Telomerase Cajal body protein 1 depletion inhibits telomerase trafficking to telomeres and induces G₁ cell cycle arrest in A549 cells

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Abstract. Telomerase Cajal body protein 1 (TCAB1) is a telomerase holoenzyme, which is markedly enriched in Cajal bodies (CBs) and facilitates the recruitment of telomerase to CBs in the S phase of the cell cycle. This recruitment is dependent on TCAB1 binding to a telomerase RNA component. The majority of cancer cells are able to grow indefinitely due to telomerase and its mechanism of trafficking to telomeres. In the present study, a certain level of TCAB1 expression in A549 human lung cells was identified and TCAB1 knockdown exhibited a potent antiproliferative effect on these cells, which was coupled with a decrease in the cell density and activity of the cellular enzymes. In addition, TCAB1-depletion was demonstrated to inhibit telomerase trafficking to telomeres in the A549 cells, leading to subsequent G₁ cell cycle arrest without inducing apoptotic cell death. Overall, these observations indicated that TCAB1 may be essential for A549 cell proliferation and cell cycle regulation, and may be a potential candidate for the development of a therapeutic target for lung adenocarcinomas.

Introduction

Approximately 85% of human cancers restore telomeric DNA base pairs, which have been lost, in order to preserve fertility by activating telomerase (1,2). Telomeres are repetitive DNA sequences (TTAGGG)n present at the termini of chromosomes, which prevent chromosomes from fusing with each other or rearranging. Telomerase is a ribonucleoprotein complex that counterbalances telomere loss by elongating telomeric DNA repeats; this maintains the chromosomal integrity and cells divide continuously in the M phase (3,4). In addition, telomerase is significantly associated with tumor size and aggressiveness, as well as genomic instability and prognosis in the majority of human cancers (5-8). Telomerase consists of two critical components, the RNA subunit (human telomerase RNA component; hTERC), which serves as a template for telomere elongation, and a catalytic subunit (human telomerase reverse transcriptase; hTERT), that provides reverse transcriptase activity (9,10). hTERC is hypothesized to be expressed ubiquitously in all tissues (11), whereas hTERT expression significantly correlates with telomerase activity and appears to be a limiting determinant of enzyme function (12,13).

The accumulation of telomerase in the nucleus is mediated by telomerase Cajal body protein 1 (TCAB1), which is a WD40 repeat protein (also denoted as wdr79 and WRAP53). TCAB1 exists in the active telomerase holoenzyme and it has been demonstrated that TCAB1 is required in HeLa and super telomerase (S-T) cells for the trafficking of telomerase to the Cajal bodies (CBs) during the S phase of the cell cycle (14-16), the phase in which telomere DNA is replicated (17). Previous studies have shown that TCAB1 is a constitutive component of CBs, which has been detected within CBs in a panel of cancer and primary cell lines, including: U2OS, osteosarcoma; H1299, lung cancer; HCT116, colon cancer; HeLa-PV, cervical cancer; HEK293, embryonic kidney cancer; MCF-7, mammary epithelial cells; and HDF, human diploid fibroblasts. Knockdown of TCAB1 impairs the growth of these cancer cells and results in mislocalization of telomerase from CBs to the nucleoli, thus inhibiting the ability of telomerase to restore the telomere length. This consequently leads to massive cell death with several morphological changes in certain cultured cancer cells (18-22).

Annually, more than one million individuals succumb to lung cancer worldwide, thus, it is the leading cause of cancer mortality in humans. Non-small cell lung cancer (NSCLC) accounts for 80% of all the lung cancer types, with adenocarcinoma as the major subtype (23). Since 1995, a paradigm shift in the management of advanced-stage NSCLC has occurred and an expanding array of molecular markers are being used to individualize cancer therapy. As a telomerase holoenzyme component in the telomere synthesis pathway, TCAB1 stably associates with the active telomerase enzyme and directs it through CBs to the telomere in the S phase (20). It was hypothesized in the present study that TCAB1 exhibits the same function in A549 lung adenocarcinoma cells; therefore, to validate this hypothesis a preliminary study was conducted. This demonstrated the function of TCAB1 small interfering (si)RNA in the downregulation of TCAB1.
expression in proliferation and enzymatic activities in A549 lung adenocarcinoma cells, without altering telomerase activity. Telomerase/telomere miscolocalization and the consequent cell cycle arrest were further investigated, and the results indicated the possibility of TCAB1 as a potential target during lung adenocarcinoma therapy.

Materials and methods

Antibodies. The following three antibodies served as the primary antibodies for western blot (WB) analysis: Monoclonal anti-β-actin (A5441; Sigma-Aldrich, St. Louis, MO, USA); anti-TCAB1 (ab99376; Abcam, Cambridge, MA, USA); and hTERT (RabMAb®; 1531-1; Abcam). The secondary antibodies used for WB analysis were as follows: Anti-rabbit IgG (#7074; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-mouse IgG horse radish peroxidase-conjugated (#7076; Cell Signaling Technology, Inc.). For immunofluorescence (IF), the hTERT (RabMAb®; 1531-1; Abcam) and anti-telomeric-repeat-binding factor (TRF)-2 (4A794; ab-13579; Abcam) antibodies served as the primary antibodies. Goat polyclonal secondary antibodies against rabbit IgG-H&L (fluorescein-isothiocyanate; ab6717; Abcam) and mouse IgG-H&L (cyanine 3; ab97035; Abcam) served as the secondary antibodies.

Cell culture, siRNA oligonucleotides, plasmids and transfection. A549 cells obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) were cultured in F-12K (1237832; Gibco-BRL, Carlsbad, CA, USA) with 10% fetal calf serum (1133067; Gibco-BRL) at 37°C in a humidified incubator with an atmosphere of 5% CO₂. Lipofectamine 2000 (11668-019; Invitrogen Life Technologies, Carlsbad, CA, USA) was used for the transfection reagent. TCAB1 siRNA (sc-93974; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used to knock down TCAB1 and control siRNA (sc-37007; Santa Cruz Biotechnology, Inc.) served as the negative control. Diluted siRNA and control siRNA in F-12K at a concentration of 40 nM were remixed and incubated for 30 min prior to each transfection.

Cell lysis and WB analysis. Protein was extracted according to the standard instructions for the radioimmunoprecipitation assay lysis buffer (P0013B; Beyotime Institute of Biotechnology, Shanghai, China). The concentration was determined using the Bio-Rad Protein Assay reagent (P0013B; Bio-Rad). For each sample, 1.5 µg cell extract was added to the TRAPEze telomerase detection kit assay (S7707; Millipore, Billerica, MA, USA). The gels were visualized with GelRed (41003; Biostain, Inc., Hayward, CA, USA) and visualized with a UV transilluminator. Appropriate positive and negative heat-inactivated cell extracts were set up with test samples and all reactions were performed in triplicate.

Telomerase activity assay. A549 cell pellets were subjected to lysis in 300 µl 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate lysis buffer (provided in the kit) and protein concentration standardization was performed using the Bio-Rad Protein Assay reagent (P0013B; Bio-Rad). For each sample, 1.5 µg cell extract was added to the TRAPEze telomerase detection kit assay (S7707; Millipore, Billerica, MA, USA). The gels were stained with GelRed (41003; Biostain, Inc., Hayward, CA, USA) and visualized with a UV transilluminator. Appropriate positive and negative heat-inactivated cell extracts were set up with test samples and all reactions were performed in triplicate.

Immunofluorescence assay. Cells were fixed in ice-cold 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 15 min at room temperature and washed twice with ice-cold PBS. The samples were incubated for 10 min with PBS containing 0.25% Triton X-100 (room temperature) and washed three times with PBS for 5 min each. The samples were immediately incubated with 1% BSA (9408-46-8; Sangon Biotech) in PBS with Tween 20 (PBST) for 30 min for blocking at room temperature, followed by two primary antibodies (1:1,000) diluted in 3% BSA in PBST (w/v). The cells were subsequently incubated with two primary antibodies at 4°C overnight, washed with PBS three times for 5 min each and incubated with the secondary antibodies (1:1,000) in 1% BSA in PBST for 1 h at room temperature in the dark. Finally, the cells were washed three times with PBS for 5 minutes and visualized at room temperature using a Carl Zeiss microscope (Carl Zeiss AG, Jena, Germany). The exposure times between treatments were consistent and the image brightness and contrast were
A

Figure 1. Effect of TCAB1 siRNA on the expression of TCAB1 and hTERT. (A) WB and RT-PCR analyses demonstrate the effect of TCAB1 siRNA transfection on TCAB1 and hTERT expression in A549 cells. (B) Densitometric quantification of the relative TCAB1 and hTERT levels of the cells. Levels of TCAB1 and hTERT were normalized to actin levels. Each bar represents triplicate analyses of the mean ± SD. *P<0.05 vs. con siRNA and WT (n=3). (C) A TRAP assay was performed to evaluate the effect of TCAB1 siRNA treatment on the activity of telomerase in A549 cells. The 56-bp bands represent the IC. (D) The bar graph presents the densitometry‑quantified data of the TRAP products in the single lane/PC (line 1) of the TRAP reaction ratios from three independent experiments. Telomerase activities were quantified by comparing the mean band intensity of each lane with the PC. The PC mean band intensity was defined as 100% telomerase-positive. Each bar represents triplicate analyses of the mean ± SD. *P<0.05 vs. con siRNA and WT (n=3). TCAB1, telomerase Cajal body protein 1; con siRNA, control small interfering RNA; WT, wild-type; WB, western blot; hTERT, telomerase reverse transcriptase; RT-PCR, reverse transcription polymerase chain reaction; PC, positive control; IC, internal control; SD, standard deviation; TRAP, telomeric repeat amplification protocol.
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Cell cycle and apoptosis analysis. For the cell cycle assay, the cells were collected and washed with PBS. The cell pellets were obtained by centrifugation at 10 x g (Maxi Mix II rocker rotator - M37610-33CN, Thermo Fisher Scientific) and the supernatant was discarded. Next, 1 ml of DNA staining solution (Multisciences Biotech Co., Ltd, China) was added and blended by vortexing (Maxi Mix II rocker rotator - M37610-33CN Thermo Fisher Scientific) for 10 sec. In preparation for flow cytometry, the cells were incubated for 30 min at room temperature in the dark. For the apoptosis assay, the cells were resuspended in 0.5 ml binding buffer (Santa Cruz Biotechnology, Inc.), containing Annexin V (1:50) and 40 ng/sample of propidium iodide (BD Biosciences, Franklin Lakes, NJ, USA) and incubated for 30 min at 37˚C in the dark, prior to flow cytometry. At least 10,000 cells were analyzed for each sample and the experiments were repeated three times.

Results

TCAB1 siRNA depletes TCAB1 protein without altering hTERT expression. TCAB1 expression was investigated in A549 cells. The protein and total RNA were isolated from A549 cells treated with TCAB1 siRNA and control siRNA and the levels of TCAB1 and hTERT were analyzed by WB analysis and RT-PCR. TCAB1 expression was downregulated by ~45% without altering hTERT expression (Fig. 1A and B). Telomeric repeat amplification protocol (TRAP) was also conducted to analyze telomerase activity and the results showed that the telomerase activity remained unchanged compared with the control groups (Fig. 1C).

TCAB1 silencing inhibits hTERT localization. The IF revealed that TCAB1 depletion suppressed telomerase trafficking to telomeres in A549 cells. hTERT and TRF-2 are essential subunits of telomerase and telomeres without which they are unable to form. In contrast to the control groups, TCAB1 depletion also suppressed hTERT accumulation at the TRF-2 of the telomeres. The hTERT signal surrounds the membrane of the nucleus in TCAB1-depleted A549 cells, however, does not enter the nucleus, therefore, almost no foci were observed with hTERT/TRF-2 colocalization (Fig. 2A). A total of 100 cells selected randomly from three random fields of each group were scored. hTERT in the cells of the control groups was found to accumulate in the nucleoli and colocalize with TRF-2 more frequently than that in the TCAB1 siRNA-treated cells (Fig. 2B). These results...
indicated that A549 cells that lack TCAB1 are less likely to transport telomerase to telomeres, which demonstrates the effect of TCAB1 depletion on telomerase recruitment to the telomere.

Inhibitory effect of TCBI siRNA on the proliferation of A549 cells. To investigate the impact of TCAB1 depletion in lung adenocarcinoma cells, A549 cells were transfected with TCAB1 siRNA and images were captured using an inverted microscope (magnification, x40; Olympus Corporation, Tokyo, Japan) separately at 24, 48 and 72 h. Notably, the graphs revealed an evident distinction between cell densities, particularly in the group treated with TCAB1 siRNA (Fig 3A). The graph and bar chart demonstrate a growth trend and cell number difference in each group (Fig 3B), which exhibited an evident weakness in the
Figure 4. Effect of TCAB1 siRNA on cell cycle and apoptosis regulation. (A) Each bar of the bar graph represents the mean percentage of cells in the G1 and S phases. Data are presented as the mean ± SD of triplicate analyses. *P<0.05 vs. con siRNA and WT (n=3). (B) The cell cycle distribution was determined by the detection of DNA degradation. A549 cells were treated with TCAB1 siRNA and stained with PI and analyzed by flow cytometry. The cell count versus PI staining is shown (n=10,000 per treatment). (C) Expression of CDK6 was determined by RT-PCR analysis of the total isolated RNA. Actin served as a gel-loading control. The densitometric quantifications of the CDK6 levels were normalized against the actin levels. Data are presented as the mean ± SD of triplicate analyses. *P<0.05 vs. control siRNA and WT (n=3). (D) Annexin V staining of TCAB1 siRNA-treated cells. Dot-plot data of FITC and PI staining measured by fluorescence-activated cell sorting analysis and graphical representation of apoptosis in cells treated with TCAB1 siRNA. *P<0.05 vs. control siRNA and WT (n=3). WT, wild-type; con siRNA, control small interfering RNA; TCAB1, telomerase Cajal body protein 1; RT-PCR, reverse transcription-polymerase chain reaction; CDK6, cyclin-dependent kinase 6; FITC, fluorescein isothiocyanate; PI, propidium iodide; SD, standard deviation.
reproductive capacity of TCAB1-depleted cells. Simultaneously, the activity of cellular enzymes was evaluated by MTT assay and the results were compared to reveal a decreased absorbance in TCAB1-depleted cells, which indicated decreased activity of cellular enzymes (Fig 3C). These results indicated that TCAB1 siRNA effectively inhibits the proliferation of A549 cells.

**TCAB1 depletion arrests the G1 cell phase without inducing apoptosis.** Further study was conducted to elucidate the mechanism of action for the antiproliferative activity of TCAB1 depletion on A549 cells. Flow cytometry was used to analyze the A549 cell cycle distribution and apoptosis. Cancer cells are generally immortal and divide uncontrollably, and the replication of telomeres occurs in the S phase (24). Thus, it was predicted that the number of A549 cells would increase in the G1 phase and decrease in the S phase as a result of TCAB1 depletion. Consistent with this hypothesis, TCAB1 siRNA was found to induce G1 cell cycle arrest, and the percentage of cells with G1 DNA content increased from 70 to 88% and the percentage of cells in the S phase decreased from 25 to 9%, when compared with that of the control groups (Fig. 4A). However, cells were not found to accumulate in the sub G1 peak (Fig. 4B). In addition, the G1 cell cycle checkpoint protein, cyclin-dependent kinase 6 (CDK6), was suppressed (Fig. 4C). Accordingly, the Annexin V staining apoptosis assay of A549 cells treated with TCAB1 siRNA demonstrated no evidence of apoptotic cell death (Fig. 4D). These observations indicated that the antiproliferative effects of TCAB1 siRNA in A549 cells is caused by cell cycle arrest without the induction of apoptosis.

**Discussion**

Up to 90% of cancers exhibit activated telomerase that permits cell immortalization and leads to tumorigenesis. Furthermore, the localization of telomerase in CBs in the S phase of the cell cycle is an indispensable step in telomere synthesis. TCAB1 is required in this step in HeLa and S-T cells, however, its depletion has not previously been investigated in A549 lung adenocarcinoma cells. In this initial study, the expression of TCAB1 in TCAB1 siRNA treated and untreated A549 cells was examined and was further integrated with the results regarding TCAB1 expression in NSCLC cell lines. As predicted, TCAB1 was present in the A549 cell line and was downregulated by transfection with TCAB1 siRNA. A previous study has identified that TCAB1 is required for the delivery of telomerase to the nucleus for telomere replication in certain human cancer cells, and that TCAB1 knockdown impairs the growth of these cancer cells (20). However, to evaluate the efficacy and to further elucidate the mechanism of TCAB1 in A549 cells, the current study investigated telomerase/telomere colocalization in TCAB1-depleted A549 cells. IF demonstrated that the hTERT (telomerase) adheres to the membrane of the nucleus and fail to associate with the TRF-2 (telomere) in TCAB1 siRNA-treated cells. Consistently, the graphs of cell density demonstrated that TCAB1 depletion exhibits a potent antiproliferative effect on A549 cells, in addition to decreasing the activity of cellular enzymes in the MTT assay. This antiproliferative effect may be due to the telomerase/telomere miscolocalization caused by TCAB1 depletion. In addition, recent studies have revealed that telomerase (hTERT) depletion in mouse lymphomas results in the emergence of the alternative lengthening of telomere (ALT) (25). ALT is a telomere maintenance mechanism, which exists in telomerase-negative neoplastic and non-neoplastic human cells, characterized by homologous recombination (26-28). The ALT mechanism exists in NSCLC and thus, leads to aggressive malignant properties and the acquisition of resistant mechanisms to counteract telomerase deficiency in late generations of tumor cells (25,29). The association between TCAB1 and telomerase is not fully understood and therefore, to exclude the possibility of telomerase alternation and emergence of ALT in TCAB1-depleted A549 cells, the current study identified the disassociation between TCAB1 depletion and telomerase activity in A549 cells by WB and TRAP. Consistent with the unaltered hTERT expression identified by WB analysis, the results of TRAP also revealed unaltered telomerase activity. Furthermore, the considerable infertility of TCAB1-depleted A549 cells showed no sign of activating the ALT mechanism. These results are consistent with a study reporting that TCAB1 only functions as a telomerase holoenzyme component in the telomere synthesis pathway following the assembly of the telomerase complex, which contains TERT, TERC and dyskerin (20).

Based on the potent antiproliferative activity, the current study investigated the mechanism of TCAB1 siRNA treatment in the regulation of cell proliferation in A549 cells. Generally, cell cycle arrest and apoptosis are the most common causes of antiproliferation of cells and thus, whether TCAB1-depletion regulates cell cycle progression or induces cell apoptosis was also investigated. Notably, the cell cycle assay revealed that a greater percentage of cells remain in the G1 phase, with a reduced percentage of cells in the S phase following TCAB1 siRNA treatment. However, the sub-G1 peak, which is indicative of apoptotic cell death, was not detected. Accordingly, Annexin V staining of TCAB1-depleted cells revealed no evidence of apoptotic cell death. Additionally, G1-acting CDK6 expression was investigated and found to be significantly reduced in response to TCAB1 siRNA treatment, indicating that the downregulation of CDK6 may result in the suppression of the cyclin D1-CDK6 complex, which eventually leads to G1 phase arrest. These results indicate that the inhibitory effect of TCAB1 depletion on the proliferation of A549 cells is evoked by cell cycle arrest at the G1 phase without causing evident apoptotic cell death.

The cell cycle is a process that involves DNA replication and cell division (30). DNA replication occurs during the S phase, and division occurs in the G2 and M phases; arrest in any of the phases may inhibit cell proliferation. Furthermore, metabolic activities of DNA at the chromosome ends, where telomeres exist, are important throughout cell cycle progression (31). Telomeric DNA is arranged into folded structures in the G1 phase, which unfold to replicate during the process of DNA replication following the recruitment of telomerase to the telomere in the S phase (32). Thus, the present study identified that the infertility of TCAB1 siRNA-treated A549 cells is evoked by G1 phase arrest due to TCAB1 depletion, which reduces the presence of telomerase at the telomer (shown by IF) in the S phase when telomere DNA replication occurs.

In conclusion, the results of the present study demonstrated the function of TCAB1 depletion via decreased telomerase/telomere colocalization proportions, infertility, and reduced activity
of cellular enzymes in A549 cells, without downregulating telomerase expression and activity. In addition, it was found that the antiproliferative effect of TCAB1 depletion is evoked by G1 phase cell cycle arrest without inducing apoptotic cell death. These results highlight the essential function of TCAB1 within A549 cells. However, the function of TCAB1 in the recruitment of the telomerase complex from the cytoplasm to CBs and restoration of telomere length remains unclear. Recent studies have identified TCAB1 as an essential factor for CBs maintenance and that TCAB1 knockdown prevents the formation of novel CBs (18). Stern et al (33) revealed that depletion of CBs also reduces telomerase foci at telomeres. These observations further establish TCAB1 as a significant factor for telomerase recruitment and telomere synthesis. Another study has revealed that the oligonucleotide-binding-fold domain of the telomere-binding protein, TPP1, recruits telomerase to the telomeres through a TERT-associated mechanism (34). The manner in which TCAB1 acts as a cofactor in this mechanism for telomerase trafficking and their connection, as well as the telomere length response in TCAB1-depleted A549 cells, remain to be identified and require further investigation.

Briefly, ‘oncogene addiction’ is defined as the dependence of a cancer cell on one pathway or overactive gene for its survival and/or growth, which provides cancer-specific weaknesses that can be targeted during anticancer therapy (35). In the present study, such properties were identified with regard to the TCAB1 protein. Specifically, TCAB1 is a potential oncogene that is essential for A549 cell survival and, thus, is a notable target for therapeutic intervention in lung adenocarcinoma.

Acknowledgements

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References