3c* shRNA lentivirus or control lentivirus

For the infection of RKO cells with *eIF3c* shRNA or control shRNA, cells were seeded onto 96-well plates (50 000 cells/well) and after 24 h of incubation, the culture medium was replaced with opti-MEM medium containing the lentivirus. After 24 h, virus-containing incubation medium was replaced with fresh medium and incubated for another 72 h. The success of infection was examined by counting the green fluorescence emitted by the green florescence protein (GFP) within the lentivirus particles under fluorescence microscopy 96 h after infection.

2.4 RNA extraction and real-time polymerase chain reaction (PCR) analysis

RNA was extracted from RKO cells infected with the lentivirus for 5 d. Cells were lysed with Trizol reagent (Invitrogen, USA) and the total RNA was extracted from the lysate using standard

procedures. The extracted RNA was used to synthesize the cDNA using the Promega M-MLV cDNA synthesis kit according to the manufacturer's instructions. For real-time PCR analysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference. The forward and reverse primers used were: for *eIF3c*, forward primer 5′-AGA TGA GGA TGA GGA TGA GGA C-3′ and reverse primer 5′-GGA ATC GGA AGA TGT GGA ACC-3′, for GAPDH, forward primer 5′-TGA CTT CAA CAG CGA CAC CCA-3′ and reverse primer 5′-CAC CCT GTT GCT GTA GCC AAA-3′. Relative gene expression level of the *eIF3c* gene in the presence or absence of *eIF3c* siRNA compared to that of GAPDH was calculated using the $2^{-\Delta\Delta CT}$ analysis method.

2.5 Reverse transcriptase PCR

RNA extraction and cDNA synthesis were carried out as described above. GAPDH was used as an internal control. The primers used for reverse transcriptase PCR were as follows: *eIF3c*, forward primer 5′-AGA TGA GGA TGA GGA TGA GGA C-3′ and reverse primer 5′-GGA ATC GGA AGA TGT GGA ACC-3′; GAPDH, forward primer 5′-TGA CTT CAA CAG CGA CAC CCA-3′ and reverse primer 5′-CAC CCT GTT GCT GTA GCC AAA-3′. The experiment was performed according to the users' manual and 1 μg cDNA was used as the template. The PCR products were loaded onto an agarose gel containing ethidium bromide (EB) for electrophoresis.

2.6 Western blotting analysis

Western blotting was carried out to evaluate *eIF3c* gene expression levels in siRNA-infected colon cancer cells (5 d after infection) compared to those of control siRNA-infected cells. To isolate the cellular protein, the cells were washed with cold phosphate buffered saline (PBS) and lysed with radio-immune precipitation assay (RIPA) buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% nonidet P (NP)-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] containing phenylmethyl sulfonylfluoride (PMSF) (1 mmol/L) and protease inhibitors (2 μg/ml; Protease Inhibitor Cocktail Set III, Calbiochem) on ice for 30 min. The protein content was measured by the Lowry method and the protein concentration of each sample was adjusted to 2 μg/μl. Then, 20 μl of collected protein was loaded onto a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrophoresed at 60 V for 4 h. The gel was transferred to polyvinylidene difluoride membrane and the proteins were detected by respective antibodies using electrochemiluminescence (ECL) kit (Amersham, USA) and exposed to X-ray film. GAPDH was used as control and detected by an anti-GAPDH antibody (Santa Cruz Biotechnology). The bands on X-ray films were quantified with an ImageQuant densitometric scanner (Molecular Dynamics, USA).

2.7 Analysis of the effect of *eIF3c* knockdown on cell proliferation

The level of RKO cell proliferation after *eIF3c* siRNA infection was analyzed by counting the number of viable cells with a Cellomics ArrayScan® VTI HCS reader (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Infected cells were seeded into a 96-well plate at a concentration of 2000 cells/well and 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added into each well once daily for 5 d. The Cellomics machine detects viable cells by their green fluorescence emission. The number of viable cells was calculated for each of five days after plating. All experiments were performed in triplicates.

2.8 Colony forming assay

RKO cells transfected with *eIF3c* siRNA or control siRNA for five days were collected and seeded in six-well plates at a density of 500 cells/well. The medium was changed every three days. After two weeks of culture, the cells were washed and fixed in 4% paraformaldehyde. The fixed cells were stained by adding freshly prepared diluted Giemsa stain for 20 min. The cells were rinsed with distilled water and colonies with more than 50 cells were counted using a fluorescence microscope.

2.9 Cell cycle analysis

The cell cycle distribution was analyzed by flow cytometry assay following propidium iodide (PI) staining. The infected cells were seeded onto a six-well plate at a density of 1×10^6 cells/well. After 24-h incubation, the cells were collected and washed with ice cold PBS. The collected cells were fixed in

70% ethanol and incubated for 30 min at 4 °C. The ethanol was discarded by centrifugation and the cell pellet was suspended in 100 μg/ml of DNase-free RNase for 30 min at 37 °C. Then PI solution (100 μg/ml) was added to the cell suspension which, after filtering through a 50-μm nylon mesh, was then analyzed using a flow cytometer (FACS Cali-bur, BD Biosciences).

2.10 Detection of apoptosis

Apoptotic cells were detected using the Annexin V-APC Apoptosis Detection Kit (BioVision, USA). *eIF3c* siRNA- and control siRNA-infected cells were seeded onto a six-well plate at a density of 5×10^5 cells/well. After five days of incubation the cells were collected and washed with ice cold PBS. Cells were resuspended in 100 μl of binding buffer followed by the addition of 5 μl of Annexin V solution and 5 μl of PI solution. The mixture was incubated for 5 min in the dark before being analyzed using a flow cytometer.

2.11 Statistical analysis

All data are expressed as mean±standard deviation (SD) of three independent experiments and the error bars represent the SD. Data were statistically analyzed using SPSS 16.0 software by a paired *t*-test analysis method. $P<0.01$ was considered as statistically significant.

3 Results

3.1 *eIF3c* siRNA infection down-regulated *eIF3c* expression in RKO cells

The expression levels of the *eIF3c* gene in different colon cancer cell models were examined and the results indicated that *eIF3c* was expressed in all models (Fig. 1a). RKO cells with higher *eIF3c* expression were selected for the further analysis. The successful infection of *eIF3c* siRNA or control siRNA into RKO cells was confirmed via a microscopic detection of green fluorescence. *eIF3c* siRNA infected cells (Lv-*eIF3c* shRNA) showed more than 80% successful infection compared to the control group (Lv-Con) (Fig. 1b). The protein expression levels of *eIF3*c in *eIF3c* siRNA-infected and in control sequence-infected cells were compared by western blotting. The results obtained (Figs. 1c and 1d) clearly indicated that siRNA treatment had down-regulated the protein expression level of eIF3c by 70% compared to that of the control group. Therefore, these results demonstrate that the higher expression level of *eIF3c* in RKO cells was suppressed by siRNA infection.

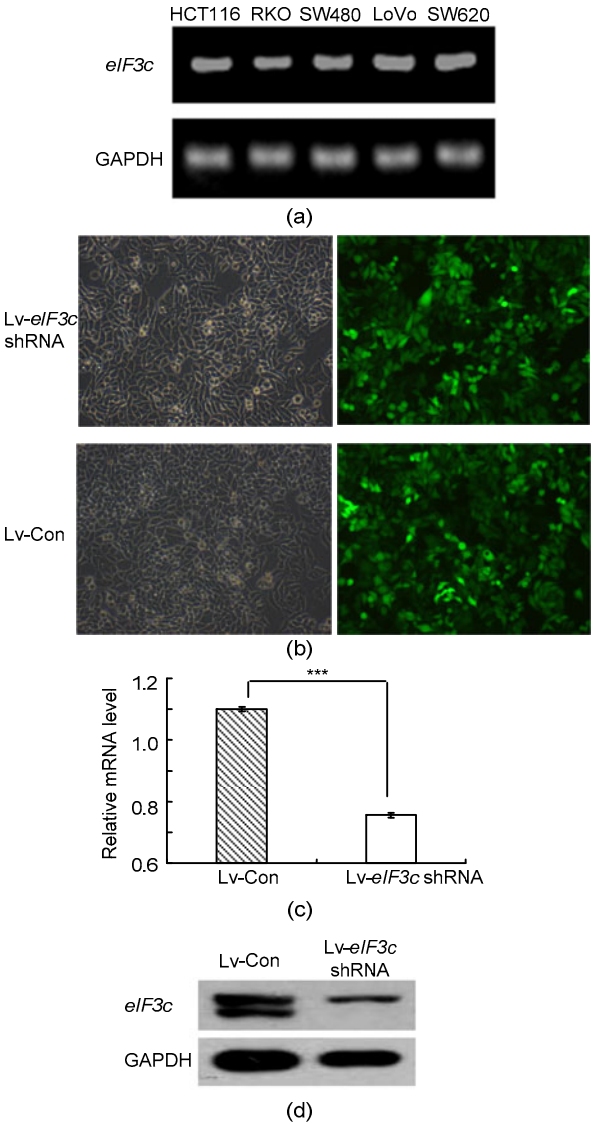


Fig. 1 Effect of knockdown of *eIF3c* on the *eIF3c* mRNA and protein expression levels in RKO cells

(a) mRNA expression levels of *eIF3c* in different colon cancer cells; (b) Light microscopic (left) and fluorescent microscopic (right) pictures of RKO cells; (c) Quantitative real-time PCR data of siRNA mRNA levels following the knockdown compared with the control group; (d) eIF3c protein expression levels in *eIF3c* siRNA-infected and control siRNA-infected lung cancer cells. *** $P<0.001$ in comparison with the control

3.2 *eIF3c* siRNA infection suppressed the cell proliferation of RKO cells

The effect of the knockdown *eIF3c* gene on cell proliferation was analyzed by counting the number of viable cells using a Cellomics Array Scan® VTI HCS reader. The results indicated that one and two days after infection cell proliferation was very similar in the *eIF3c* siRNA-infected group and the control group (Fig. 2).

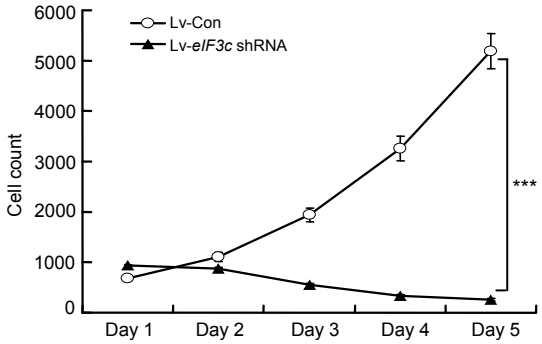


Fig. 2 Effect of *eIF3c* knockdown on the proliferation of RKO cells

*** $P<0.001$ in comparison with the control

However, cell proliferation significantly ($P<0.001$) decreased from the third day onwards, such that after five days, the number of cells in the control group was 60 times higher than that in the *eIF3c* siRNA-infected group. These results indicated that deletion of *eIF3c* had a significant influence on the survival of RKO colon cancer cells.

3.3 *eIF3c* siRNA infection suppressed colony formation in RKO cells

RKO colon cancer cells tend to grow in colonies under normal conditions. Therefore, the effect of *eIF3c* deletion on colony formation was determined by performing a colony formation assay. The number and size of colonies were observed in *eIF3c* siRNA infected-cells and control siRNA-infected cells. The number of the colonies decreased significantly ($P<0.01$) with the *eIF3c* deletion, showing a 3.5-fold decrease compared to the control group (Figs. 3a and 3c). The size of the colonies in *eIF3c* siRNA-infected cells was smaller than that of those in the control group (Fig. 3b). It appears that because of the decrease in cell proliferation in the absence of the *eIF3c* gene, the number of colonies decreased.

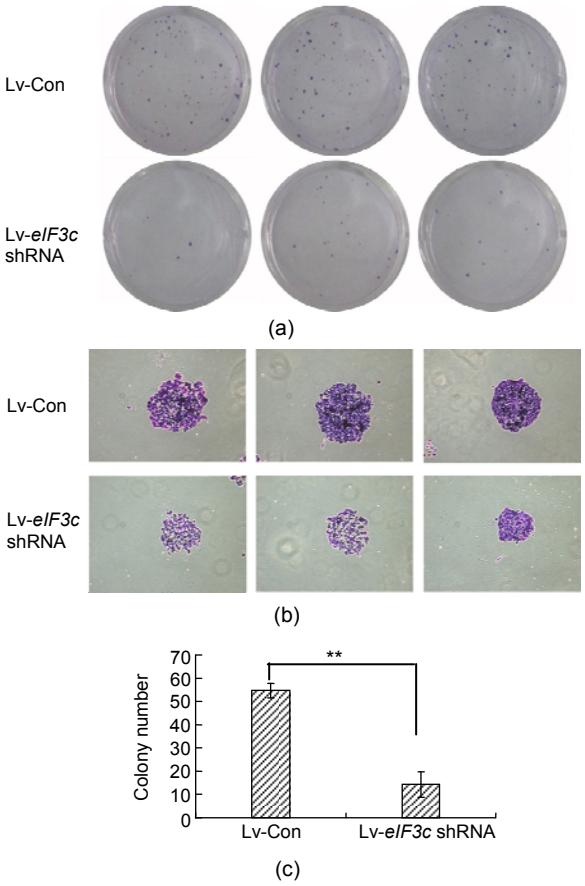
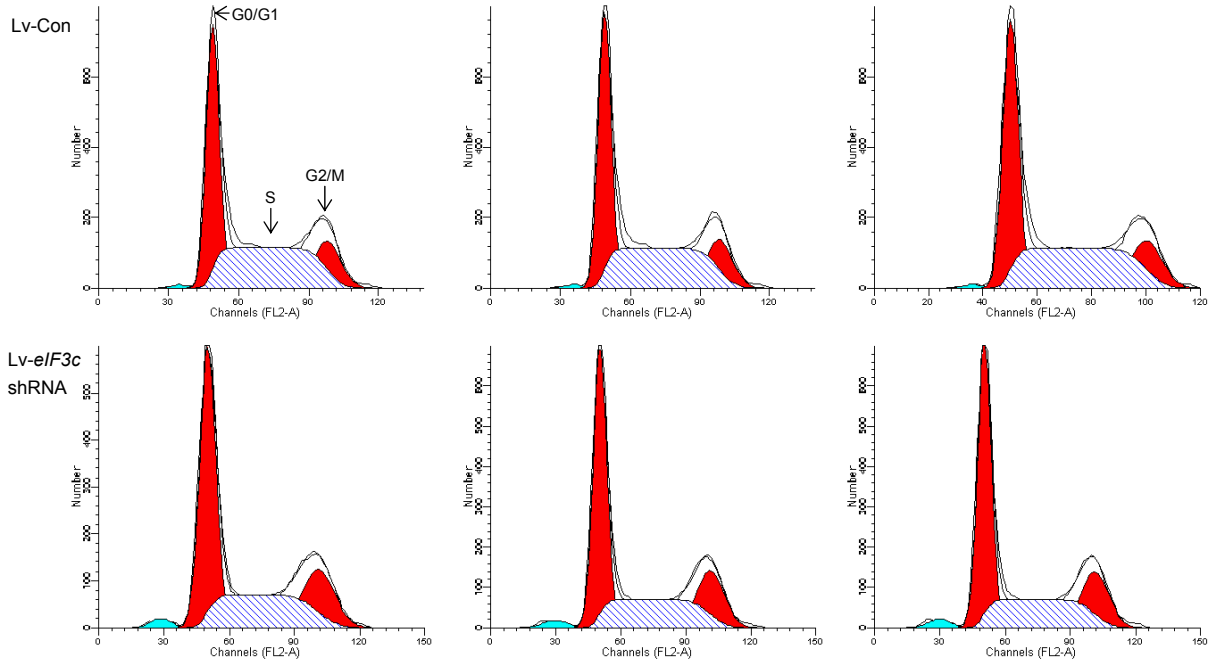


Fig. 3 Effect of *eIF3c* knockdown on the colony forming ability of RKO cells

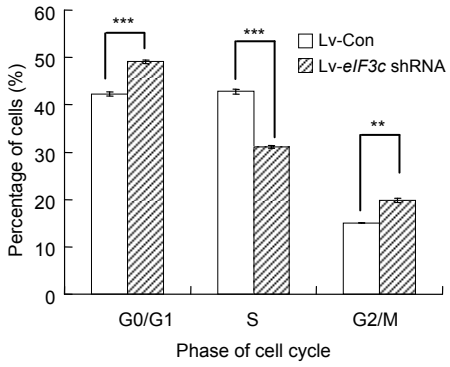
(a) Giemsa staining of the cancer cell colonies under light microscope; (b) Images showing the reduction in the number of colonies following Giemsa staining; (c) Numerical representation of the number of colonies in the *eIF3c*-suppressed group and the control group. ** $P<0.01$ in comparison with the control

3.4 *eIF3c* gene deletion arrested the cell cycle of RKO cells

As the *eIF3c* gene was found to be essential for cell proliferation, the effect of *eIF3c* deletion on the cell cycle distribution of colon cancer cells was analyzed using a flow cytometer. The cell cycle distribution of *eIF3c* siRNA-infected cells was different from that of control cells (Fig. 4a). The *eIF3c*-deleted group showed an 8% increase in cells in G0/G1 phases ($P<0.001$) and a 5% increase in cells in the G2/M phases (Fig. 4b). However, cell numbers in the S phase decreased by 12% ($P<0.001$) with the *eIF3c* deletion (Fig. 4b). Collectively, these results indicate that the *eIF3c* gene and the RKO cell cycle distribution were significantly correlated.



(a)



(b)

Fig. 4 Effect of *eIF3c* knockdown on the cell cycle distribution of RKO cells

(a) The histograms obtained from flow cytometric analysis of *eIF3c* deleted and non-deleted cells from three independent trials; (b) Percentages of RKO cells at different phases of the cell cycle following *eIF3c* siRNA or control siRNA infection. *** $P<0.001$, ** $P<0.01$ in comparison to the control

3.5 *eIF3c* gene knockdown induced apoptosis in RKO cells

The effect of *eIF3c* gene deletion on apoptosis was analyzed by comparing the apoptosis levels in *eIF3c* knockdown and control cells. *eIF3c* gene suppression induced apoptosis in RKO cells ($P<0.05$) (Fig. 5).

The percentage of apoptotic cells doubled in the absence of the *eIF3c* gene compared to that of the control group (Fig. 5b). Therefore, the *eIF3c* gene may have a strong influence on the survival of RKO cells and in the absence of *eIF3c*, the survival rate decreases via induction of apoptosis.

4 Discussion

Targeted treatment methods have not yet been discovered for the treatment of colon cancer despite it being one of the most prominent causes of malignancy-related deaths worldwide (Karaayvaz *et al.*, 2011). Therefore, this study was focused on the identification of possibilities for the manipulation of cancer-related genes as a mode of colon cancer therapy. *eIF3c* is over-expressed in some tumors, including seminomas and meningiomas, where it facilitates cell survival. It has been found that *eIF3c* can interact with the NF2 tumor suppressor merlin/

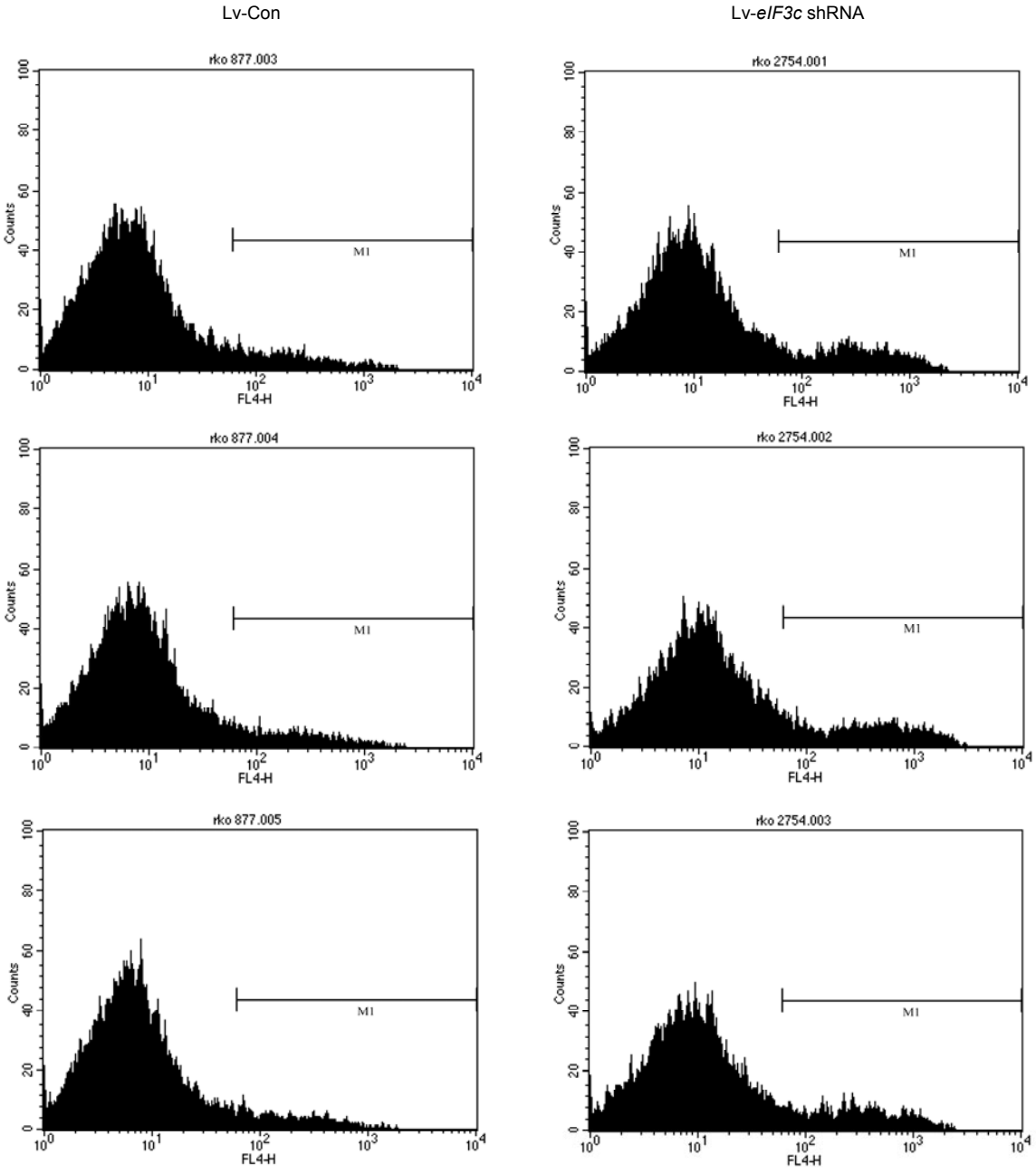


Fig. 5 Effect of *eIF3c* knockdown on apoptosis in RKO cells

(a) The histograms obtained from PI-Annexin V analysis of *eIF3c* knockdown and control cells from three independent trials; (b) Percentage of apoptotic cells in control group and *eIF3c* knockdown group. * $P<0.05$ in comparison with control

schwannomin, where merlin can inhibit *eIF3c*-mediated cell proliferation (Zhang *et al.*, 2007). However, in malignant conditions *eIF3c* expression is inversely related to merlin expression, as increased expression of *eIF3c* down-regulates merlin expression. This results in increased clonogenicity, increased viability, facilitated S-phase entry, and decreased apoptosis levels (Scoles, 2008). All these factors contribute to increased proliferation rates of cancer cells.

Thus, we investigated whether specifically reducing the levels of *eIF3c* protein in established colon cancer cell lines, in which this protein was over-expressed, might result in decreased cell proliferation levels. To achieve this objective, we used RNAi with siRNAs designed for use against *eIF3c*. RNAi is a conserved mechanism operating in insects, nematodes, plants, and mammalian cells for gene manipulation (Caplen *et al.*, 2001; Elbashir *et al.*, 2001). In this process, sequence specific post transcriptional knockdown is initiated by the introduced RNAi. The RNAi consists of double-stranded annealed sense and antisense RNAs which have sequences similar to those of the gene to be silenced (Bass, 2000). In experimental conditions lentivirus-based vectors have been used successfully to deliver siRNA efficiently with prominent gene knockdown effects (Devi, 2006).

The results clearly indicated that RKO colon cancer cells over-expressed the *eIF3c* gene under normal conditions and siRNA-mediated knockdown significantly decreased ($P<0.001$) the expression level. In the absence of the *eIF3c* gene, the survival rate of colon cancer cells drastically decreased by 60-fold. Furthermore, the cell cycle was arrested as the number of cells entering the S phase was significantly reduced ($P<0.05$) compared to the control, and the induction of apoptosis was prominent in the absence of the *eIF3c* gene. These results indicated a strong relationship between expression of the *eIF3c* gene and colon cancer cell survival. According to the literature, *eIF3c* is strongly associated with the maintenance of cell viability and it functions in the same manner in colon cancer cells. Over-expression of the *eIF3c* gene due to its higher tendency to double or undergo other mutations would positively contribute to colon cancer cell survival. Negative effects on colon cancer cell survival upon knockdown of *eIF3c* further support this correlation. Therefore, *eIF3c* could be regarded as a gene that promotes tumor progression, and regulation of its expression via siRNA knockdown would be an interesting and novel therapeutic tool for the treatment of colon cancer.

5 Conclusions

Over-expression of the *eIF3c* gene was observed in RKO colon cancer cells and this over-expression was suppressed by lentivirus-mediated infection of *eIF3c* siRNA. The deletion of the *eIF3c* gene resulted in a significant decrease in RKO cell survival and colony formation. Furthermore, the RKO cell cycle was arrested by inhibiting entry into S phase, and apoptosis was induced in the absence of the *eIF3c* gene. Collectively, these results show that the *eIF3c* gene has a positive effect on colon cancer cell survival and progression. Therefore, siRNA-mediated knockdown of the *eIF3c* gene could be considered as a novel therapeutic tool for colon cancer treatment.

Compliance with ethics guidelines

Ning SONG, Yan WANG, Xiao-dong GU, Zong-you CHEN, and Liu-bin SHI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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