siRNA Mediated-hTERT Knockdown Impedes Proliferation of Mammalian Cancer MCF7 and HepG2 Cells

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Abstract: Telomerase is an attractive molecular target for cancer therapy because it is present in most malignant cells but is undetectable in most normal somatic cells. Human telomerase consists of two subunits, an RNA component (hTR) and a human telomerase reverse transcriptase component (hTERT). Small interfering RNA (siRNA), one kind of RNA interferences, has been demonstrated to be an effective method to inhibit target gene expression in human cells. We investigated the effects of siRNA targeting both hTERT mRNA and protein expression on the inhibition of proliferation and growth of human breast carcinoma cells (MCF-7) and liver carcinoma cells (HEPG-2). Here we used two siRNAs sequences (siRNA#1 and siRNA#2) that differentially target hTERT. Our results revealed that treatment of MCF7 and HepG2 cells with either of hTERT siRNAs resulted in significant decrease in both mRNA ($p<0.05$) and hTERT protein expression ($p<0.05$). Summary, our results clearly demonstrate that siRNA mediated knockdown of telomerase has efficiently suppressed proliferation rate of MCF7andHepG2cells. From these findings, we propose that targeting telomerase using siRNA might be a rational approach in cancer therapy.

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1. Introduction:

Telomeres cap the ends of eukaryotic chromosomes protecting them from being recognized from DNA repair system which may lead to degradation, fusion, and recombination (1). Hence telomeres are important for maintaining genomic stability (2). In mammalian cells telomeres maintain their length stable generally through the action of telomerase (2). Telomerase enzyme complex have two major subunits contributing to enzymatic activity: a structural RNA component (*hTER*) and a catalytic subunit with reverse transcriptase activity (*hTERT*) (3). In most normal cells, telomerase activity is absent or very low, however, telomerase is active in most malignant tumor cells (3). Telomeres shorten with each cycle of cell division during replication due to a so called "end-replication-problem" (1). When telomeres reach a critical short length, cell arrest /death will ensue. This is happened in normal cells because of the absence of telomerase. However in cancer cells, the reactivation of telomerase is thought to stabilize telomere length, thereby compensating for the cell division-related telomere shortening and providing unlimited proliferative capacity to malignant cells (4). Consistently, inhibition of telomerase in tumor cells should disrupt telomere length maintenance causes a proliferative crisis for malignant cells which leads to cell death. Previous studies have been developed to inhibit telomerase activity in tumor cells using antisense oligonucleotides (5-7). Although these strategies have been shown to effectively inhibit telomerase activity, further investigation of PNAs as gene therapy has been hampered by the poor intrinsic absorbability of PNA in living cells.

RNAi is a sequence-specific, post-transcriptional gene silencing mechanism that uses the introduction of small interfering RNA (siRNA), a hybrid consisting of a sense and antisense strand homologous in sequence to the silenced gene(8). siRNA, 21-nt RNA with 2-nt 3′ overhang, can mediate strong and specific suppression of gene expression. RNAi technology is currently evaluated as a potentially useful method to develop highly specific gene-silencing therapies (9,10).

The main aim of the current study is to verify whether or not the use of siRNA can target hTERT and hence inhibit the proliferation/growth rate in breast and liver cancer cell lines.

2. Material and Methods

Cell culture

Breast cancer cell line (MCF7), liver cancer cell line (HepG2) and normal melanocytes cell line (HFB4) were maintained in RPMI medium (SIGMA ALORICH#R8758) supplemented with 10% fetal bovine serum (SIGMA, USA#F2442) in the presence of penicillin (100 units/ml) and streptomycin (100 µg/ml). These cells were cultured in 5% CO_2 incubator in a humidified incubator at 37 C.

siRNA transfection

Two siRNA sequences (siRNA#1 and siRNA#2) (Table 1) and negative control siRNA (scRNA, silencer negative control siRNA, Sigma) were transfected independently into cell lines using the N-TER Nano particle siRNA Transfection System (Mirus, USA) according to manufacturer's protocol. Briefly, cells were seeded in 96-well plates at densities of 5000 cells per well and were then incubated under normal growth condition for approximately 24h. The cells were then combined with 50 nmol of either of siRNAs (siRNA#1, siRNA#2) or scRNA complexed with N-TER at a ratio of 10:1. The transfected cells were then incubated at 37 °C, 5% CO_2 for 24h, 48h and 72h. Following transfection, the cells were harvested and assayed for gene expression and viability.

Table 1: Sequences of siRNA#1 and siRNA#2 that target hTERT.

siRNA	Sequence
siRNA#1	F: 5'GUGUCUGUGCCCGGGAGAA dTdT3' R: 5'UUCUCCCGGGCACAGACAC dTdT3'
siRNA#2	F: 5'GAGCAAGUUGCAAAGCAUU dTdT3' R: 5'AAUGCUUUGCAACUUGCUC dTdT3'

Analysis of hTERT mRNA expression by semi-quantitative RT-PCR

mRNA expression of hTERT was quantified using semi-quantitative RT-PCR. Total RNA was extracted from approximately $3x10^6$ cells using TRIZOL (Invitrogen). 1 µg of total RNA was used to synthesize cDNA using M-MIV reverse transcriptase (Promega) according to manufacturer's protocol. The cDNA was added to a final volume of 20µl PCR reaction including 5 pmol of each primer (Table 2). PCR conditions were performed as follows: 25 cycles of 94°C/45 sec, 60°C/45 sec, 72°C/90 sec.

Table 2 : Sequences of hTERT and b-actin primers used for RT-PCR

primer	Sequence
hTERT	F: 5'-TCTACCGGAAGAGTGTCTGGAGCAA-3' R: 5'-GCTCCCACGACGTAGTC-CATGTTCA-3'
b-actin	F: 5'-TTCAGGTTTACTCACGTCATCC-3' R: 5'-CCAAATGCGGCATCTTCAAACCC-3'

The RT-PCR products were electrophorized using 2% agarose gel with ethidum bromide. The signals were then quantified by densitometer analysis using UNSCAN-IT software. The relative expression of hTERT was calculated according to the following formula:

$$\text{Relative hTERT mRNA expression} = \frac{(\text{hTERT signal - background signal}) / (\text{b-actin signal - background signal})_{siRNA}}{(\text{hTERT signal - background signal}) / (\text{b-actin signal - background signal})_{scRNA}} \times 100$$

Western blot analysis

About $3X10^6$ transfected cells were suspended in RIPA lysis buffer in the presence of proteinase inhibitors (Roche) for at least 30 min on ice. 40 µg of total proteins were electrophorized on 8% SDS-polyacrylamide gel and transferred onto PDVF membrane (Amersham Biosciences). After blocking the membrane in 5% non fat milk for 2h at room temperature (RT), the membrane was incubated for 1h at RT with anti-hTERT antibody at final concentration of 1:1000 (Alpha Diagnostic). The probed membrane was then washed 3 times with TBST (0.1 % Tween-20 in TBS) and then incubated for 1h at RT with horse-raddish peroxidase (HRP)-conjugated secondary antibody (1:2000). Then the

membrane was washed 3 times in TBST buffer. The bands were then visualized by chemoluminescence using ECL-PLUS reagent (Amersham Biosciences). β-actin was used as a loading control. The inhibition rate was calculated in the same manner as RT-PCR. The relative expression of hTERT was calculated according to the following formula:

$$\text{Relative hTERT protein expression} = \frac{(\text{hTERT signal - background signal}) / (\text{b-actin signal - background signal})_{siRNA}}{(\text{hTERT signal - background signal}) / (\text{b-actin signal - background signal})_{scRNA}} \times 100$$

Estimation of cell viability by TACS MTT Cell Proliferation Assay

Cellular proliferation was assayed using a sensitive in vitro TACS MTT assay. This assay is a colorimetric assay system that measures the reduction of a tetrazolium component into an insoluble formazan product by the mitochondria of viable cells. Briefly, cells were transfected with either siRNAs (1 or 2) or scRNA. 72 h-post transfection, 0.5 mg/ml MTT was added to the cells and incubated under appropriate growth condition for 4h. The absorption as an indication for cell proliferation was then measured at 570 nm.

3. Results:

Effect of siRNA treatment on mRNA expression of hTERT

Using RT-PCR we analyzed mRNA expression of hTERT after transfection with 50 nmol of siRNA of both MCF7 and HepG2 cell lines. Here we used two different siRNAs (siRNA#1 and siRNA#2) that differently target mRNA of hTERT in two different sites. Our results revealed that hTERT mRNA expression was significantly reduced (~75 % for both siRNA#1 and siRNA#2) after 24h in MCF7 (Fig. 1 A&B). Importantly, this decrease persisted for 48h- and 72h-post transfection (~70% & 74% and ~70% & 75% for siRNA#1 and siRNA#2, respectively). Indeed, no effect on hTERT mRNA expression was reported after transfection of MCF7 cells with control siRNA (scRNA, data not shown). Essentially, similar results have been reported for HepG2 cells (Fig. 1 C&D). Compared to scRNA, about 75% and 70% hTERT down-regulation was observed after 24h for siRNA#1 and siRNA#2 respectively. 48h- and 72h- post transfection, hTERT was down-regulated to similar extent in hepG2 to that in MCF7 cells (73% & ~72% and 72% & 69% for siRNA#1 and siRNA#2 respectively). Altogether, these data suggest that targeting hTERT with either of siRNA #1 or #2 has effectively suppressed hTERT mRNA expression level in both MCF7 and HepG2 cell lines.

Effect of siRNA treatment on protein expression of hTERT

Down-regulation of mRNA does not necessarily mean protein down-regulation. In order to verify that, we analyzed hTERT protein expression after transfecting both strains with 50 nmol of either siRNA#1 or siRNA#2. As shown in Fig. 2 A&B, protein expression of hTERT in MCF7 was significantly decreased 24h-post transfection (~94% for both siRNAs compared to scRNA). 48h- and 72h-post transfection of either of siRNAs has almost completely inhibited hTERT protein expression in MCF7 cells (Fig.2 B). On the other hand, hTERT protein expression was not affected after scRNA treatment (Fig. 2A, compare lane 1 and 2). Basically, similar findings have been reported in HepG2 cells (Fig. 2 C&D). hTERT protein expression was significantly down-regulated (~95%) 24h-post siRNA#1 treatment and almost no protein was detected after 48h and 72h of siRNA#1 treatment. Of note, siRNA#2 treatment was more effective in hTERT protein down-regulation in HepG2 as almost no hTERT protein was detected 24h-, 48h- and 72h-post transfection. Collectively, our results implicated that both siRNA#1 or siRNA#2 treatment have effectively down-regulated hTERT expression.

Effect of Telomerase knock-down on proliferation

Telomerase was shown to impact cellular proliferation and regulates cell growth. In order to verify that, cellular proliferation was measured using MTT assay after hTERT knockdown. Our results demonstrated that hTERT knockdown did not affect proliferation rate of normal HFB4 melanocyte cells (Fig. 3, first 3 columns). In addition, cell proliferation was not affected after treatment of either MCF7 or HepG2 cells with scRNA (Fig. 3 columns 4 and 7). Importantly, Knocking down hTERT using siRNA#1 showed a dramatic inhibitory effect on cell proliferation in both MCF7 and HepG2 cells (Fig. 3, ~36% and ~34% respectively). Similar results have been reported for siRNA#2 (~34% and ~33% in MCF7 and HepG2 respectively). These results clearly revealed that hTERT down-regulation suppresses cell proliferation in tumor but not normal cells.

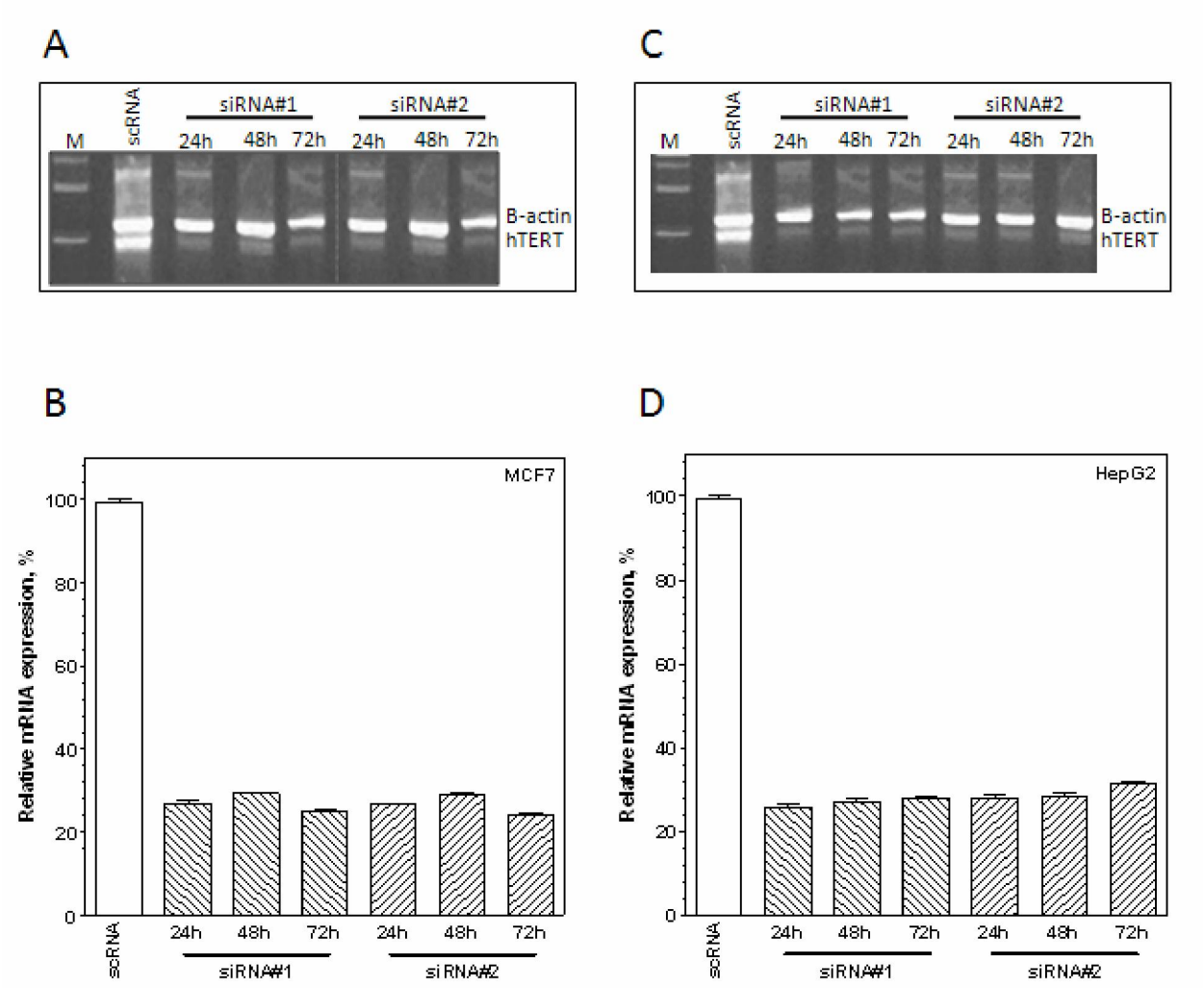

Fig.1: Down-regulation of hTERT mRNA expression in MCF7 and HepG2 cells by siRNA#1 and siRNA#2. (A) Representative examples of hTERT mRNA expression (upper bands) after transfecting MCF7 cells with 50 nmol of scRNA (lane 1), siRNA#1 (lanes 3-5) or siRNA#2 (lanes 6-8). Compared with scRNA- transfected MCF7 cells, hTERT mRNA expression was down-regulation after 24h, 48h and 72h. (B) Relative hTERT mRNA expression. Shown are mean values hTERT expressions in MCF7 of at least 3 independent RT-PCR experiments. (C) Representative examples of hTERT mRNA expression (upper bands) after transfecting HepG2 cells with 50 nmol of scRNA (lane 1), siRNA#1 (lanes 3-5) or siRNA#2 (lanes 6-8) showing hTERT downregulation after the indicated time points. (B) relative hTERT expression. Shown are mean values hTERT expressions in HepG2 of at least 3 independent experiments. M: 1kb DNA Molecular marker; sc: scRNA, #1: siRNA#1, #2: siRNA#2. B-actin was used as internal control (lower bands).

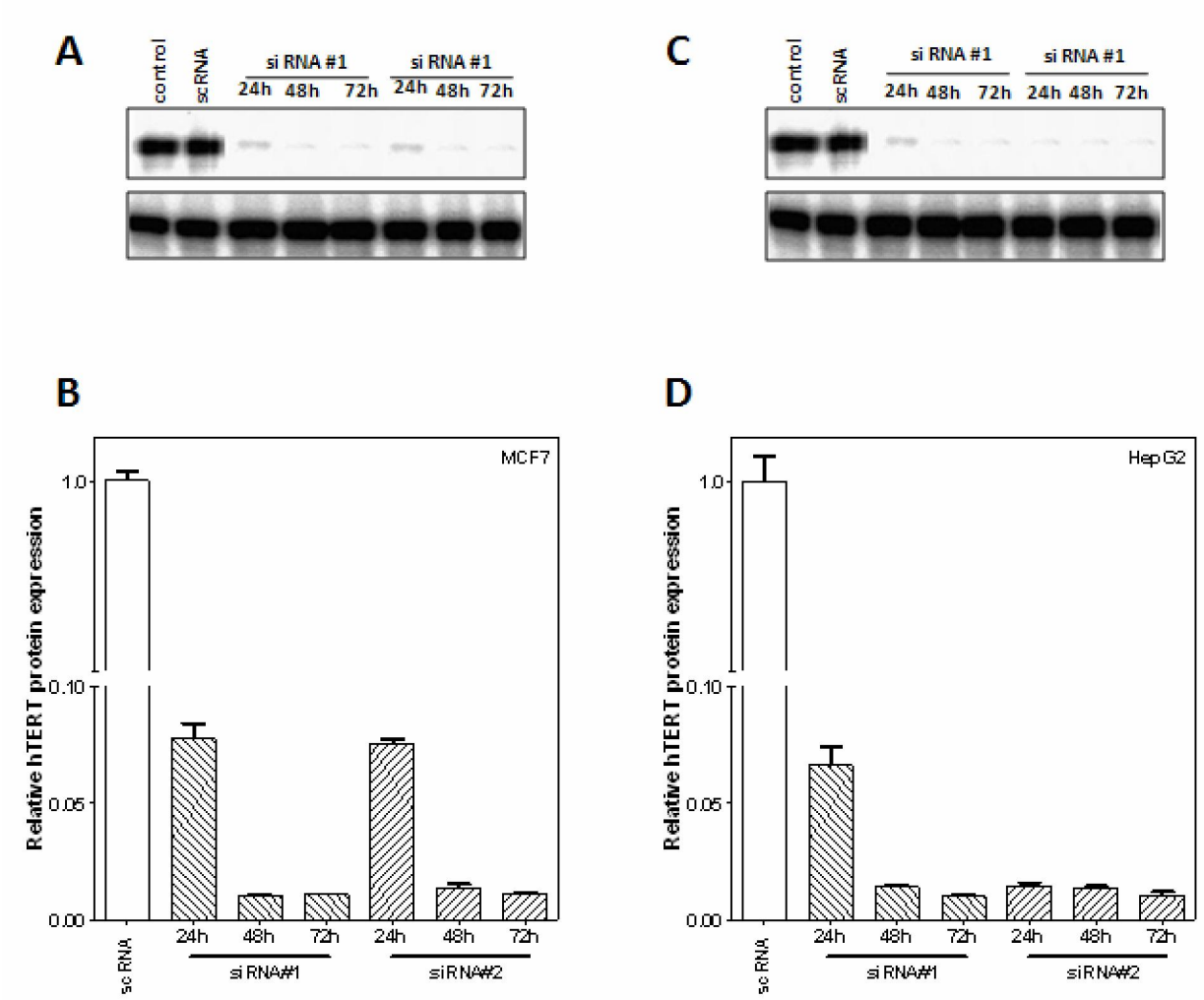

Fig.2: Down-regulation of hTERT protein expression in MCF7 and HepG2 cells by siRNA#1 and siRNA#2. (A) Representative examples of hTERT protein expression using Western blot after transfecting MCF7 cells with 50 nmol of scRNA (lane 1), siRNA#1 (lanes 3-5) or siRNA#2 (lanes 6-8). hTERT protein expression is efficiently down-regulated after treatment of MCF7 cells with either of siRNA#1 or siRNA#2. (B) Relative hTERT protein expression. Shown are mean values hTERT expressions in MCF7 of at least 3 independent blots. (C) Representative examples of hTERT protein expression after transfecting HepG2 cells with 50 nmol of scRNA (lane 1), siRNA#1 (lanes 3-5) or siRNA#2 (lanes 6-8) showing hTERT downregulation after the indicated time points. (B) Relative hTERT protein expression. Shown are mean values hTERT expressions in HepG2 of at least 3 independent experiments. control: untreated cells; sc: scRNA, #1: siRNA#1, #2: siRNA#2. b-actin was used as a loading control.

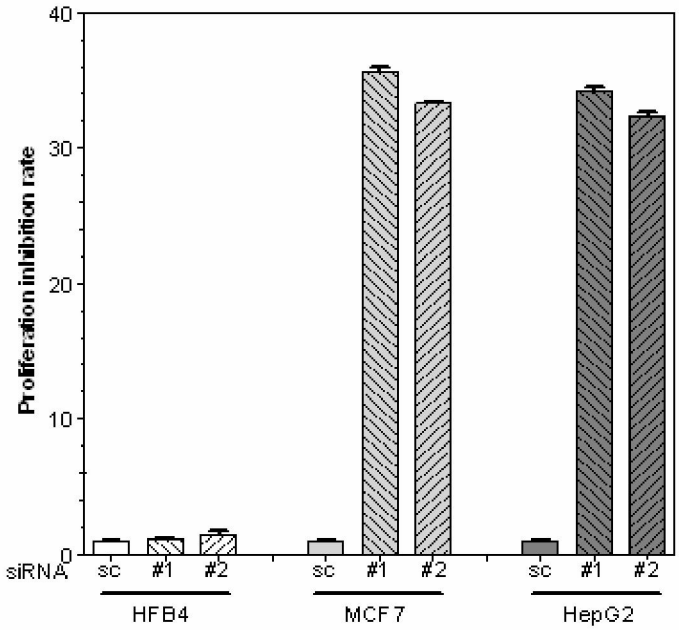

Fig.3: Inhibition of cell proliferation after hTERT knockdown by siRNA. Inhibition of cell proliferation was measured using MTT assay after transfecting HBF4, MCF7 and HepG2 cells by 50 nmol of scRNA, siRNA#1 or siRNA#2. hTERT Knock-down by either siRNA#1 or #2 showed a significant higher inhibitory ($p<0.05$) effect on tumor cells (MCF7 and HepG2) but not on normal HBF4 cells. Shown are mean values of at least 3 independent experiments.

4. Discussion

Telomerase maintains telomere length which in turn protects the ends of chromosomes from being detected as DNA damage by DNA repair systems. In the absence of telomerase telomeres lose nucleotides during replication which leads to shortening in the chromosomes and finally cell death (1-3,11). Telomerase activity has been reported as a valuable prognostic factor in many tumor types including neuroblastomas, bladder cancer, lung cancer and colorectal cancer (6,12-17). Giving the almost universal expression of telomerase in cancer cells but not in most normal cells, we hypothesized that targeting telomerase might be a powerful approach to cancer therapy.

Many strategies have been developed to target telomerase such as antisense nucleotides and specific inhibitors for the reverse transcriptase activity of telomerase (5,7,11,18). Although, these strategies showed a significant efficiency for inhibiting telomerase activity in cancer cells, their significant toxicity limited their clinical use. RNAi is characterized by high efficiency, high specificity and low toxicity. In the current study we used two siRNAs (siRNA#1 and siRNA#2) to target hTERT in MCF7 and HepG2 tumor cells. Our results have clearly showed that both siRNAs were efficiently down-regulated hTERT mRNA (Fig.1) and protein (Fig.2) expression in both MCF7 and HepG2 cells. In consistent with our results, several studies have demonstrated that siRNA is a powerful method to target telomerase (8,19). Down regulation of hTERT indeed impedes telomerase activity which results in preventing telomere length from being maintained. A number of genetic validation experiments indicate that telomere maintenance by the enzyme telomerase is a key event in the immortalization process and the continuous proliferation of a large proportion of human cancers (2,4,20,21). Consistently, targeting hTERT should affect cell proliferation/growth. Using MTT assay we reported in the current study that hTERT down-regulation has an efficient inhibitory

effect on cell proliferation in both MCF7 and HepG2 tumor cells (Fig.3) but not on normal cells. This indicates that telomerase plays an essential role in cell proliferation and viability control of tumor cells but properly not normal cells. Previously, it was shown that inhibition of telomerase sensitizes tumor cells to several insulting agents such as ionizing radiation (22-24). The mechanism of this sensitization is not clear. Based on our present data, we can explain this sensitization as telomerase supports proliferation. Consequently, inhibition of telomerase impedes the proliferation rate and hence leads to cell death.

Overall, this study presents a proof of principle for the use of RNA interference system to target *hTERT* as a powerful method to inhibit telomerase and hence inhibit the growth of tumor cells.

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