Telomerase expression in normal human fibroblasts stabilizes DNA 5-methylcytosine transferase I (DNMT1)

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Running title: Telomerase upregulates DNMT1 promoter
Summary

The finite proliferative potential of normal human fibroblasts can be overcome, a process commonly called immortalization, by the introduction of the catalytic subunit of telomerase (hTERT). In contrast to malignant transformation, the pattern of gene expression remains largely unmodified in telomerase-induced immortalization. Here we show evidence that suggests that the maintenance of a “young” pattern of gene expression by telomerization is mediated, at least in part, by a novel function of hTERT that involves regulation of DNA methyltransferase I (DNMT1) gene expression.

Introduction

Primary cultures of normal human diploid cells have a limited proliferative lifespan (1,2). After completing a finite number of divisions, the culture enters a state called replicative senescence that is characterized by a growth arrest refractory to further mitogenic stimulation and by altered gene expression (3). Introduction of the catalytic subunit of human telomerase (hTERT), the enzyme that elongates telomere ends, into pre-senescent cells extends their proliferative life span (4). This observation is consistent with the hypothesis that telomere attrition, resulting from the incomplete replication of chromosome ends by DNA polymerase α, triggers cellular senescence (5). One of the most interesting aspects of lifespan extension by hTERT, is that in addition to preventing irreversible growth arrest, it also prevents the changes in
gene expression observed to occur during cellular aging (3). Additionally, there are no signs of transformation or changes in the differentiation state of the telomerized cultures (6,7).

The pattern of gene expression of a particular cell type is determined mainly by chromatin architecture (the concerted pattern of cis DNA sequences, DNA methylation and nucleosomal protein modifications) and a particular set of transcription factors. Faithful heritability of gene expression patterns is critical to the maintenance of a particular differentiated state. Although the effects of hTERT re-expression in normal human fibroblasts have been regarded mostly as rejuvenation, the experimental evidence indicates that reactivation of telomerase more likely freezes the culture in its current state, possibly by fixing the pattern of gene expression.

Here we investigated the mechanisms by which telomerase expression in fibroblasts maintains a fixed epigenomic state that prevents age-related changes in gene expression and senescence.

**Experimental Procedures**

*Cells and Plasmids* - The human foreskin fibroblast cell strains Wi38, HCA2 and ImR90 and their derivatives expressing the catalytic subunit of human telomerase (hTERT) were a kind gift of J. Campisi (Lawrence Berkeley National Laboratory, Berkeley, CA). The human embryonic lung fibroblast cell strains HuLF and HuLF-hTERT were established and cultured as described previously (8). pCMV-hTERT and pMet-CAT were kindly provided by R. A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, Ma) and M. Szyf (McGill University, Montreal, Quebec) respectively.
Determination of the 5mC Content- Genomic DNA was isolated using the DNAZol DNA extraction kit (Molecular Research Center, Inc.) according to manufacturer's directions. RNA was removed by alkaline hydrolysis (0.5 M NaOH, 37 °C for 1 h). The 5mC content in DNA samples was determined by HPLC analysis of enzymatic hydrolysates of DNA. 10 µg of DNA were digested at 37 °C for 3 h using 2 units of microccocal nuclease (United States Biochemical, Cleveland, OH) and 2 µg of spleen phosphodiesterase II (Roche Diagnostics Corp., Indianapolis, IN) in 10 mM CaCl2 and 20 mM sodium succinate, pH 6.0. The resulting 3'-deoxymonophosphate nucleosides were further hydrolyzed by overnight incubation at 37 °C with 20 units of alkaline phosphatase (Amersham Pharmacia Biotech). Samples were injected into a Beckman Ultrasphere ODS, 4.6 mm x 25 cm (5 µm particle size) column at room temperature, programmed as follows: 100% buffer A (2% methanol in 50 mM potassium phosphate, pH 4.5) for 10 min, injection of the samples, elution for 20 min in 100% buffer A, followed by buffer B (9% methanol in 50 mM potassium phosphate, pH 4.5) over a 5-min linear gradient up to 100% buffer B for 10 min. HPLC mobile phase was delivered to the column at 0.3 ml/min. Absorbance at 280 nm was recorded. The percent methylcytosine in the genome was determined as a ratio of the area of the 5mC peak to the total area of methylcytosine and cytosine residues in the sample.

DNA Methyltransferase Activity- Cells were harvested by trypsinization, washed twice in phosphate-buffered saline, and resuspended in 0.1 ml of hypotonic lysis buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 0.01% NaN₃, 10% glycerol, 1% Tween 80, 60 µg/ml phenylmethanesulfonyl fluoride, and 100 µg/ml RNase A. The cells were lysed by four cycles of freezing in dry ice-ethanol and thawed at 37 °C. Protein concentration of the supernatant, after centrifugation of the cell lysates at 15,000 x g for 20 min, was determined.
by the Bradford method (Bio-Rad). The enzyme activity was measured as described previously (9), with slight modifications. Briefly, a 20-µl reaction mixture containing 5 µg of cell extract protein, 0.5 µg of a hemimethylated oligonucleotide duplex corresponding to the imprinted locus SNRPN (small nuclear riboprotein-associated peptide N) exon-1, and 3.3 µCi of S-adenosyl [methyl-³H] methionine (92 Ci/mmol; Amersham Pharmacia Biotech) was incubated at 37 °C for 2 h and the reaction terminated by the addition of 300 µl of a solution containing 1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 3% 4-aminosalicylate, 5% butanol, 125 mM NaCl, 0.25 mg/ml salmon sperm DNA, and 1 mg/ml proteinase K. After incubating for a further 30 min at 37 °C, the reaction mixture was extracted with an equal volume of phenol/chloroform/isoamyllic alcohol and ethanol-precipitated. DNA was dissolved in 0.3 N NaOH and incubated at 37 °C for 2 h. DNA was collected on a glass fiber filter disc, saturated with 1 mM non-labeled AdoMet, and washed with 5% trichloroacetic acid followed by 70% ethanol. The filter was air-dried and the radioactivity measured by scintillation counting in 5 ml of scintillation fluid. After subtracting background, the radioactivity incorporated into the DNA as a measure of DNAMeTase activity was determined. Reactions were performed in triplicate and the results expressed as the mean ± S.D.

**Oligonucleotides**- 2'-O-Methylphosphorothioate oligonucleotides were used in the antisense experiment: DNAMeTase antisense (MG88), AAGCATGAGCACCCTCTCC and unrelated control oligonucleotide, ATACAACATGACAATAGATCG (boldface nucleotides are 2'-O-methyl-modified). The following oligonucleotides were used as substrate for the DNAMeTase activity determinations. The hemimethylated duplex was prepared by annealing equimolar amounts of SNRPN-meth,
CTTGCCMGCTCCATMGMGTCACTGACMGCTCCTCAGACAGATGMGTCAGGCATCTCG
MGGMGGCMGCTCCACTCTG  (methylated single strand oligonucleotide), and SNRPN-
unmeth,
CAGAGTGGAGCGGAGCGGGAGATGCCTGACGCATCTGTCTGAGGAGCGGTCAGTG
ACGCGATGGAGCGGGCAAG (unmodified single strand oligonucleotide).
The complementary oligonucleotides were incubated in annealing buffer (50 mM Tris-HCl, pH 7.4, 1 mM Na₂EDTA, and 100 mM NaCl) at 95 °C for 5 min followed by 65 °C for 15 min and 37 °C for 15 min (10). The full-length duplexes were purified on a 15% polyacrylamide gel, dissolved in TE (10 mM Tris-HCl, pH 7.8, 1 mM EDTA), and stored at 20 °C.

*Northern Blot Analysis-* Northern analysis was performed according to standard procedures. 32P-Labeled DNA probes for human DNMT1, p21 and actin mRNAs were prepared by random priming.

*Western Blot Analysis-* Samples containing equal amounts of protein (20 µg) from cell lysates were separated by SDS-polyacrylamide gel electrophoresis and then transferred to a nylon membrane. Proteins were detected by incubation with the indicated antibodies and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

*Transient Transfections-* CMV-hTERT and pMet-CAT or p21-CAT were cotransfected into HCA2 cells (PD 19) using LipofectAMINE Plus (Life Technologies, Inc.). Transfection medium (0% fetal bovine serum) was replaced with 10% fetal bovine serum medium 3 h after plasmid delivery. All transfections were done in triplicate. 0.1µg of pCMV-β-gal was included as an
internal control. 24 h after transfection, cells were collected and divided into two aliquots of 20% and 80% for protein and RNA extracts respectively. Samples from the 80% aliquots were pooled for RNA preparation.

**Oligonucleotide Treatment**- Cells (5 x 10⁵) were electroporated on three sequential days (300 V, 960 microfarads) with a 100 nM concentration of the hybrid 2'-O-methylphosphorothioate DNAMeTase antisense or unrelated oligonucleotide in freshly made buffer (cytomix) (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄, 10 mM KH₂PO₄, 25 mM Heps, 2 mM EGTA, 5 mM MgCl₂, 2 mM ATP, 5 mM glutathione, pH 7.6). After each electroporation the cells were incubated in complete medium for 48 h in the presence of 40 nM oligonucleotide. 2.5 µg of CMV-hTERT was added to the last electroporation, along with 0.1µg of pCMV-β-gal.

**Telomere restriction fragment analysis**- Mean telomere length was evaluated by using terminal restriction fragment (TRF) analysis, a variation of standard Southern blotting. DNAs isolated from the different cell preparations were digested with a mixture of restriction enzymes and resolved on a 0.7% agarose gel (11). The dried gel was hybridized with a ³²P-labeled oligonucleotide, (TTAGGG)₃, exposed to a Phosphorimager screen, and TRFs were calculated as described in detail elsewhere (11) using two or three separate measurements for each sample.

**Measurement of telomerase activity by telomerase repeat amplification protocol (TRAP) assay**- The TRAP assay was performed using the TRAP-EZE kit (Intergen, Purchase, NY) as indicated by the manufacturers.
Results

Telomerase-induced lifespan extension prevents the decrease in DNA methyltransferase activity normally seen upon serial subcultivation

It has been suggested that decreasing DNA methylation level plays a role in cellular aging (12,13). Thus, we measured DNA methylation levels, known to decrease during in vitro cellular aging of normal human fibroblasts (14-16). We examined normal control cells (HCA2) and cells that had been immortalized by introduction of the catalytic subunit of telomerase (HCA2-hT).

The DNA methylation level of control cultures decreases as the cultures age. However, telomerized cells maintained constant levels of global DNA methylation throughout their extended life span (figure 1). The observed halt in the decrease in methylation levels suggests the possibility that the methylcytosine content of normal fibroblasts reaches an equilibrium point and "bottoms out" at or shortly prior to senescence. This would not be apparent under normal circumstances because the cells stop dividing, and only revealed when the cells bypass senescence due to expression of telomerase activity. However, the level of DNA methylation exhibited by telomerized cultures was similar to the level in the cultures just before hTERT introduction (at PD 25 or at PD 45, white and black bars in figure 1, respectively), favoring an active maintenance of DNA methylation at “young” levels.

Since the age-related decrease in DNA methylation levels is at least partially a consequence of a reduction in DNA methyltransferase (DNAMeTase) activity (16,17), we analyzed the DNAMeTase activity of different telomerized cell cultures. Interestingly, we observed a transient increase in DNAMeTase activity shortly after telomerization (figure 2) that
lasted only a few population doublings. Nevertheless, after decreasing to normal levels the DNAMeTase activity was stabilized at a relatively constant level, while non-telomerized control cells exhibited a decline with increasing PD (figure 2). This telomerization-dependent maintenance of DNAMeTase was observed in three independent fibroblast cultures, including HCA2, ImR90 and HuLF, that have been telomerized at different population doubling levels. However, telomerized Wi38 cells did not exhibit this effect. Notably, in this particular culture of telomerized Wi38 cells telomerase activity is very low compared to the other telomerized cell cultures. In addition, determination of telomere length in the same Wi38-hT cells at different PD indicated that telomere attrition persisted in these cells even after telomerization and apparent immortalization (data not shown). Thus, we suggest that the maintenance of DNAMeTase activity as a consequence of hTERT expression may stabilize DNA methylation levels.

**Expression of telomerase stimulates the DNMT1 promoter**

In order to test whether DNA methyltransferase is regulated at the transcriptional level by telomerase, we examined the expression of a mouse DNMT1 promoter construct (18) in normal human fibroblast cells in the presence and absence of telomerase expression. Figure 3b shows that transient transfection of the mouse DNMT1 promoter and CMV-hTERT (a construct shown previously to express telomerase activity, [(19) and figure 3a] in normal hTERT negative HCA2 cells stimulated the promoter in a dose dependent manner. The observed hTERT induction of expression of the mouse promoter suggested that telomerase might also activate the endogenous DNMT1 promoter, as mouse and human somatic cells seem to regulate the expression of DNMT1 similarly. To test this possibility we measured DNMT1 mRNA levels and DNAMeTase activity in HCA2 cells transiently transfected with a CMV-hTERT construct. Northern blot analysis indicated that endogenous DNMT1 mRNA was also upregulated in the
hTERT transfected cells compared to cells transfected with empty vector (fig 3c). Moreover, DNMT1 activity was also increased in the transfected cells compared to control cells (data not shown). These data together indicated that telomerase reactivation modified the steady state levels of DNMT1 mRNA by transcriptional activation.

**Telomerization blocks the age-related increase in p21**

It has been suggested that telomerase expression suppresses the senescence-associated induction of the CDK inhibitor p21 (7), one of the main effectors of cellular senescence. In addition, inhibition of DNMT1 results in increased transcription from a p21 promoter/reporter construct (20) and from the endogenous p21 promoter (16), which probably accounts for the reported tumor-suppressive effects of decreased DNMT1 expression (21,22). Since p21 mRNA and protein are significantly increased as fibroblasts enter senescence (23), we determined whether p21 mRNA and protein levels in high passage telomerized cultures were maintained at the level found in young cells. Western analysis indicated that p21 protein (in all cultures analyzed) was maintained at levels exhibited by the cultures just prior to introduction of telomerase (at PD 45) (fig 4A and B). However, a marked increase in p21 levels was observed in cultures at confluence (fig 4C) indicating that the ability of telomerized cells to respond to stress by upregulation of p21 was still intact. In agreement with the protein content, hTERT immortalized cells displayed p21 steady state mRNA levels comparable with the young proliferating parental cells (fig 4D).

**p21 promoter activity is repressed by telomerase expression**

We next analyzed the effect of transient transfection of hTERT on endogenous p21 levels. Northern blot analysis indicated that endogenous p21 mRNA levels were slightly decreased in hTERT transiently transfected cells, suggesting that the increase in DNMT1 (fig
3D) could repress the p21 endogenous promoter. However, regulation of p21 levels had also been shown to be dependent on stabilization of mRNA (24). To identify the mechanism of telomerase-induced prevention of p21 upregulation in response to repeated cell division, we analyzed the effects of transiently co-transfecting hTERT along with a p21-reporter construct (25). Cotransfection of hTERT resulted in a dose-dependent inhibition of expression from the p21 promoter, confirming the transcriptional control of p21 expression by hTERT (Figure 5). Cotransfection of 1 ug of pCMV-β-gal did not affect p21 promoter activity, as normalized by protein content (data not shown), eliminating the possibility of promoter competition.

To further elucidate the pathway by which expression of hTERT results in repression of the p21 promoter, we tested the effects of transient transfection of DNMT1 antisense (or an unrelated control) oligonucleotides along with hTERT (or pGL3-control vector) on the expression of the p21-luciferase construct. Expression of hTERT did not reverse the activation of the p21 promoter by antisense inhibition of DNMT1 (data not shown). This indicates that the inhibition of p21 by DNMT1 is downstream of the direct effect of hTERT.

Discussion

The data of this study together suggest that the hTERT dependent prevention of the age-related decrease in DNMT1 expression could participate in the maintenance of a "young" phenotype in two ways. First, by freezing the epigenomic state of young, proliferating cells, by stabilizing DNA methylation; and second, by maintaining low levels of the cell cycle inhibitor p21, mediated at least partially by DNMT1's transcriptional repressor activity.
The data are consistent with a model in which both, modification of telomere dynamics and DNMT1 upregulation, are components of the output pathway leading from telomerase reactivation to indefinite replicative capacity (figure 6). In this model, telomerase introduction counteracts telomere attrition, preventing the cellular response to telomere shortening (cellular senescence) and also regulates the expression of DNMT1. It is not clear yet if telomerase regulation of DNMT1 is via a direct effect of the telomerase protein or if it is mediated by its telomerase activity. Damm et al reported that pharmacological inhibition of telomerase processivity produced downregulation DNMT1 (and upregulation of p21), favoring the latter hypothesis (26). However, in the same study a 5-fold reduction of hTERT mRNA was also observed, precluding a definitive resolution of this issue. The age related increase in p21 is blocked by complementary pathways, one involving the elimination of the positive signal coming from short telomeres and another involving a negative signal coming from the DNMT1 repressive activity. DNMT1 in turn, also has another role in the maintenance of a young proliferative state, which is to preserve the pattern of DNA methylation. In conjunction, these processes participate in the maintenance of an indefinite proliferative potential.

Our data suggest the existence of previously unknown functions of hTERT, namely, activation of the DNMT1 promoter and maintenance of DNMT1 activity during serial subcultivation of normal human fibroblasts. The mechanism by which this occurs has yet to be determined. However, one consequence of this function of hTERT appears to be the prevention of changes in gene expression related to cellular aging. The finding that normal cells that have been subjected to telomerase-induced life span extension maintain an epigenomic state that resembles the pre-immortalization state increases the
possibility of using such cells in therapeutic processes. The reports of oncogenic effects of increased DNMT1 expression (27-29) are only partially consistent with our data, as maintenance of DNMT1 activity seems to be a requisite for extension of the proliferative potential of cultured cells, but oncogenic transformation does not seem to be the obligatory outcome of this upregulation.

References


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Figure legends

**Figure 1.** Global DNA methylation levels in telomerized cells. Genomic methylation levels were measured in unmodified HCA2 (gray bars) and hTERT expressing HCA2 cells that have been telomerized at PD 45 (black bars) or at PD 25 (white bar) at the indicated PDs, utilizing reverse phase HPLC at pH 4.5. Data shown are means and standard deviations, \( n \geq 3 \).

**Figure 2.** DNAMeTase activity in telomerized cells. DNAMeTase activity was measured in control (gray bars) and telomerized cells (black bars) at the indicated population doublings using a hemimethylated substrate and protein extracts obtained from HCA2 (telomerized at PD 45), ImR90, HuLF and Wi38 cells. Data shown are means and standard deviations, \( n \geq 3 \).

**Figure 3.** Transient transfection of hTERT induced expression of the DNMT1 promoter. HCA2 cells were transiently cotransfected with increasing amounts (0.0 to 2.0 µg) of pCMV-hTERT and 0.55 µg of pMet-CAT. A, Telomerase activity in each transfected sample was detected by the TRAP assay. The 35-base pair internal standard was used as a control in the assay (IC). B, Total RNA (10µg) prepared from the indicated transfected cells was subjected to northern blot analysis using a DNMT1 (top) radiolabeled probe. After stripping, the membrane was hybridized with an actin probe as loading control (bottom). C, CAT activity was measured 48 hs after transfection and normalized to β-gal units. Data shown are means and standard deviations, \( n \geq 3 \). D, Phosphoimager quantitation of the Northern blot, normalized to the actin signal.
**Figure 4.** hTERT immortalization prevents the increase in p21 expression that results from cellular aging. A-C, Protein extracts (20 µg) obtained from control and telomerized (hT) HCA2 (A and C) ImR90 (B, left) and HuLF (B, right) cells at the indicated PD were analyzed by immunoblotting for the detection of p21. 1: subconfluent, 2: confluent. D, Total RNA (10 µg) extracted from control (HCA2) and telomerized (HCA2-hT) HCA2 cells at the indicated PD was subjected to northern analysis with DNMT1, p21 and actin probes.

**Figure 5.** hTERT expression represses p21 promoter activity. HCA2 cells were transiently transfected with increasing amounts (0.22-0.45 µg) of a CAT reporter gene construct containing the –2400 to +14 fragment of the human p21 promoter, p21-CAT (grey bars). 0.45 µg of p21-CAT was co-transfected with increasing amounts (0.12 to 0.62 µg) of the pCMV-hTERT plasmid in HCA2 cells (black bars). An internal control pCMV-β-gal plasmid was added to all transfections along with the appropriate amount of pGL3-control vector to equilibrate for the amount of DNA transfected. After 24 h, CAT activity was measured and normalized to β-gal units. Transfections were performed in triplicate (data shown are means and standard deviations). Inset: *in vitro* telomerase activity of the pCMV-hTERT transfected cells.

**Figure 6.** Model for hTERT regulation of cellular immortalization.
Figure 5

![Graph showing CAT activity with p21-CAT and hTERT concentrations. The graph includes error bars for each data point.](image)

- **p21-CAT (μg):**
  - 0.22
  - 0.45
  - 0.90
  - 0.45
  - 0.45
  - 0.45

- **hTERT (μg):**
  - 0.00
  - 0.00
  - 0.00
  - 0.12
  - 0.31
  - 0.62
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