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EVIDENCE OF EXTRA-TELOMERIC EFFECTS OF hTERT AND ITS REGULATION INVOLVING A FEEDBACK LOOP*

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Abstract

The human telomerase reverse transcriptase (hTERT) is the catalytic subunit of the enzyme telomerase which is responsible for telomeric maintenance and extension. Using RNA interference to knock down hTERT mRNA expression, we provide evidence that hTERT exerts extra-telomeric effects on the cell cycle and on its own regulatory proteins, specifically: *p53* and *p21*. We tested our hypothesis that hTERT regulates its own expression through effects on upstream regulatory genes using transformed human embryonic kidney (HEK 293) cells, *p53* and $p16^{INK4a}$ null human ovarian cancer SKOV-3 cells, and *p53*-null MDA-MB-157 human mammary cancer cells. In HEK 293 cells, hTERT knock down resulted in elevated *p53* and *p21* transcription and a decrease in cellular proliferation. Similar results were observed in the MDA-MB-157 cell line where *p21* was up-regulated, correlating with cell growth inhibition. In contrast, we observed a decrease in expression of *p21* in SKOV-3 cells with *hTERT* knock down and cell growth appeared to be unaffected. These findings suggest that *hTERT* may be involved in a feedback loop system, thereby playing a role in its own regulation.

Keywords

Telomerase; Telomere; hTERT; Cell cycle; p53; p21

Introduction

Telomerase is a DNA polymerase that uses an RNA template in a reverse transcription process to synthesize telomeric ends on linear chromosomes during replication to counteract the “end-replication problem”. Telomerase activity is barely detectable in somatic cells but is reactivated in immortalized cell lines and human cancers [1,2]. Due to this repression of telomerase, normal somatic cells experience telomeric attrition at a mean loss of 30-150 bps of telomeric DNA with each cell replication [3] until a critical minimum telomeric length is reached, at which time the cells experience cellular senescence [4]. Telomeres serve to preserve chromosomal integrity by preventing rearrangements, nuclease degradation, and end-to-end chromosomal

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fusions [5,6]. The telomerase holoenzyme is a ribonucleoprotein complex; although it is comprised of many components, only two subunits are essential to its function. In humans, the catalytic subunit of telomerase, hTERT, confers its reverse transcriptase activity, adding 5'-TTAGGG-3' repeat sequences onto telomeres; the RNA component, *hTR*, is complementary to the telomeric repeat sequence, serving as a template for elongation of the telomeres [7-9]. The *hTR* component of telomerase is found to be ubiquitously expressed in most cell types including telomerase-negative cells, such as differentiated somatic cells [10,11]. hTERT, on the other hand, is tightly regulated during differentiation and is almost undetectable in most somatic cells [12]. A positive correlation has been found between the amount of mRNA of *hTERT* and the activity of telomerase, suggesting that telomerase activity is regulated at the gene transcriptional level of *hTERT* [13-16]. Therefore, inhibition of *hTERT* expression usually results in decreasing of telomeric length.

For the more than 20 years since Carol Greider discovered telomerase [7], scientists have believed that telomerase is a terminal effector and that its role is mainly to extend and maintain the length of telomeres. However, recent studies have shown that the expression of *hTERT* affects a variety of other genes. In a recent study in which microarray analysis of 19,000 genes was performed in bovine adrenocortical cells ectopically expressing *hTERT*, 284 genes were found to be either positively or negatively affected by changes in hTERT, suggesting that hTERT may have extra-telomeric effects. Interestingly, most of the genes affected were involved in cell-cycle regulation, signaling, and metabolism [17]. Further, overexpression of telomerase due to the ectopic expression also resulted in a decrease in the mRNA of *tp53bp1*, a gene involved in the induction of the p53/p21 system [17]. In this study, we hypothesize that hTERT exerts its effects not only on telomeres and other genes, but also specifically on its upstream regulators, mainly p53 and p21, thereby partially regulating itself.

p53 is a tumor suppressor protein that functions in differentiation, apoptosis, DNA repair and control of cell cycle progression, by regulating the transcription of many genes, both negatively and positively. The *hTERT* gene has two p53 binding motifs at -1877 and -1240 relative to the start of transcription, upstream of the 5' core promoter region (18). Overexpression of *p53* and binding of the protein with the assistance of transcription factor Sp1 at these two motifs represses the *hTERT* promoter [18]. In mammary epithelial cells, abrogation of p53 function induces cellular immortality, probably through the reactivation of telomerase [18].

The $p21^{WAF1/Cip1/SDI1}$ (*p21*) gene is ubiquitously expressed in mammalian cells, and as a major player in cell cycle arrest, is critical for the control of differentiation, senescence, and apoptosis [19,20]. Over-expression of *p21* induces a correlative repression of *hTERT*, which has been demonstrated in human glioma cells [21] and in squamous cell carcinoma where telomerase expression is abnormally elevated [22]. *p21* contains a p53 responsive element at -2281/-2262 in the distal region of its promoter. Full transactivation of the *p21* promoter is enabled by the binding of p53 protein, Sp1 proteins and related factors at the promoter region [23,24]. The downstream effect of p53 transactivation of the *p21* promoter is the negative regulation of the cell cycle, thereby preventing progression past the G1/S checkpoint [25]. At the same time, *p21* activation leads to the repression of the *hTERT* promoter, thereby effectively shutting down the cell cycle, as we will show in this study.

We analyzed human embryonic kidney (HEK 293), human ovarian cancer (SKOV-3), and human mammary cancer (MDA-MB-157) cell lines, to assess the effects of *hTERT* knock down the levels of p53 and p21, and the subsequent effect on cell proliferation. HEK cells are adenovirally transformed and express adenoviral E1A and E1B proteins [26]. A transformed cell line was chosen to show the effects of *hTERT* knock down on the proliferation of transformed cells and to investigate the potential of RNA interference (RNAi) of *hTERT* for therapeutic purposes. Also, because these cells are embryonic in origin, proliferate indefinitely

in vitro and do not show contact inhibition, they were appropriate for the short-term dsRNA knock down of *hTERT* and for proliferation studies. Further, studies have indicated that mammalian cells of embryonic origin lack the interferon response upon treatment with double-stranded RNA [27-29]. Adenoviral E1B and E1A proteins inactivate p53 either by physically binding p53 or by binding its downstream effectors such as mdm-2 and other apoptotic components [30-32]. However, adenovirally transformed cells can accumulate p53 to an extent where excess p53 is transcriptionally active [33]. Thus, HEK cells are not G1-arrested, have high levels of telomerase [34], and have transcriptionally active *p53* [33].

P53 and $p16^{INK4a}$ null SKOV-3, and *p53*-null MDA-MB-157 cell lines [35] were utilized in this study as *p53* deficient model systems to determine the effects of long-term hTERT knock down on p21 levels and cell proliferation. We demonstrate how hTERT levels can directly affect p21, since the mechanism of p53-dependent transactivation of p21 is eliminated in these cell lines.

Using specific RNAi knock down of hTERT in a variety of different cell types, our studies collectively demonstrate for the first time that the level of hTERT in the cell may regulate the expression of its gene.

Materials and methods

dsRNA Synthesis

A DNA template for the synthesis of dsRNA was first generated by PCR amplifying a 219 bp fragment of HEK cDNA specific to the *hTERT* gene transcript with primers: ElmoreF (5'-GACTCGACACCGTGTTCACCTAC-3') and ElmoreR (5'-ACGTAGAGCCCGGCGTGACAG-3'), each constructed with T7 promoter sequences attached to their 5'-ends (5'-TAATACGACTCACTATAGGGAGA-3') as indicated by Ambion MEGAscript RNAi kit. dsRNA was generated using MEGAscript RNAi Kit [Ambion, Austin, TX], according to the manufacturer's instructions. The generated single-stranded RNAs were heated to 75°C for 5 min and allowed to slowly anneal at room temperature until cooled.

Plasmid Construct

pSilencer 3.1 H1-neo [Ambion Inc.] plasmid was digested with restriction enzymes *BamHI* and *HindIII* at 37°C for 1 h and the enzymes were inactivated at 65°C for 15 min. Oligonucleotide fragments were removed from the reaction using the Qiaquick DNA Cleanup Systems, Nucleotide Removal protocol [Qiagen, Valencia, CA]. A plasmid construct, pS2-3, targeting the upstream section of *hTERT* mRNA beginning at +863, was used to determine the effects of long-term *hTERT* knockdown. The insert sequence for pS2-3 is as follows: *Sense*: 5'-GATCCGTTCTGTGTGGTGTCACCTGTTCAAGAGACAGGTGACACCACACAGAATT-TTGAAA-3'; *Antisense*: 5'-AGCTTTTCCAAAAAATTCTGTGTGGTGTCACCTGTCTCTTGAACAGGTGACACCA-CACAGAAC-3'. The oligonucleotides for the insert were designed according to Ambion's recommendations for short hairpin RNA (shRNA) design. Each insert contains a *BamHI* and a *HindIII* restriction site overhang, a 19 bp target sequence, a 9 bp loop sequence, and the reverse complementary sequence of the 19 bp target. The insert also includes an RNA polymerase III terminator sequence. The oligos for the insert were annealed at 1 μg/μl and 37° C for 1 h as specified in the kit.

A clone, pSCR, provided by Ambion, which expresses a shRNA scrambled sequence, was used as a control (*Sense*: 5'-

GATCCACTACCGTTGTTAAGGTGTTCAAGAGACACCTATAACAACGGTAGTTTT TG GAAAA-3'). pSCR clones were transformed into competent DH5α *E. coli* cells and selected by growing in Luria Broth medium supplemented with 100 μg/ml of ampicillin. Plasmid DNA was isolated and purified using the Maxiprep kit (Qiagen) according to manufacturer's instructions. The presence of plasmid constructs and insert sequences were confirmed by DNA sequencing.

Cell Culture and Maintenance

Human embryonic kidney (HEK 293) cells were obtained from American Type Culture Collection (ATCC) [cat #CRL1573] and cultured with DMEM supplemented with 10% heat-inactivated FBS, 1% 200 mM L-glutamine, and 1% sodium pyruvate. The cells were maintained in 100 mm tissue culture dishes and passaged 1:10 - 1:12 with trypsin/EDTA at 80% confluency. Human ovarian (SKOV-3) cells [ATCC; cat #HTB-77] were cultured with McCoy′s 5a medium supplemented with 10% FBS, 2 mM L-glutamine. Human mammary (MDA-MB-157) cancer cells [ATCC cat #HTB-24] were cultured with DMEM F12 50/50 medium supplemented with 10% FBS and 2 mM L-glutamine. All cultures were incubated at 37°C in 5% CO_2.

Transfection and Selection; HEK cells

For dsRNA transfection, cells were trypsinized 24 h before transfection and viable cells were counted with a hemacytometer using the Trypan blue dye exclusion method. 4.0×10^5 cells were plated per well of a 6-well plate and incubated for 24 h to provide experimental cultures of approximately 60% confluency for transfection the next day. dsRNA (2.5 μg) was transfected in each well with FuGENE 6 transfection reagent [Roche, Indianapolis, IN] at a ratio of 1:5 μg to μl according to manufacturer's recommendations. The FuGENE-dsRNA complex was allowed to incubate with the cells until harvesting and counted with a hemacytometer.

For plasmid DNA transfection, cells were transfected with pS2-3, and pSCR using FuGENE 6 transfection reagent [Roche] at a ratio of 1:5 μg to μl, according to manufacturer's recommendations. The FuGENE-DNA complex was allowed to incubate with the cells for 24 h before removal and replenishing with fresh culture medium supplemented with antibiotics. Antibiotic resistant cells were selected with 300 μg/ml of G418 antibiotic in HEK medium. Cells were selected for at least 4 weeks before harvesting for analysis.

Transfection and Selection; SKOV-3 and MDA-MB-157 cells

Cells were transfected with pS2-3, and pSCR using FuGENE 6 transfection reagent [Roche] at a ratio of 1:3 μg to μl, according to manufacturer's recommendations. The FuGENE-DNA complex was allowed to incubate with the cells for 24 h before removal and replenishing with fresh culture medium supplemented with antibiotics. Antibiotic resistant cells were selected with 800 μg/ml and 400 μg/ml of G418 antibiotic for SKOV-3 and MDA-MB-157 cells, respectively. Cells were selected for at least 4 weeks before harvesting for analysis.

Harvesting and Cell Counting

HEK cells transfected with dsRNA were harvested and counted at days 1, 3, and 5. HEK and SKOV-3 cells transfected with plasmid constructs were allowed to recover and selected for at least 4 weeks before harvesting for analysis and setting up of cell proliferation assays. Cell-proliferation assays were performed by trypsinizing the cells, using the Trypan blue dye exclusion method and counting with a hemacytometer.

RT-PCR Analysis

The expression of *hTERT*, p53, and *p21* were assessed by PCR using cDNA libraries generated from treated cells. PCR analysis of transcripts for housekeeping genes, *GAPDH* and *β-actin*, were performed as loading controls and were used as standards to normalize data. Total RNA was extracted using the Qiagen RNeasy kit (Qiagen). Extracted RNA was quantified with a UV spectrophotometer and equal amounts of RNA per experiment were used to generate cDNA with the Superscript First-Strand Synthesis System (Invitrogen). Synthesized cDNA was subjected to PCR amplification with primers targeting the gene products for *hTERT* (F: 5'-CAAGAACGCAGGGATGTCGCTG-3'; R: 5'-CTGCGTCTGGGCTGTCCTGAGT-3'), *p53* (F: 5'-ATTTGCGTGTGGAGTATTTG-3'; R: 5'-GGAACAAGAAGTGGAGAATG-3'), *p21* (F: 5'-CAGGGTCGAAAACGGCGGCA-3'; R: 5'-AGGAGCCACACCCCTCCAGA-3'), *GAPDH* (F: 5'-GAAGGTGAAGGTCGGAGTC-3'; R: 5'-GAAGATGGTGATGGGATTTC-3') and *β-actin* (F: 5'-GCTCGTCGTCGACAACGGCTC-3'; R: 5'-CAAACATGATCTGGGTCATCTTCTC-3'). The PCR reactions were analyzed by 2% agarose gel electrophoresis. For each primer set, the number of amplification cycles was predetermined to in order to be in the exponential phase.

Statistical Analysis

Cell counts were subjected to a two-tailed T-test, assuming equal variances. Mock transfected and transfected samples were compared to the control untransfected samples to determine significant differences ($p<0.05$) in proliferation rate.

Results

hTERT knock down using long dsRNA and shRNA

To assess the short-term effects of *hTERT* knock down, a 219 bp long dsRNA complementary to *hTERT* mRNA beginning at +863, was synthesized and transfected into HEK cells. *hTERT* mRNA in the cells was significantly reduced to 55% by 24 h of treatment and the RNAi slowly decreased in effectiveness thereafter (Fig. 1A). *GAPDH* was used as a loading control and its consistent expression levels show that the RNAi effect was specific for *hTERT*. To determine the effects of *hTERT* knock down on the cell-cycle, a growth curve was generated. Untreated HEK cells and cells incubated with only FuGENE 6 tranfection reagent were used as controls to ensure that there is no toxicity due to the transfection reagent. The proliferation studies showed parallel linear growth between the untreated and mock-treated control cells while treated cells were growth inhibited (Fig. 1B and C). Growth inhibition peaked on day 3 of treatment, and was sustained through day 6 after treatment (Fig. 1C).

The cellular mRNA levels of *p53* and *p21* were analyzed following the introduction of RNAi of *hTERT* on days 1, 3, and 6 post-treatment with the 219 bp dsRNA. *p53* mRNA levels were elevated within 24 hours of treatment, correlating with the decreased levels of *hTERT* mRNA in the cell (Fig. 2A). *p53* mRNA levels showed a marked 4-fold elevation in treated HEK cells on day 1 and remained elevated through day 6 (Fig. 2B), while the untreated HEK control cells showed a steady incline of *p53* mRNA levels to 3.1 fold by day 6. This suggests that the increase in *p53* levels in the control HEK cells probably resulted from cell-to-cell contact as opposed to a response to lowered hTERT levels. Following the induction of *p53* in the treated HEK cells, *p21* mRNA levels progressively increased, consistent with findings that p53 transactivates the p21 promoter [23,24] (Fig. 2A). In the treated HEK cells, *p21* mRNA levels were increased 1.7 fold on day 1 and 4.6 fold by day 6 (Fig. 2C). In contrast, *p21* levels in the untreated HEK control cells were slightly elevated to 1.5 fold and remained relatively stable (Fig. 2C). Further, since HEK cells do not elicit an interferon response when transfected with long dsRNA [27,28] and the *GAPDH* loading control does not show a variable expression, the

results indicate that the RNAi response is target specific and appropriated to the dsRNA treatment, instead of off-target RNAi effects. Taken together, the findings suggest that reduced levels of *hTERT* mRNA in HEK cells resulted in a profound growth inhibitory response induced by the p53/p21 pathway.

The plasmid construct, pS2-3, was transfected into HEK cells and selected in G418 antibiotic for at least 4 weeks to analyze the long-term effects of *hTERT* knock down. The experiment was repeated twice to ensure reproducibility of results. Non-transfected HEK cells and HEK cells transfected with a construct expressing a scrambled sequence (pSCR) were used as controls. Proliferation studies showed comparative linear growth in both the control HEK and pSCR cells. HEK cells expressing pS2-3 showed slower growth rates as compared to the controls (Fig. 3E). To determine whether the constructs had successfully knocked-down *hTERT* and the effects on other genes of interest, total RNA was extracted from untransfected HEK cells and HEK transfected with pS2-3. cDNA was synthesized via reverse transcriptase reactions and PCR was performed with primers specific to *hTERT*, *p53*, *p21*, *GAPDH*, and *β-actin*. Cells transfected with pS2-3 showed decreased levels of *hTERT* mRNA as compared to the control cells, indicating that the construct was able to, but not fully knock-down *hTERT* (Fig. 3A).

The effect of *hTERT* knock down on the other genes of interest paralleled that of the short-term studies with dsRNA transfection. As expected, HEK cells transfected with pS2-3 showed elevated levels of *p53* and *p21* (Fig. 3C). Further, the invariability in expression of the housekeeping gene, *GAPDH*, indicates that the knock down is target specific. Although *hTERT* was not fully knocked-down, it is important to note that it is adequate to affect changes on the expression of *p53* and *p21*. Taken together, the results of long-term knock down of *hTERT* in HEK cells suggest that telomerase may directly or indirectly affect the cell cycle or the genes regulating the cell cycle.

hTERT knock down in p53 null SKOV-3 and MDA-MB-157 cells

To investigate the knock down of hTERT in *p53*-null systems in order to eliminate the variable of p53-induced effects on p21, pS2-3 and pSCR plasmid constructs were transfected into SKOV-3 ovarian cancer cells and MDA-MB-157 breast cancer cells. Cells transfected with plasmid constructs were subjected to G418 antibiotic selection for at least a month before harvesting for analysis.

Untransfected SKOV-3 cells (denoted as SKOV) and SKOV-3 cells transfected with the plasmid expressing a scrambled sequence (SKOVpSCR) were used as controls. However, in our proliferation studies, SKOVpS2-3 cells did not show a marked retardation in growth, as compared to SKOV and SKOVpSCR. By day 3 of the proliferation studies, SKOV, SKOVpSCR, and SKOVpS2-3 cells seemed to reach a plateau in the rate of proliferation after becoming confluent and experiencing contact inhibition in culture. There was no obvious growth in these cultures between day 3 and day 5 (Fig. 4B). This is consistent with a previous study that showed that inhibition of telomerase activity in SKOV-3 cells neither resulted in telomeric erosion nor growth inhibition [36].

To determine if *hTERT* had been successfully knocked-down in the transfected SKOV-3 cells, reverse transcriptase PCR analysis was performed and showed *hTERT* knock down in SKOVpS2-3 (Fig. 4A). Corresponding to decreased levels of *hTERT* mRNA in SKOVpS2-3 cells, expression of *p21* also appeared to be down-regulated (Fig. 4A), while *p21* expression was not affected in the controls SKOV and SKOVpSCR. The invariability of the expression of *GAPDH* also indicates that the effect of RNAi is target specific.

MDA-MB-157 cells transfected with pS2-3 (MDA-pS2-3) showed a significant reduction in proliferation rate as compared to control untransfected MDA-MB-157 cells and cells expressing the scrambled sequence construct (MDA-pSCR) (Fig. 5C). This indicates that hTERT protein or *hTERT* mRNA is able to exert control on cell cycle progression either directly or indirectly.

Reverse transcriptase PCR using primers specific to the genes of interest was performed to determine effect of *hTERT* knock down on the levels of transcription for *hTERT* and *p21*. The knock down was confirmed by lower levels of *hTERT* transcript in MDA-pS2-3 cells but not in control MDA-MD-157 or MDA-pSCR cells (Fig. 5A). The downstream effects of the knock down resulted in an increase in *p21* expression in MDA-pS2-3 while expression of *p21* in control cells MDA-MB-157 and MDA-pSCR remained at basal levels (Fig. 5B).

Discussion

Telomerase has only recently been found to exert extra-telomeric effects [17]. Here, we show specific effects of hTERT expression and telomerase activity on the cell cycle regulators, *p53* and *p21*. Both of these genes are known upstream regulators of *hTERT*, suggesting the concept that one of the extra-telomeric effects of hTERT may be in a feedback loop, thereby regulating itself. As there are multiple regulatory factors that act on the *hTERT* promoter, such as epigenetic modifications, p53, p21, c-Myc/Mad/Max, estrogen-receptor complexes, Sp1, WT1, E2F [37-42], it is the culmination of all factors exerting their effects on the *hTERT* promoter that eventually determines the transcriptional output of *hTERT*. Several studies have also successfully knocked down hTERT and shown that RNAi of hTERT decreases telomerase activity, indicating that RNAi if hTERT is a viable system to study the downstream effects of telomerase [43-45]. Our results suggest that the effects of hTERT and telomerase activity on extra-telomeric targets vary in different cellular systems.

Since HEK cells are of embryonic origin, we were able to use two different RNAi systems to knock down hTERT. Namely, dsRNA and a plasmid construct expressing an shRNA, were used to knockdown *hTERT* mRNA. Since both RNAi systems in HEK cells generated similar results, we are able to conclude that the HEK cells did not experience interferon response due to the transfection of long dsRNA, and that the RNAi effects were target specific. Moreover, in all experiments, we observed no variability in expression of housekeeping genes, *GAPDH* and *β-actin*, further supporting that the RNAi was target-specific.

The knock down of *hTERT* using dsRNA targeting a 219 bp region of the *hTERT* mRNA resulted in a significant reduction of *hTERT* mRNA in the cell, and subsequently caused the cells to be temporarily growth arrested. The RNAi effect on *hTERT* caused an immediate increase in *p53* mRNA levels, which in turn induced the transcription of *p21* [23,24] (Table 1), suggesting that some specific telomeric-effects of hTERT include the regulation of *p53* and *p21*. It is also highly unlikely that changes in telomeric lengths alone affected cell proliferation rate and induced the upstream regulators of *hTERT*. Instead, it is probable that the knock down of *hTERT* itself induced such effects, because *p53* and *p21* levels were elevated in the transient dsRNA knock down of *hTERT* within 24 h of treatment, before telomeric changes are detectable. Thus, telomerase is able to exert an effect on the cell cycle independent of telomere length.

When 19-mer shRNAs targeting *hTERT* mRNA were used to stably knock down *hTERT* in long-term studies, we observed differential responses to the RNAi effect. In HEK cells, the pS2-3 construct expressing shRNA specific to *hTERT* retarded growth of the cells and caused an elevation of *p53* and *p21* mRNA in response to *hTERT* knock down. The finding that the cells were not growth-arrested could be attributed to the fact that the RNAi knock down effect using a single short target was not as effective as targeting *hTERT* with the 219 bp dsRNA.

Also, the residual *hTERT* levels after knock down may provide enough telomerase activity to sustain proliferation, despite its slowed rate. It is also significant to note that although the knock down of *hTERT* was not 100%, the slight reduction in *hTERT* mRNA levels was adequate to produce changes in proliferation rate and exert extra-telomeric effects on *p53* and *p21*.

To further evaluate how hTERT might affect *p53*-null and different neoplastic cell systems, *hTERT* was knocked down in SKOV-3 ovarian cancer and MDA-MB-157 breast cancer cells. Proliferation studies showed a significant growth decrease in MDA-pS2-3 cells expressing shRNA specific to *hTERT* mRNA as compared to the untreated control cells as well as the cells expressing a scrambled shRNA sequence (MDA-pSCR). This cell growth reduction was coupled with an increase in *p21* mRNA levels, consistent with the HEK study. It is very probable that the growth reduction is due to the upregulation of *p21*. This also indicates that *hTERT* is able to regulate *p21* independent of *p53*.

SKOV-pS2-3 cells expressing shRNA specific to *hTERT* mRNA resulted in a decreased expression of *p21*, supporting the evidence that *hTERT* can regulate *p21* in a *p53*-independent manner. It is interesting that we did not observe a significant retardation of growth in SKOVpS2-3 cells as compared to the untreated control cells and the cells expressing a scrambled shRNA sequence (SKOVpSCR). Further, since SKOV-3 cells do not undergo alternate lengthening of telomeres (ALT)[36], a probable explanation for the lack of growth reduction could be that since SKOV-3 cells are null for both *p53* and $p16^{INK4a}$, a decrease in *p21* due to the knock down may result in insufficient cell cycle inhibitory machinery. However, the exact mechanisms of how *hTERT* up or down regulates *p21* in these systems remain unclear.

Our findings support the novel model that telomerase, via the regulation of *hTERT* (the gene encoding its catalytic subunit), is involved in a feedback loop that may engage various pathways. Our studies clearly indicate that the knock down of *hTERT* is able to affect its own regulatory gene components, namely, *p53* and *p21*. In cell systems such as HEK cells where the *p53* gene is intact, the feedback loop is *p53* dependent resulting in *p21* transcriptional activation and cell growth inhibition. In *p53*-null cell systems such MDA-MB-157 cells, the knock down of *hTERT* results in a *p53*-independent upregulation of *p21*, with similar growth retardation. In contrast, the knock down of *hTERT* in *p53*/$p16^{INK4a}$-null and ALT-negative SKOV-3 cells resulted in a decreased expression of *p21* and did not result in growth inhibition.

These findings indicate that perhaps the titer of *hTERT* mRNA or telomerase activity in the cell may signal the cell to induce a feedback regulation system, also further supporting the hypothesis that telomerase has extra-telomeric functions. The precise mechanisms of the feedback system need to be refined and further detailed. Future studies involving other cell types and the use of a p21-null model would be useful to clarify how other genes are affected after *hTERT* knock down.

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References

[1]. Nakayama J, Tahara H, Tahara E, Saito M, Ito K, Nakamura H, Nakanishi T, Ide T, Ishikawa F. Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. Nat. Genet 1998;18:65–68. [PubMed: 9425903]

[2]. Armelin HA, Armelin MCS, Kelly K, Stewart T, Leder P, Cochran BH, Stiles CD. Functional role for c-myc in mitogenic response to platelet-derived growth factor. Nature (London) 1984;310:655–660. [PubMed: 6088986]

[3]. Vaziri H, Benchimol S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr. Biol 1998;8:279–282. [PubMed: 9501072]

[4]. Wright WE, Shay JW. Two-step mechanism controlling cellular senescence and immortalization. Exp. Geront 1992;27:383–389.

[5]. Greider CW. Telomeres. Curr. Opin. Cell Biol 1991;3:444–451. [PubMed: 1892656]

[6]. Greider CW, Blackburn EH. Telomeres, telomerase and cancer. Sci. Am 1996;274:92–97. [PubMed: 8560215]

[7]. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 1985;43:405–413. [PubMed: 3907856]

[8]. Greider CW, Blackburn EH. The telomere terminal transferase of tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell 1987;51:887–898. [PubMed: 3319189]

[9]. Greider CW, Blackburn EH. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature 1989;337:331–337. [PubMed: 2463488]

[10]. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, Ziaugra L, Baijersbergen RL, Davidoff MJ, Liu Q, Bacchetti S, Haber DA, Weinberg RA. hEST2, the putative human telomerase catalytic subunit gene, is upregulated in tumor cells during immortalization. Cell 1997;90:785–795. [PubMed: 9288757]

[11]. Nakamura TN, Morin G, Chapman KB, Wienrich RL, Andrews WH, Lingner J, Harley CB, Cech TR. Telomerase catalytic subunit homologs from fission yeast and humans. Science 1997;277:955–959. [PubMed: 9252327]

[12]. Matsutomi K, Yu EY, Khurts S, Ben-Porath I, Currier JL, Metz GB, Brooks MW, Kaneko S, Murakami S, DeCaprio JA, Weinberg RA, Stewart SA, Hahn WC. Telomerase maintains telomere structure in normal human cells. Cell 2003;114:241–253. [PubMed: 12887925]

[13]. Yi X, Tesmer VM, Savre-Train I, Shay JW, Wright WE. Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels. Mol. Cell. Biol 1999;19:3989–3997. [PubMed: 10330139]

[14]. Li M, Wu Y, Liang YR, Wu XY. Correlation between expression of human telomerase subunits and telomerase activity in esophageal squamous cell carcinoma. World J. Gastroenterol 2003;9:2395–2399. [PubMed: 14606063]

[15]. Cong Y-S, Wen J, Bacchetti S. Human telomerase and its regulation. Microbiol. Molec. Biol. Rev 1999;66:407–425. [PubMed: 12208997]

[16]. Ducrest AL, Szutorisz H, Lingner J, Nabholz M. Regulation of the human telomerase gene. Oncogene 2002;21:541–552. [PubMed: 11850779]

[17]. Perrault SD, Hornsby PJ, Betts DH. Global gene expression response to telomerase in bovine adrenocortical cells. Biochem. Biophys. Res. Commun 2005;335:925–936. [PubMed: 16105662]

[18]. Kanaya T, Kyo S, Hamada K, Takakura M, Kitagawa Y, Harada H, Inoue M. Adenoviral expression of p53 represses telomerase activity through down-regulation of human telomerase reverse transcriptase transcription. Clin. Cancer Res 2000;6:1239–1247. [PubMed: 10778946]

[19]. Dotto GP. More than a break to the cell cycle? Biochim. Biophys. Acta 2000;1471:M43–M56. [PubMed: 10967424]

[20]. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature 1993;366:634–635. [PubMed: 8259207]

[21]. Harada K, Kurisu K, Sadamoto T, Tahara H, Tahara E, Ide T, Tahara E. Growth inhibition of human glioma cells by transfection-induced p21 and its effects on telomerase activity. J. Neurooncol 2000;47:39–46. [PubMed: 10930098]

[22]. Hendersen YC, Breau RL, Liu TJ, Clayman GL. Telomerase activity in head and neck tumors after introduction of wild-type p53, p21, p16, E2F-1 genes by means of recombinant adenovirus. Head Neck 2000;22:347–354. [PubMed: 10862017]

[23]. Koutsodontis G, Tentes I, Papakosta P, Moustakas A, Kardassis D. Sp1 plays a critical role in the transcriptional activtation of the human cyclin-dependent kinase inhibitor p21$^{WAF1/Cip1}$ gene by the p53 tumor suppressor protein. J. Biol. Chem 2001;276:29116–29125. [PubMed: 11384995]

[24]. Prowse DM, Bolgan L, Molnár A, Dotto GP. Involvement of the Sp3 transcription factor in induction of p21$^{Cip1/WAF1}$ in keratinocyte differentiation. J. Biol. Chem 1997;272:1308–1314. [PubMed: 8995437]

[25]. Young JI, Smith JR. DNA methyltransferase inhibition in normal human fibroblasts induces a p21-dependent cell cycle withdrawal. J. Biol. Chem 2001;276:19610–19616. [PubMed: 11259405]

[26]. Louis N, Evelegh C, Graham FL. Cloning and sequencing of cellular viral junctions from the human adenovirus type 5 transformed 293 cell line. Virology 1997;233:423–429. [PubMed: 9217065]

[27]. Ali S, Kukolj G. Interferon regulatory factor 3-independent double-stranded RNA-induced inhibition of hepatitis C virus replicons in human embryonic kidney 293 cells. J. Virology 2005;79:3174–3198. [PubMed: 15709037]

[28]. Yang S, Tutton S, Pierce E, Yoon K. Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. Mol. Cell. Biol 2001;21:7807–7816. [PubMed: 11604515]

[29]. Billy E, Brondani V, Zhang H, Müller U, Filipowicz W. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. PNAS 2001;98:14428–14433. [PubMed: 11724966]

[30]. Yew PR, Berk AJ. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature 1992;357:82–85. [PubMed: 1533443]

[31]. Chen J, Lin J, Levine AJ. Regulation of transcription functions of the p53 tumor suppressor by the mdm-2 oncogene. Mol. Med 1995;1:142–152. [PubMed: 8529093]

[32]. Chen J, Wu X, Levine AJ. Mdm-2 inhibits G1 arrest and apoptosis functions of the p53 tumor suppressor protein. Mol. Cell. Biol 1996;16:2445–2452. [PubMed: 8628312]

[33]. Löber C, Lenz-Stöppler C, Dobbelstein M. Adenovirus E1-transformed cells grow despite the continuous presence of transcriptonally active p53. J. Gen. Virol 2002;83:2047–2057. [PubMed: 12124469]

[34]. Wick M, Zubov D, Hagen G. Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT). Genes Dev 1999;232:97–106.

[35]. Ramirez PT, Gershenson DM, Guillermo T-L, Ramondetta LM, Fightmaster D, Wharton JT, Wolf JK. Expression of cell-cycle mediators in ovarian cancer cells after transfection with p16INK4a, p21WAF1/Cip-1, and p53. Gyn. Onc 2001;83:543–548.

[36]. Gan Y, Mo Y, Johnston J, Lu J, Wientjes MG, Au JL-S. Telomere maintenance in telomerase-positive human ovarian SKOV-3 cells cannot be retarded by complete inhibition of telomerase. FEBS Lett 2002;527:10–14. [PubMed: 12220625]

[37]. Shats I, Milyavsky M, Tang X, Stambolsky P, Erez N, Brosh R, Kogan I, Braunstein I, Tzukerman M, Ginsberg D, Rotter V. p53-dependent down-regulation of telomerase is mediated by p21^{waf1}. J. Biol. Chem 2004;279:50976–50985. [PubMed: 15371422]

[38]. Xiang H, Wang J, Mao Y, Liu M, Reddy VN, Li DW. Human telomerase accelerates growth of lens epithelial cells through regulation of the genes mediating RB/E2F pathway. Oncogene 2002;21:3784–3791. [PubMed: 12032846]

[39]. Dean M, Levine RA, Campisi J. *c-myc* regulation during retinoic acid-induced differentiation of F9 cells is posttranscriptional and associated wtih growth arrest. Mol. Cell. Biol 1986;6:518–524. [PubMed: 3785153]

[40]. Won J, Yim J, Kim TK. Opposing regulatory roles of E2F in human telomerase reverse transcriptas (hTERT) gene expression in human tumor and normal somatic cells. FASEB J 2002;16:1943–1945. [PubMed: 12368233]

[41]. Liu L, Lai S, Andrews LG, Tollefsbol TO. Genetic and epigenetic modulation of telomerase activity in development and disease. Genes Dev 2004;340:1–10.

[42]. Poole JC, Andrews LG, Tollefsbol TO. Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). Gene 2001;269:1–12. [PubMed: 11376932]

[43]. Li Y, Li M, Peng Y, Jiang Z, Li W, Li H. Suppression of telomerase acitivty by plasmid-mediated RNA interference. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi 2006;23:615–619. [PubMed: 16856401]

[44]. Zheng JN, Sun YF, Pei DS, Liu JJ, Li CJC,W, Sun XQ, Shi QD, Han RF, Ma TX. Inhibition of proliferation and induction of apoptosis in human renal carcinoma cells by anti-telomerase small interfering RNAs. Acta Biochim Biophys Sin (Shanghai) 2006;38:500–506. [PubMed: 16820866]

[45]. de Souza Nascimento P, Alves G, Fiedler W. Telomerase inihibition by an siRNA directed against hTERT leads to telomere attrition in HT29 cells. Oncol. Rep 2006;16:423–428. [PubMed: 16820926]

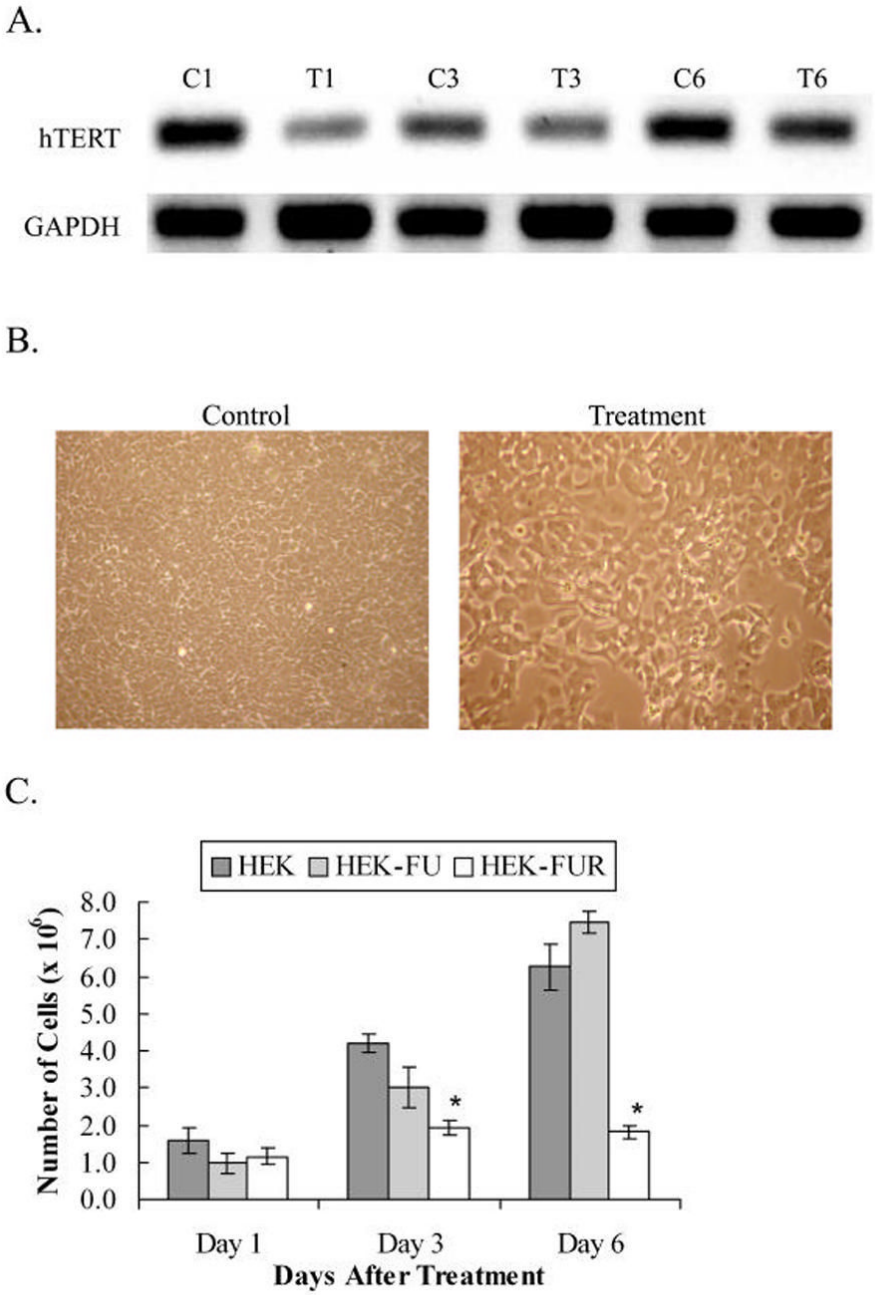

Fig. 1.

Knock down of *hTERT* in HEK cells from transfecting cells with dsRNA complementary to *hTERT* mRNA. *A*, PCR analysis of *hTERT* and *GAPDH* expression were performed using cDNA from treated and untreated HEK cells. *C1*, *C3 and C6*, correspond to HEK untreated control cells on treatment days 1, 3 and 6, respectively. *T1, T3 and T6*, correspond to HEK cells treated with 2.5 μg dsRNA complementary to a 219 bp section of *hTERT* mRNA on treatment days 1, 3 and 6, respectively. Levels of *hTERT* mRNA are reduced within 24 hours of treatment due to RNAi and the levels begin to slowly recover thereafter. *GAPDH* was used as a loading control. *B*, HEK control and dsRNA-treated HEK cells at a total magnification of 100X on day 6 after transfection. The panel with the control HEK cells shows a more densely

populated culture than the treated cells. *C*, Cells plated at a density of 4×10^5 cells and counted on days 1, 3, and 6 after dsRNA transfection. HEK: untreated control cells. HEK-FU: control cells incubated with Fugene 6 transfection reagent. HEK-FUR: dsRNA-treated cells transfected using Fugene 6 transfection reagent. Y-axis error bars represent ± standard error of the mean (SEM). * denotes statistical significance ($n = 3$, $p < 0.05$).

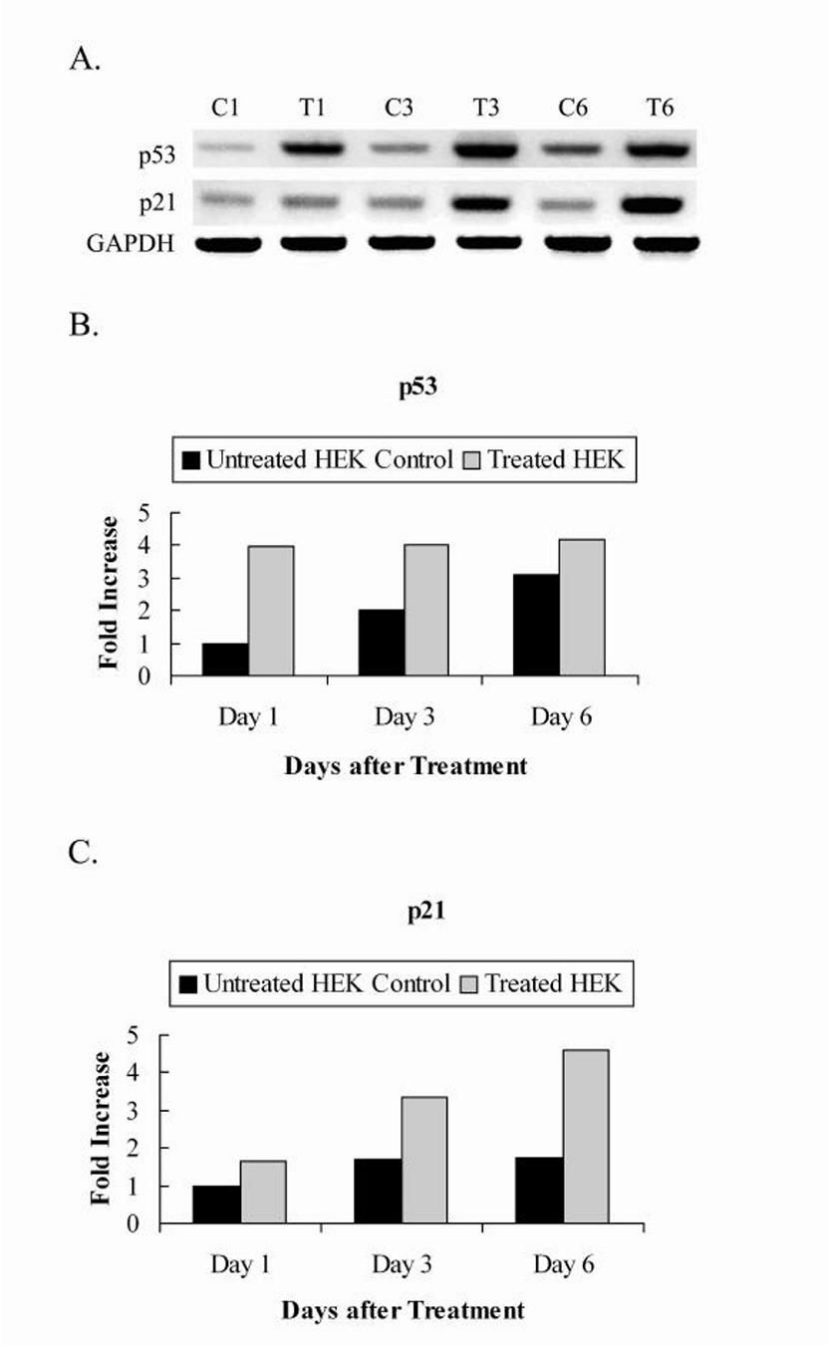

Fig. 2.

PCR analysis of *p53*, *p21* and *GAPDH* expression using cDNA from HEK cells treated and untreated with synthesized dsRNA. *A*, 2% agarose gel electrophoresis showing *p53* and *p21* mRNA levels after induction of RNAi of *hTERT*. *GAPDH* was included as a loading control. Lanes C1, C3, and C6: untreated HEK control cells at days 1, 3, and 6 after treatment respectively. Lanes T1, T3, and T6: treated HEK cells at days 1, 3, and 6 after treatment respectively. *B*, *C*, Quantitations of *p53* and *p21* mRNA levels respectively, normalized to housekeeping gene, *GAPDH*. Values are from a representative gel.

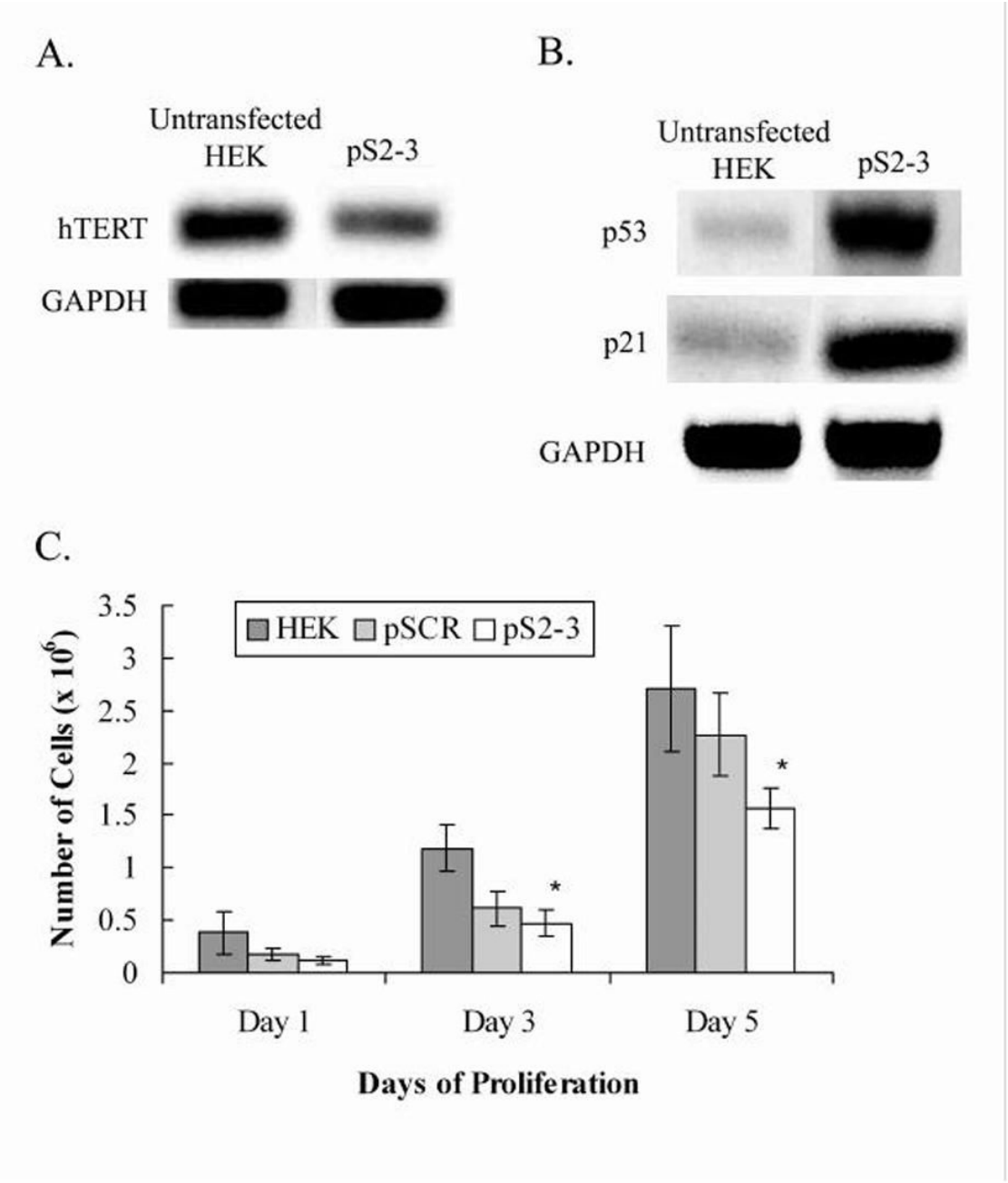

Fig. 3.

Gene expression and proliferation assays of control HEK cells and HEK cells transfected with plasmid constructs pSCR and pS2-3. *A*, *hTERT* mRNA levels showing *hTERT* knock down in cells transfected with pS2-3 as compared to the untransfected HEK. *GAPDH* was used as a loading control. *B*, Increased transcription of *p53* and *p21* in cells transfected with pS2-3 as compared to the untransfected HEK cells. *GAPDH* was used as a loading control. *C*, The graph shows linear growth of controls HEK and pSCR, while pS2-3 shows a significantly slower growth rate. Y-axis error bars represent ± standard error of the mean (SEM). * denotes statistical significance ($n = 3$, $p < 0.05$).

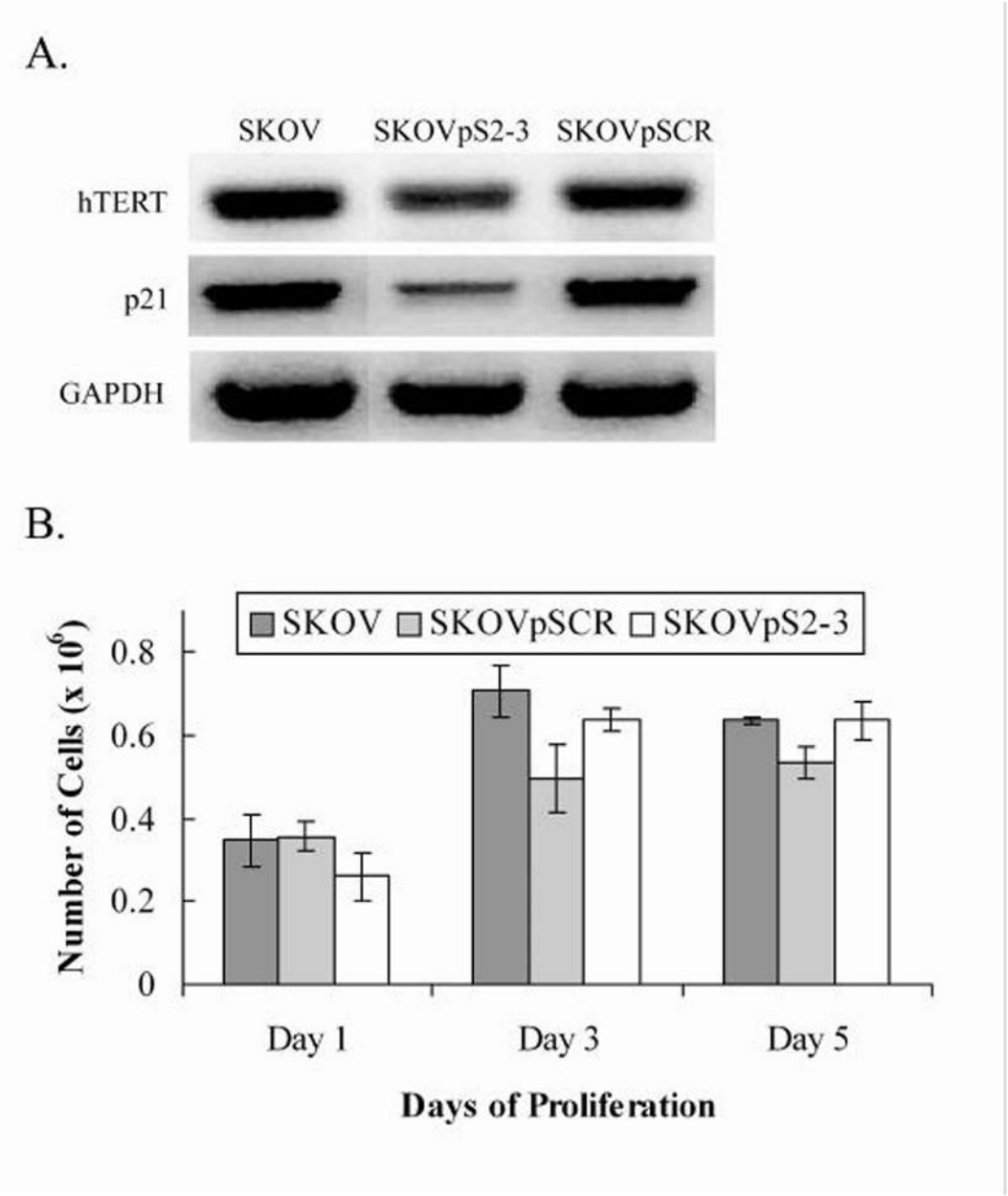

Fig. 4.

Gene expression and proliferation assays of SKOV, SKOVpS2-3, and SKOVpSCR. *A*, Reverse transcriptase PCR of RNA extracts from untransfected SKOV, SKOVpS2-3, and SKOVpSCR. SKOV and SKOVpSCR were used as controls. PCR was performed with primers specific to the gene of interest: *hTERT*, *p21*, and *GAPDH. GAPDH* was used as a loading control. *B*, No significant inhibition of growth was observed in association with *hTERT* knock down in SKOVpS2-3 ($n = 3$, $p < 0.05$).

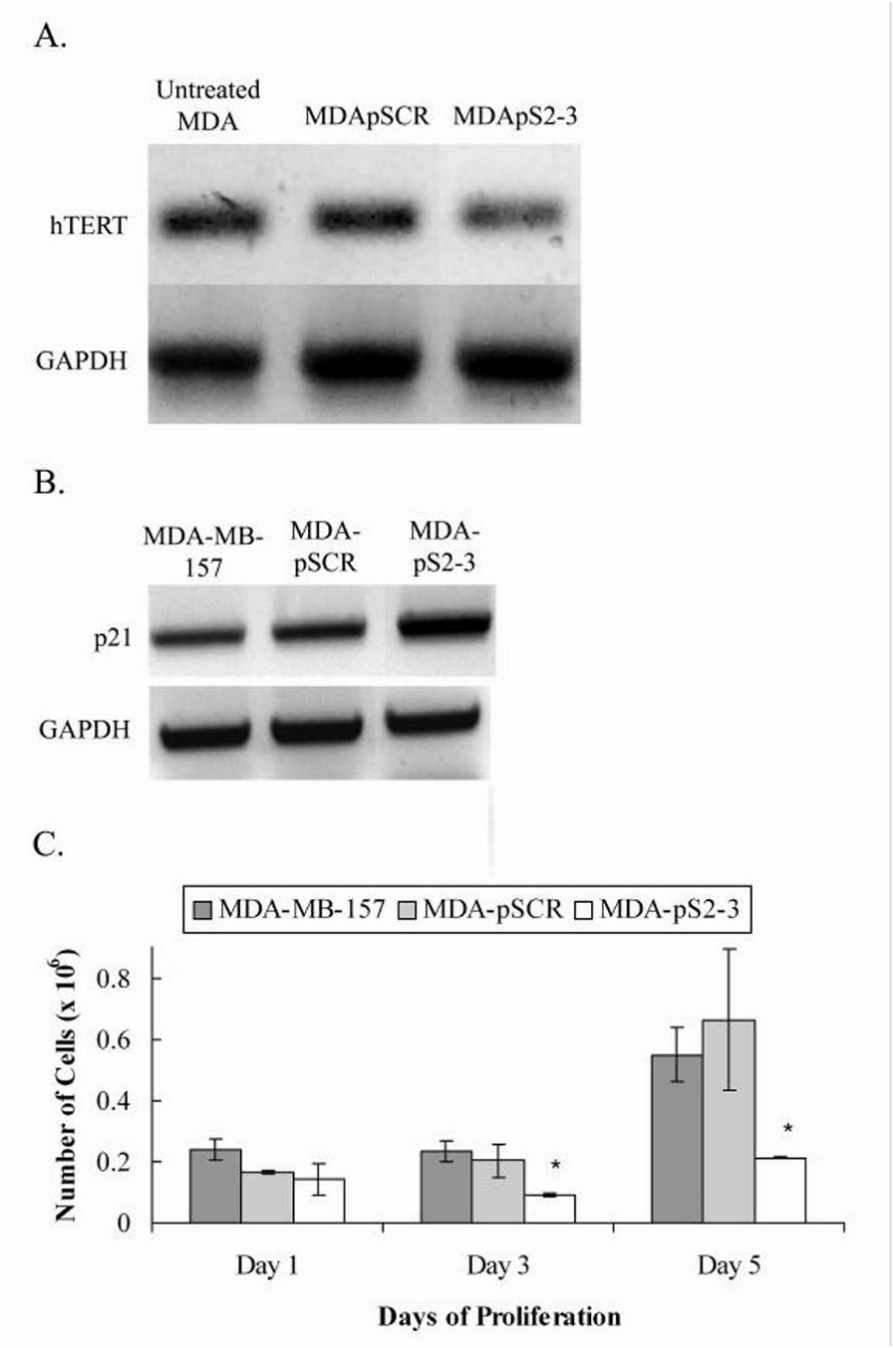

Fig. 5.

Gene expression and proliferation assays of MDA-MB-157 untreated control cells and cells transfected with plasmid constructs pSCR and pS2-3 (MDA-pSCR and MDA-pS2-3 respectively). *A*, Reverse transcriptase PCR of RNA extracts from treated MDA-MB-157 cells and untreated cells at 74 days after transfection. PCR was performed with primers specific to the gene of interest. *hTERT* mRNA levels showing *hTERT* knock down in MDA-pS2-3 as compared to the untransfected MDA-MB-157 and MDA-pSCR control cells. *GAPDH* was used as a loading control. *B*, Increased transcription of *p21* was observed in pS2-3 as compared to the untransfected controls MDA-MB-157 and MDA-pSCR. *GAPDH* was used as a loading control. *C*, The graph shows parallel growth of controls MDA-MB-157 and MDA-pSCR, while

MDA-pS2-3 shows growth inhibition. Y-axis error bars represent ± standard error of the mean (SEM). * denotes statistical significance ($n = 3$, $p < 0.05$).

Table 1

Summary of proliferation changes and expression of p53 and p21 in cell lines HEK, SKOV, and MDA-MB-157.

Cell Line	Genotype	Effects of *hTERT* knock down		
		Proliferation	p53	p21
HEK	Adenoviral transformed	↓	↑	↑
MDA-MB-157	*p53-/-*	↓	-	↑
SKOV-3	*p53-/-; p16*$^{INK4a-/-}$	-	-	↓