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Full Length Research Paper

siRNA-mediated silencing of translationally controlled tumor protein induces apoptosis and inhibits cell growth

Zeng-Chun Ma, Qian Hong, Yu-Guang Wang, Hong-Ling Tan, Cheng-Rong Xiao, Qian-De Liang, Bei-bei Lu and Yue Gao*

Beijing Institute of Radiation Medicine, Tai-Ping Road 27, Beijing, 100850, P. R. China.

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Translationally controlled tumor protein (TCTP) is an evolutionally highly conserved protein which has been implicated in many cellular functions that are related to cell growth, death and tumorigenesis. We used a DNA vector-based RNA interference approach by expressing shRNA to knockdown TCTP in MCF7 cells for evaluating its antitumor effect and its molecular mechanism. TCTP shRNA treatment MCF7 cells suppressed cell growth and proliferation. In addition, cell cycle arrest and apoptotic cell death were observed from TCTP shRNA-treated MCF7 cells. Myeloid cell leukemia 1 (MCL-1) was identified to be related with TCTP in cell growth and apoptosis cell death. These results suggested that vector-based TCTP RNA interference could be an efficient molecular therapeutic method to kill MCF7 cells, and TCTP played pivotal roles in cell growth and apoptosis cell death.

Key words: TCTP, RNA interference, apoptosis, cell cycle.

INTRODUCTION

Translationally controlled tumor protein (TCTP) is widely expressed in many tissues and cell types, and its protein levels are highly regulated in response to a wide range of extracellular signals and cellular conditions (Bommer et al., 2004; Thaw et al., 2001). Over-expression or down-regulation of TCTP perturbs cell growth (Kim et al., 2008; Tuynder et al., 2008), suggesting that TCTP has a growth related function. On the other hand, the human homolog of TCTP (termed histamine-releasing factor) was found to be the component present in patients' biological fluids which is an IgE-binding antigen and associated with disease severity (Rid et al., 2008). Many cellular proteins have been reported to interact with TCTP. Studies on TCTP's interaction with tubulin, mitotic polo-like kinases (Plk) and translation elongation factors suggest that TCTP plays a role in cell cycle progression (Johnson et al., 2008) and protein synthesis (Andree et al., 2006; Schmidt et al., 2007). Accumulating evidence indicates that TCTP functions as an antiapoptotic protein, which may be related to its interaction with myeloid cell leukemia 1 (Mcl-1), Bcl-XL, bax (Yang et al., 2005; Liu et al., 2005; Susini et al., 2008). TCTP is known as a target of artemisinin and its derivatives, it is assumed that artemisinin is opened up by heme to form a free radical, which then eventually alkylates the Cys14 of TCTP (Chae et al., 2005).

RNA interference (RNAi) has emerged as a powerful new technique which targeted post-transcriptional gene silencing. Sequence-specific inhibition of gene expression can be achieved by transfecting 19 to 21 nucleotide small interfering (si)RNAs, which are complementary to the mRNA sequence of a given target gene (Elbashir et al., 2001). In order to circumvent the transient nature of transfected siRNAs, a pSilencer™ 3.1-H1 system is currently used for knockdown of a particular gene expression to identify the functions of a targeted gene and to examine the potential usage of a targeted gene as a therapeutic method (Ji et al., 2008).

Our previous reports suggested the protein level of TCTP was increased by the radiation, the mRNA level of TCTP also increased (Ma et al., 2007). Radiation could induce tumorigenesis by activation of genes and the loss of cell-cycle checkpoints (Kim et al., 2003). Tuynder

*Corresponding author. E-mail: gaoyue@yahoo.com, mazengchun2008@gmail.com.

previously reported a significant down-regulation of TCTP in the revertants of tumor cells (Tuynder et al., 2008). In the present article, we investigate the effects of TCTP RNA interference in cancer cells using a DNA vector-based siRNA approach, in order to investigate the role of TCTP in radiation induced tumorigenesis. Our results demonstrated that the silencing of TCTP expression using a DNA vector-based siRNA approach induced apoptotic cell death, and Mcl-1 was identified to be related with TCTP, and TCTP was identified as a target to kill MCF7 cells.

MATERIALS AND METHODS

Cell lines and culture

The human metastatic carcinoma cell lines MCF7 provided by the American Type Culture Collection (Manassas, VA, USA) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Construction of the TCTP siRNA expression vector

To knock down TCTP expression in MCF7 cells, three TCTP siRNA-expressing vectors (TCTP shRNA-273, TCTP shRNA-317 and TCTP shRNA-693) were constructed by using the pSilencer™ 3.1-H1 plasmids for DNA vector-based siRNA synthesis (Ambion, TX, US). These three constructs were designed according to the manufacturer's protocol in such a way that a duplex siRNA corresponding to the following three regions of the human TCTP gene 5'-AAGGTACCGAAAGCACAGTAA-3' (TCTP shRNA-273), 5'-AAGCCTACAAGAAGTACATCA-3' (TCTP shRNA-317) and 5'-AAGACAAATGGGACTGATGTC-3 ' (TCTP shRNA-693) could be synthesized inside mammalian cells transfected with these vectors. The oligonucleotide pairs were designed to contain a terminal BamHI or HindIII restriction site for subcloning into the BamHI or HindIII site of vector to generate pSilencer™ 3.1-H1 TCTP siRNA vectors (siRNAs). These vectors produce a siRNA with a TTCAAGAGA linker sequence that forms looped structures. This linker is processed with Dicer to generate a TCTP-specific siRNA. A negative scrambled siRNA (Ambion) control with no significant homology to mouse or human gene sequences was designed to detect nonspecific effects.

Transfection

For transfection, cells were plated on 6-well plates (2×10^5 cells per well), 24-well plates (5×10^4cells per well), 96-well plates (2×10^4 cells per well) or 100 mm dishes (2×10^6 cells) and were allowed to adhere for 24 h. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for the transfections. pSilencer™ 3.1-H1 TCTP siRNA vectors or pSilencer™ 3.1-H1 scramble siRNA vector were transfected into the cells. Transfected cells were then cultured for 4 h and the culture media were replaced with fresh media supplemented with 10% FBS. The cells were harvested at 6 to 48 h after transfection.

Cell count and proliferation assay

5×10^4 cells were plated into a 24-well plate. At the exponential growth stage, the cells were transfected with respective pSilencer™ 3.1-H1 siRNA vector. At the indicated times, the cells were washed and trypsinzed, and diluted with isotonic solution. Cells were stained with trypan blue and the viable cells were counted using a hemocytometer. 2×10^4 cells were plated into a 96-well plate, after 48 h of transfection, 20 µl of MTS was added to each well. The cultures were incubated for a further 1.5 h, following which the absorbance was read in a multiplate reader (Wallac, Turku, Finland).

Cell cycle analysis

The cells transfected with pSilencer™ 3.1-H1 siRNA vector were trypsinized, counted, centrifuged and fixed in ethanol for 24 h. These cells were then washed twice in PBS and centrifuged. Pellets were resuspended with a solution containing RNase (0.02 mg/ml) (Sigma), incubated at 37℃ for 30 min and were stained with PI (0.02 mg/ml) (Sigma). The cells were analyzed by flow cytometry (FCM, FACSCalibur, BD Company).

Apoptosis and DNA fragmentation assays

Approximately 2×10^5/ml MCF cells were transfected with respective vector for the indicated times. The transfected cells were harvested, washed twice with PBS and were then incubated for 20 min at room temperature with a solution of annexin V conjugated with fluorescence isothiocyanate (2.5 µg/ml) and propidium iodide (PI) (5 µg/ml) (BD Pharmingen, San Diego, CA, USA) for flow cytometry (FCM, FACSCalibur, BD Company) to detect the levels of apoptosis.

Reverse transcription-polymerase chain reaction

At the indicated times, the cells were collected and total RNA was extracted using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. For RT-PCR analysis, 1 µg total RNA was reverse-transcribed using RT-PCR kits (Promega, Madison, WI, USA). PCR was used to amplify target cDNA with the following conditions: 28 cycles of 94℃ for 45 s, 55℃ for 45 s and 72℃ for 45 s. The PCR products were analyzed using standard agarose gel electrophoresis. Semiquantitative PCR was performed with oligonucleotides: TCTP(forward), 5'-ACCGAAAGCACAGTA ATCACT-3'; TCTP(reverse), 5'-GTCACACCATCCTCACGG TA-3'; Mcl1(forward), 5'-TAGCCAGGCAAGTCATAGAAT-3'; Mcl1(reverse), 5'-TTACTGAGCCTTCCG TCA-3'; Bcl2(forward), 5'-CAAGTGTTCCGCGTGATTGAA-3'; Bcl2(reverse), 5'-ATCTCCCGCATCCCACTCGTA-3'; Bax(forward), 5'-GGCGTGAAATGGCGT GATCTG-3'; Bax(reverse), 5'-TGGTGAGTGAGGCGGTGAG-3'; Bak(forward), 5'-GGACGACATCAACCGACGCT-3'; Bak(reverse), 5'-AGTCAGGCCATGCTGGTA GAC-3'; Bad(forward),5'-CCCAGAGTTTGAGCCGAGTGA-3'; Bad(reverse), 5'- CCCTTCGTCGTCCTCCGTCC-3';β-actin(forward), 5'-GGGAAATCGTGCGTGACATT-3'; β-actin(reverse), 5'-AAATAAAGCCATGCCAATCTC-3'.

Western blotting

For western blot analysis, the following antibodies were used: Anti-Mcl-1 (Santa Cruz, USA), β-anti-actin (Santa Cruz, USA) and Anti-TCTP (Medical and Biological Laboratories, Nagoya, Japan). For western blot analyses, cells were harvested and lysed with lysis buffer. After centrifugation at 12,000×*g* for 30 min, the concentrations of supernatant proteins were analyzed by Bradford reagent (Bio-Rad, Hercules, USA). For the analysis of protein contents, 50 µg total proteins was electrophoresed in 10% SDS-PAGE gel, transferred to polyvinylidene difluoride membranes

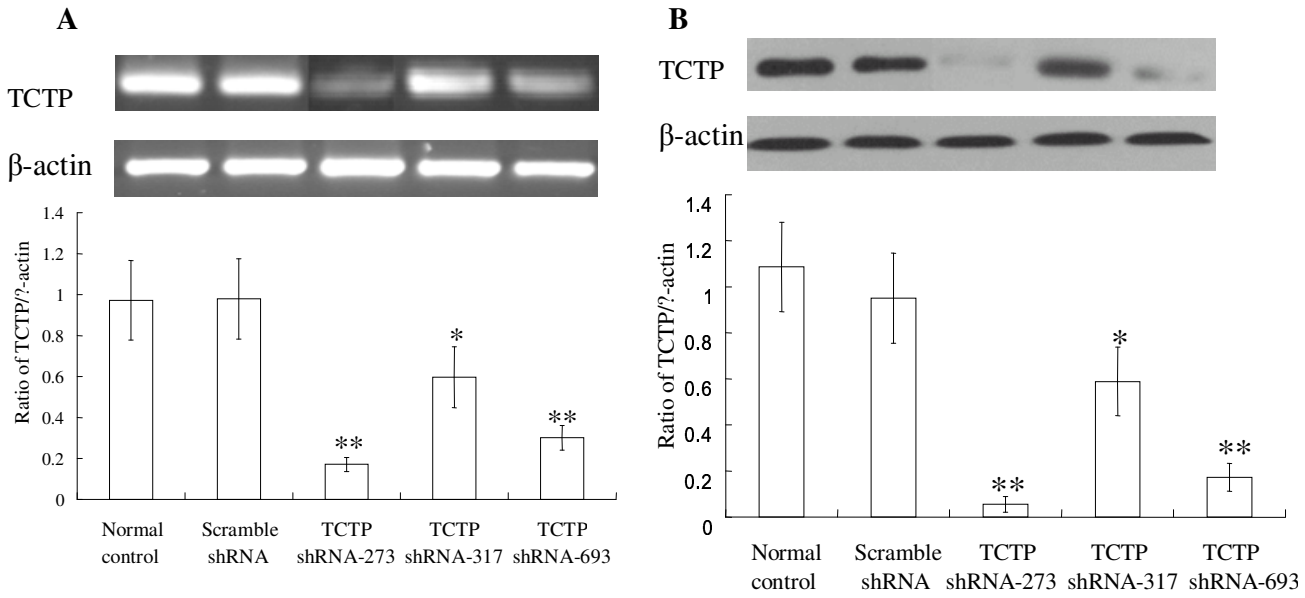

Figure 1. Expression of TCTP and the silencing efficiency of TCTP shRNA in MCF7 cells. (A) RT-PCR analysis for detecting the level of TCTP repression mediated by TCTP shRNA. To assess the extinction of endogenous human TCTP mRNA in MCF7 cells due to TCTP RNA interference, cells were transfected with TCTP shRNA, scramble shRNA for 48 h. Total RNA was then extracted from respective samples and subjected to RT-PCR using specific primers for human TCTP or β-actin. Results are shown as means ± SEM of three independent experiments.* $p<0.05$ and **$p<0.01$ compared to the value of scramble shRNA. (B) Western blotting analysis for detecting the level of TCTP repression mediated by TCTP shRNA. To assess the decreased TCTP protein in MCF7 cells due to TCTP RNA interference, cells were transfected with TCTP shRNA, scramble shRNA for 48 h. Total protein was then extracted from respective samples and subjected to perform western blotting with anti-TCTP and anti-β-actin antibodies. Results are shown as means ± SEM of three independent experiments.* $p<0.05$ and **$p<0.01$ compared to the value of scramble shRNA.

(Millipore, Bedford, USA) and were then incubated with the respective antibodies indicated above. Immunoblots were visualized using an enhanced chemiluminescence detection system (Thermo scientific, Rockford, USA).

RESULTS

TCTP expression in MCF7 cells effectively suppressed by RNA interference

To evaluate the functions of TCTP in cancer development and radiation induced tumorigenesis, TCTP-specific knockdown experiments were performed with human cancer cells. Initially, DNA oligonucleotides representing the siRNA duplex were cloned into pSilencer™ 3.1H1 vector to produce a high-level silencing effect based on a DNA vector system. The synthesized siRNA derived from DNA templates was composed of two identical 21-nucleotide sequence motifs in an inverted orientation, separated by a 9 base pair nonhomologous spacer (siRNA). To confirm the silencing efficacies of pSilencer™ 3.1H1-TCTP siRNA vector, MCF7 cells were transfected with the above samples and pSilencer™ 3.1H1-scramble shRNA (scramble shRNA) as negative controls. After culturing for 48 h, their efficacies in extinguishing TCTP mRNA expression were evaluated by RT-PCR. As shown in Figure 1A, treatment with TCTP shRNA-273 or shRNA-693 effectively downregulated the TCTP mRNA level. Treatment with TCTP shRNA-273 or shRNA-693 for 48 h decreased TCTP mRNA expressions in MCF7 cells by over 80% compared with the cells transfected with control vectors. The total protein levels of TCTP were also downregulated by TCTP shRNA-273 or shRNA-693, indirectly indicating that the knockdown of TCTP reduced the protein levels of TCTP (Figure 1B). These results suggest that TCTP shRNA-273 or shRNA-693 was effective for downregulating TCTP mRNA as well as protein levels.

TCTP depletion inhibited MCF7 cells growth and proliferation

To explore the potential function of TCTP shRNA-273 or shRNA-693, the diverse phenotypic changes of MCF7 cells affected by TCTP shRNA were investigated. Firstly the cell survival and proliferation rate were investigated. The viable cells in transfected TCTP shRNA-273 or shRNA-693 decreased significantly compared with the cells transfected with scramble shRNA (Figure 2A), and the proliferation rate of TCTP shRNA-273 or shRNA-693 transfected cells was also obviously reduced (Figure 2B).

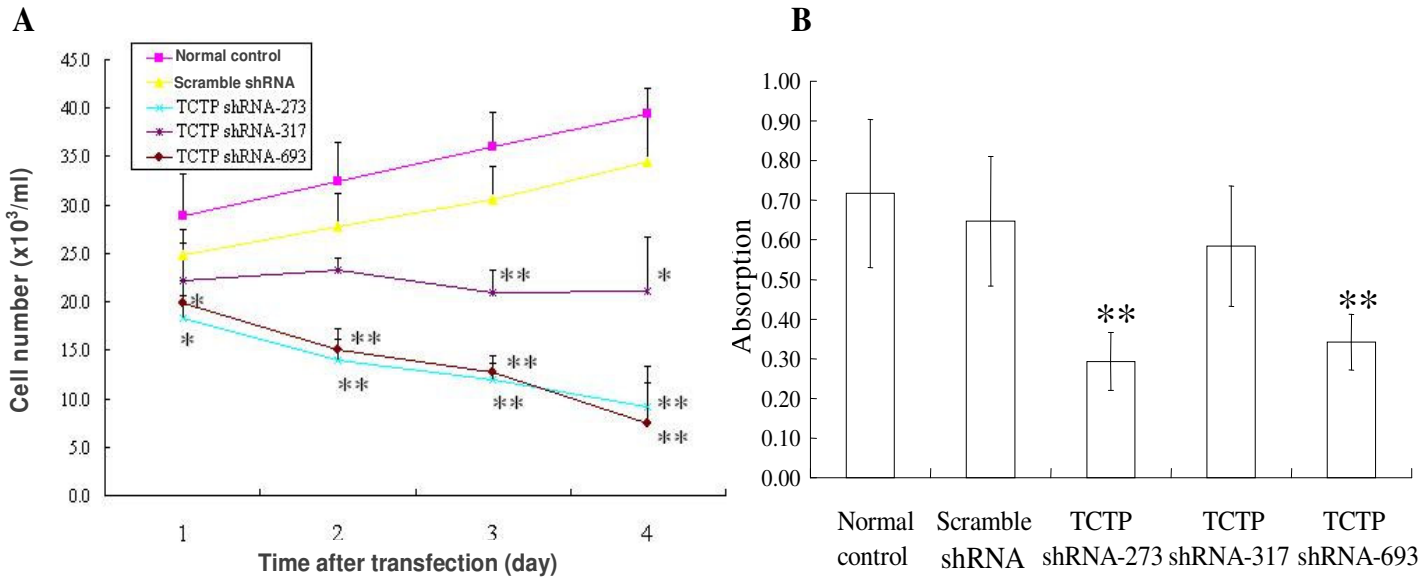

Figure 2. Cell proliferation of MCF7 cells transfected with TCTP shRNA. (A) Cell count assay: 5×10^4 cells were plated into a 24-well plate. At the exponential growth stage, the cells were transfected with TCTP shRNA, scramble shRNA. At the indicated times, the cells were washed, trypsinzed, and diluted with isotonic solution. Cells were stained with trypan blue and the viable cells were counted using a hemocytometer. Results are shown as means ± SEM of three independent experiments. *p<0.05 and **p<0.01 compared to the value of scramble shRNA. (B) 2×10^4 cells were plated into a 96-well plate, after 48 h of transfection with TCTP shRNA and scramble shRNA, 20 µl of MTS was added to each well. The cultures were incubated for a further 1.5 h, following which the absorbance was read in a multiplate reader. Results are shown as means ± SEM of three independent experiments.*p<0.05 and **p<0.01 compared to the value of scramble shRNA.

Taken together, these results suggested that growth and proliferation were inhibited in TCTP specific siRNA transfected MCF7 cells.

TCTP depletion induced cell cycle arrest (G2/M arrest) and apoptotic cell death

In order to analyse the cellular consequences of siRNA-mediated silencing of the TCTP gene, the cell cycle status in transfected cells were investigated. Cell cycle analysis indicated that the G1 or S populations of TCTP shRNA-273 or shRNA-693 treated MCF7 cells decreased; while G2/M populations increased (Figure 3), indicating TCTP shRNA-273 or shRNA-693 induces G2/M arrest in MCF7 cells. Based on annexin V and PI staining, both TCTP shRNA-273 and TCTP shRNA-693 increased apoptotic cells compared with control groups after 48 h of transfection (Figure 4).

TCTP depletion induced cell apoptosis by reducing MCL-1 expression

To understand the mechanism of cell cycle arrest and apoptosis induced by TCTP shRNA, TCTP was reported to interact with Bcl-2 family member, we examined the changes of Bcl-2 family member gene by RT-PCR. The transfection of TCTP shRNA-273 or shRNA-693 after 24 h resulted in the down-regulation of Mcl-1, and no obvious changes found in other genes (Figure 5). To confirm the relation of TCTP and Mcl-1, the expression changes of TCTP and Mcl-1 at the indicated times were investigated, the decreased TCTP coincided with Mcl-1. The total protein levels of TCTP were also down-regulated coincided with Mcl-1 (Figure 6).

DISCUSSION

TCTP was identified in a high throughput screening, while searching for differentially expressed genes between tumor cells and their revertant counterparts (Tuynder et al., 2008). This analysis revealed that TCTP was significantly down-regulated in tumor revertant cells, which have a suppressed malignant phenotype. Moreover, TCTP protein levels were clearly augmented in a variety of human tumor tissues compared with their normal tissue controls (Tuynder et al., 2008). Our previous studies demonstrated the protein level of TCTP was increased by the radiation, and radiation could induce tumorigenesis by activation of genes and the loss of cell-cycle checkpoints (Ma et al., 2007). However, the underlying mechanisms of TCTP in tumorigenesis still remain largely uncharacterized.

Inhibitory siRNAs provides new powerful tools for the

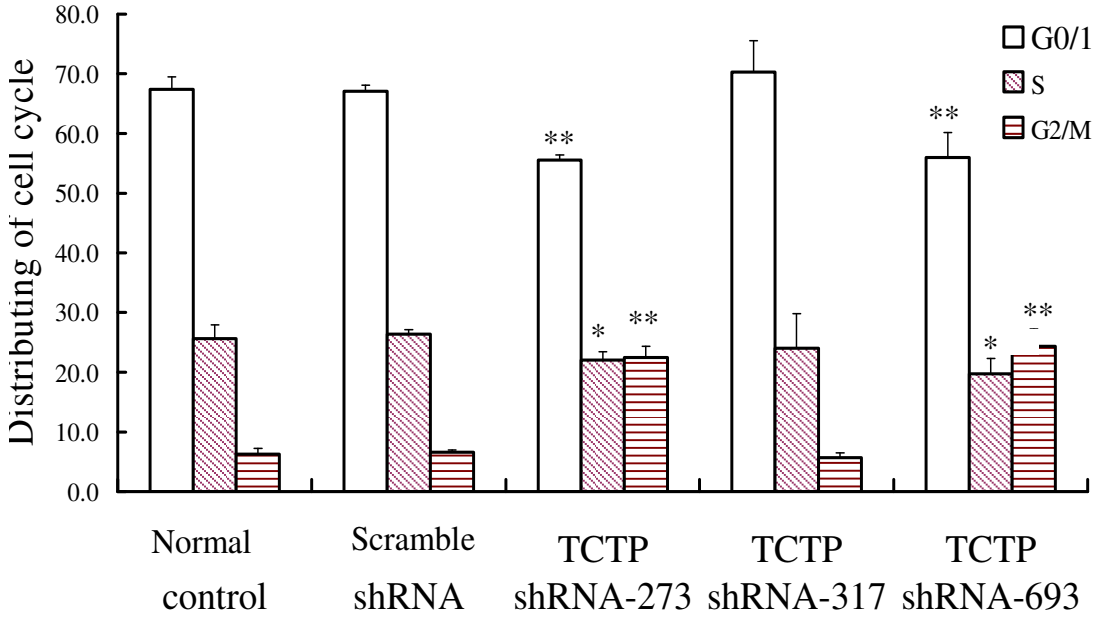

Figure 3. The cell cycle distribution of MCF7 cells transfected with TCTP shRNA. The cells transfected with pSilencer™ 3.1-H1 siRNA vector were trypsinized, counted, and fixed in ethanol for 24 h. These cells were then washed twice in PBS and resuspended with a solution containing RNase, and were stained with PI. The cells were analyzed by flow cytometry. Results are shown as means ± SEM of three independent experiments.*p<0.05 and **p<0.01 compared to the value of scramble shRNA.

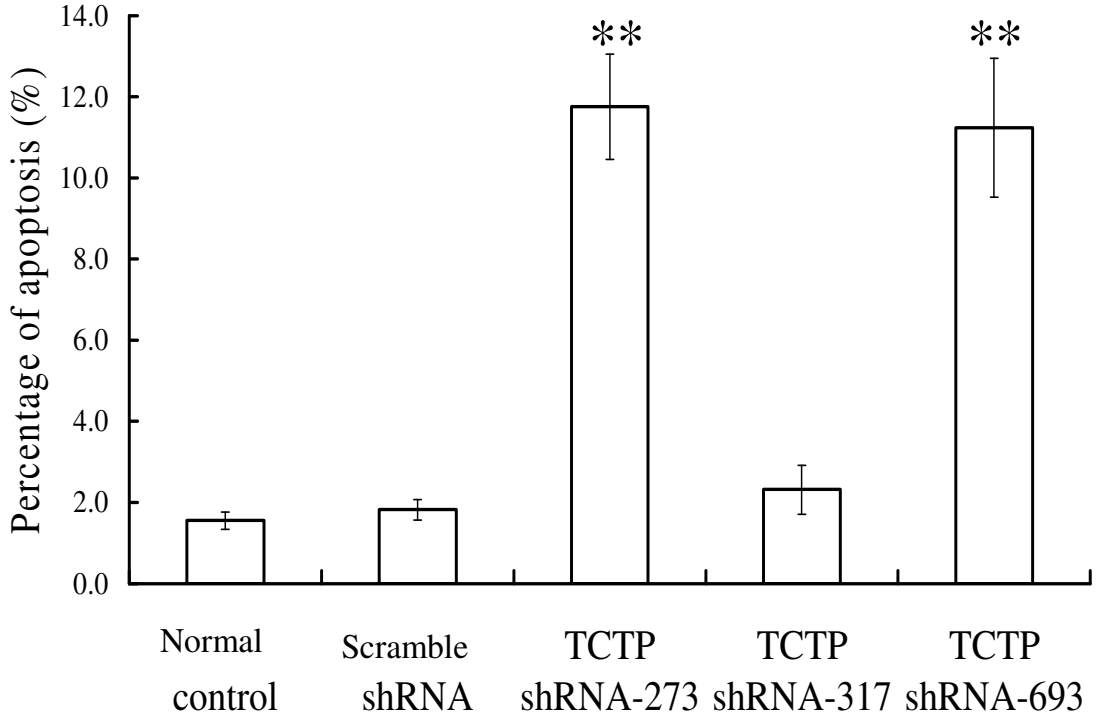

Figure 4. Apoptotic cell death of MCF7 cells transfected with TCTP shRNA. Approximately 2 × 10^5/ml MCF cells were transfected with respective vector for 48 h. The transfected cells were harvested, washed twice with PBS and were then incubated for 15 min at room temperature with a solution of annexin V conjugated with fluorescence isothiocyanate and propidium iodide for flow cytometry to detect the levels of apoptosis. Results are shown as means ± SEM of three independent experiments.*p<0.05 and **p<0.01 compared to the value of scramble shRNA.

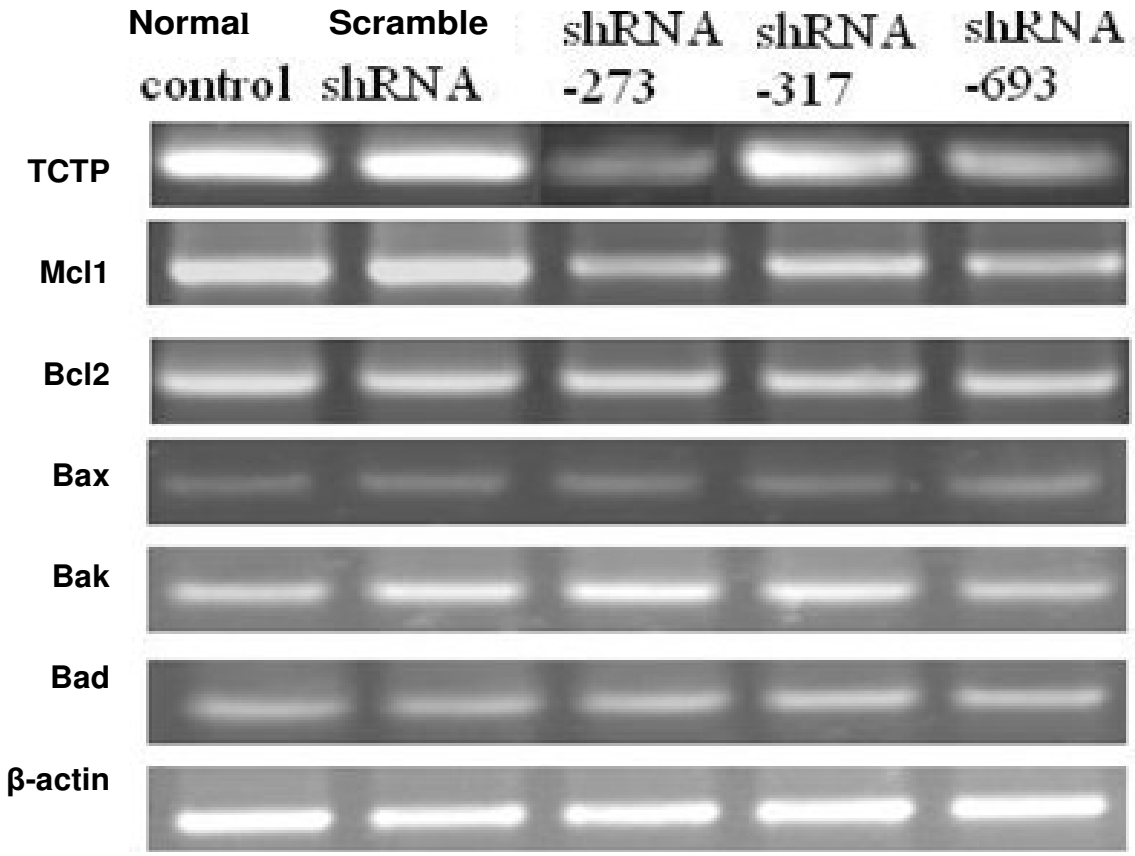

Figure 5. RT-PCR analysis for detecting the mRNA level mediated by TCTP shRNA. Cells were transfected with TCTP shRNA, scramble shRNA for 48 h. Total RNA was then extracted from respective samples and subjected to RT-PCR using specific primers.

analysis of gene function at the intracellular level. In the present study, a DNA vector-based siRNA approach was used to investigate the role of TCTP in tumorigenesis. The results demonstrated that the silencing of TCTP expression induced apoptotic cell death and growth inhibition, and Mcl-1 was identified to be related with TCTP.

Mcl-1, a Bcl-2 family member, has since been found to be induced in cells at various stages (Nijhawan et al., 2003; Craig et al., 2002). The level of Mcl-1 is a determinant of whether cells remain viable and differentiate along various pathways within the differentiation continuum (Cuconati et al., 2002; Derenne et al., 2002). Alterations that affect the normal pattern of Mcl-1 expression can contribute to the development of cancer, cell viability is prolonged when Mcl-1 expression is sustained unnecessarily rather than being induced in a regulated or transient fashion, and this predisposes cells to transformation because, with unlimited survival, they can acquire additional changes that contribute to tumorigenesis (Wuilleme et al., 2005; Vrana et al., 2006; Le et al., 2004). TCTP was identified as one cellular factor interacted with Mcl-1 and modulated Mcl-1 stability. While overexpression of TCTP augmented the protein stability of Mcl-1, knockdown expression of TCTP by RNA interference destabilized Mcl-1 (Liu et al., 2005). N-terminal region of TCTP, is required for its binding to Bcl-xL and for its antiapoptotic activity (Yang et al., 2005). Previous studies demonstrate loss of TCTP expression in mice leads to increased spontaneous apoptosis during embryogenesis, and TCTP antagonizes apoptosis by inserting into the mitochondrial membrane and inhibiting bax dimerization (Susini et al., 2008).These data suggested TCTP was associated with Bcl-2 family member proteins, in the present study, Mcl-1 was identified to be related with TCTP, all these data suggested TCTP played key roles in apoptosis.

TCTP, a mitotic spindle protein, has been implicated in important cellular processes, such as cell growth, cell cycle progression (Kubiak et al., 2008). Plk have been implicated in the formation and function of bipolar spindles. TCTP was identified as a putative Plk-interacting clone by a two-hybrid screen. Plk phosphorylates TCTP on two serine residues *in vitro* and cofractionates with the majority of kinase activity toward TCTP in mitotic cell lysates (Yarm, 2002). TCTP also interacts with both

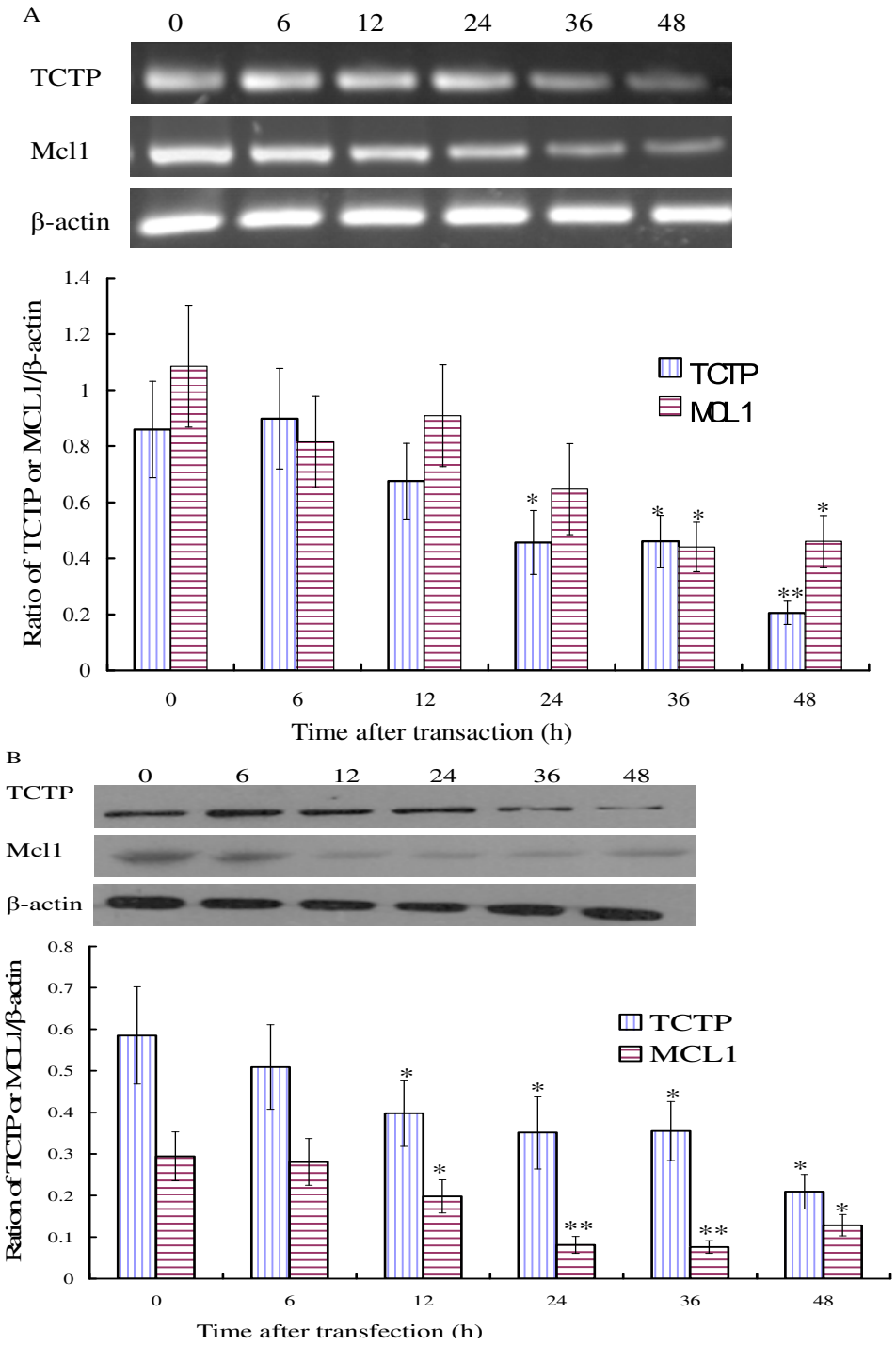

Figure 6. The expression changes of TCTP and Mcl1 in MCF7 cells transfected with TCTP shRNA. (A) RT-PCR analysis for detecting the level of TCTP and Mcl1. To assess the relation of TCTP and Mcl1 in MCF7 cells due to TCTP RNA interference, cells were transfected with TCTP shRNA-273. At the indicated times, total RNA was then extracted from respective samples and subjected to RT-PCR using specific primers for human TCTP, Mcl1 or β-actin. Results are shown as means ± SEM of three independent experiments.* $p<0.05$ and **$p<0.01$ compared to the value of 0h. (B) Western blotting analysis to assess the relation of TCTP and Mcl1 in MCF7 cells due to TCTP RNA interference; cells were transfected with TCTP shRNA-273. Total protein was then extracted from respective samples and subjected to perform western blotting with anti-TCTP, anti-Mcl1 and anti-β-actin antibodies. Results are shown as means ± SEM of three independent experiments.* $p<0.05$ and **$p<0.01$ compared to the value of 0h.

microtubules and mitochondria during cell division (Rinnerthaler et al., 2006). Our results suggested TCTP played pivotal roles in cell growth and cell cycle progression.

In summary, the knockdown of TCTP using TCTP shRNA systems in MCF7 cells induces apoptotic cell death, G_2/M stage arrest and growth inhibition. TCTP played pivotal roles in tumorigenesis, and TCTP silencing might offer a novel therapeutic strategy.

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