

Telomerase and gene expression

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December 2, 2005

Pathology 209

Abstract

While telomerase is known to extend the length of telomeres and prevent the onset of telomeric cell senescence, it has recently come to light that it has other lengthening-independent properties. Resurrection of telomerase expression in tumor cells leads to cell “immortalization”. Ectopic expression of telomerase has been shown to alter expression of growth-promoting genes and enhance cell proliferation. We would like to investigate the breadth of genes affected by telomerase, how telomerase might act as a transcription factor, and look for other proteins that might interact with telomerase.

Background

As maintained by Dr. Robert Weinberg, malignant transformation of cells occurs as a series of genetic alterations. These multiple transforming steps allow cancer cells to overcome homeostatic barriers. One step is the “immortalization” of the cell – the ability to replicate an infinite number of times (Hahn and Weinberg 2002). It has been shown that an unlimited replicative lifespan is required for transformation (Newbold and Overell 1983). One pathway to replicative immortalization is the upregulation of telomerase, the enzyme that lengthens the telomeres that cap the ends of chromosomes.

Telomeres are composed of TTAGGG tandem repeats and a number of associated proteins (Blackburn 2001, Chan and Blackburn 2002). They have a 3' G-rich overhang that forms a protective cap structure with different protein complexes (de Lange 2002). Telomere maintenance is essential for proliferating cells. Incomplete genome replication by DNA polymerases and end processing by nucleases result in the loss of 50-100 base pairs with each cell division of cultured human somatic cells (Collins and Mitchell 2002). This attrition eventually results in critically short telomeres that bring about three possible fates: proliferative senescence, apoptosis, or continued proliferation accompanied by genomic instability (Stewart and Weinberg 2000).

The main regulator of telomere length is telomerase, a reverse transcriptase that adds TTAGGG repeats onto telomeres (Collins and Mitchell 2002). The telomerase ribonucleoprotein (RNP) complex consists of two components: the telomerase reverse transcriptase (TERT) catalytic subunit and the telomerase RNA component (TERC) (Blasco EMBO 2005). Telomerase recognizes the 3' end of the G-rich telomeric overhang, and elongates telomeres by extending from the 3'OH using the RNA component as a template (Blasco Nat Rev Genet. 2005). Some correlation can be made between telomerase inactivation, differentiation and increased rate of telomere loss with time. Inactivity of the telomerase RNP is correlated with the absence of TERT mRNA, not the RNA component (Collins and Mitchell 2002). Little seems to be known about regulation of telomerase, but it has been observed that VEGF gene transfer into rats increased expression of TERT and telomerase activity (Zaccagnini, et al. 2005).

A second telomere-length regulation pathway, alternative lengthening of telomeres (ALT), has also been described as a means of telomere elongation in human cell lines and tumors that lack telomerase (Henson, et al. 2002). Factors that can influence telomere length in the absence of telomerase activity include the homologous recombination DNA repair pathway proteins such as Rad54 and XRCC3, modifiers of telomeric heterochromatin, and cell cycle regulators (Blasco EMBO 2005).

Telomerase activity and mRNA is undetectable in most adult cells, except for germ cells, proliferating epithelial cells, lymphocytes, and stem cells, which need to divide continuously. The activity in these cells is at least an order of magnitude lower than that found in cancer cells *in vitro* and is insufficient for telomere length maintenance *in vivo* (Collins and Mitchell 2002).

Telomerase is reactivated or upregulated in 90 percent of human tumors (Shay and Bacchetti 1997). Without telomerase upregulation, cancer cells would be limited in how many cell divisions they could undergo before telomere-induced senescence (Hahn et al Nat. Med.1999). Ectopic expression of hTERT in combination with two oncogenes (SV40 large-T and an oncogenic allele of H-ras) resulted in direct tumorigenic conversion of normal human epithelial and fibroblast cells (Hahn, et al. Nature 1999). Inhibition of telomerase in tumor cells leads, after time, to telomere shortening and cell death (Hahn, et al. Nat. Med.1999, Chen, et al. 2003, Corey 2002). K5-mTert transgenic mice that overexpress telomerase linked to the keratin 5 promoter have high levels of telomerase activity and have long telomeres. These mice have a higher incidence of both induced and spontaneous tumors and have increased mortality in the first year of life (Gonzalez-Suarez, et al. 2001).

Forced telomerase expression in cells and mice with normal telomere length has shown that telomerase promotes growth and survival in a manner that is telomere length-independent. Telomerase activity is upregulated in mouse tumorigenesis, despite the fact that mice have very long telomeres (Broccoli, et al. 1996). First generation telomerase-deficient mice, which lack telomerase activity but still have very long telomeres, have fewer induced skin tumors than wild-type mice (Gonzalez-Suarez, et al. 2000). These results indicate that telomerase activation might contribute to tumor progression even when telomeres are sufficiently long.

Stewart, et al. showed that the ALT mechanism of telomere maintenance could not substitute for telomerase in the process of transformation *in vitro*. The immortal ALT GM847 cell line could not be transformed by expression of oncogenic H-Ras. However, subsequent expression of hTERT in these cells resulted in transformation (Stewart, et al. 2002). This work further suggests that the tumorigenic properties of telomerase are telomere length-independent.

Smith, et al. showed that ectopic expression of telomerase in human mammary epithelial cells (HMECs) results in a diminished requirement for exogenous mitogens. By microarray analysis of 7000 genes, it was shown that telomerase expression correlates with an induction of genes that promote cell growth. Telomerase-expressing cells also showed downregulation of genes such as TRAIL, TNF-related apoptosis inducing ligand. The upregulated genes include basic fibroblast growth factor (FGF2), a wide-spectrum mitogenic, angiogenic, and neurotrophic factor, and the receptor for epidermal growth factor (EGFR), which has been implicated in human breast cancer (Kim and Muller 1999). When EGFR expression was inhibited, the enhanced proliferation of the cells was reversed. The authors conclude that telomerase may contribute to proliferation not only by stabilizing telomeres, but also by affecting the expression of growth-promoting genes (Smith, et al. 2003).

Smith, et al. also found that cells expressing a catalytically inactive dominant negative form of hTERT (DN-hTERT) did not have increased mitogenic gene expression, so catalytic activity is essential for induction of growth promoting genes (2003). VEGF-dependent angiogenesis in rats, which was found to be TERT-dependent, was abrogated

by coordinate gene transfer of DN-hTERT suggesting catalytic activity is involved (Zaccagnini, et al. 2005). However, it remains to be determined whether telomerase-mediated gene regulation depends on its interaction with telomeres or whether it is a telomere-independent mechanism (Smith, et al. 2003).

p16^{INKa} deficient HMECs were used in the studies by Smith, et al. (2003). p16^{INK4a}, also known as cyclin-dependent kinase inhibitor-2A (CDKN2A), is a tumor suppressor frequently deleted or mutated in tumor cells. It was found previously that Rb or p16^{INK4a} inactivation was required for the efficient immortalization of epithelial cells by hTERT (Kiyono, et al. 1998). It remains to be examined whether telomerase-mediated gene expression could be related to the status of the p16^{INK4a} gene. Further, it would be important to look at the effect of telomerase in other model systems and *in vivo* (Smith, et al. 2003).

Specific Aims

The purpose of this study is to determine how telomerase affects gene expression. It has been shown that telomerase can transform cells by a telomere length-independent mechanism, and that it can induce growth-promoting gene expression. It is important to identify the telomere length-independent mechanism behind transformation because length-dependent telomerase inhibition treatments take months to see an effect and could affect healthy dividing cells (Corey 2002, Shay and Wright 2002). Elucidating the growth factor-telomerase link could lead to more effective cancer treatments.

Smith, et al. (2003) showed by microarray analysis that telomerase alters the expression of genes that control growth and increases cell proliferation. They identified genes with altered expression in a telomerase-expressing mammary cell line. We would like to expand upon their research by looking for additional genes that show modified expression levels due to telomerase, using both p16^{INK4a} positive and negative cells. Using this data, we would like to determine how telomerase modulates gene expression. It is known that telomerase requires a functional catalytic site in order to transform cells and induce EGFR overexpression, but it has yet to be determined whether template addition of nucleotides to the telomeres is necessary for transformation. We hypothesize that telomerase works as a transcription factor or as part of a pathway leading to modifications of transcription factors.

Hypothesis 1: Telomerase modifies expression of growth-promoting genes and will do so regardless of the presence of the p16^{INK4a} gene.

Specific Aim 1: Examine the effect of telomerase expression on p16^{INK4a}-positive mammary cell mitogen dependence, including a verification of the previous study on p16^{INK4a} deficient cells.

Both cells expressing p16^{INK4a} and lacking p16^{INK4a} will be derived from reductive mammaplasty and cultured in medium with EGF and bovine pituitary extract. hTERT expressing cell lines will be created using pBabe-puromycin-hTERT, pBabe-puromycin-DN hTERT and pBabe-puromycin vectors using Fugene. Transduced cells will be selected in puromycin. TERT expression will be verified using the telomerase activity assay kit from Roche. Cell growth assays will be performed to measure cell dependence on mitogens, which should decrease with increased hTERT expression.

Potential pitfalls: p16^{INK4a}-expressing cells may not change their growth pattern upon expression of telomerase. It is possible that loss of the p16^{INK4a} gene, an important transforming step in tumorigenesis, must precede the induction of telomerase in order for it to decrease cell dependence on mitogens. If this is the case, the study will be continued through Aim 2 to test growth-controlling gene expression in these cells. All methods have been previously described.

Hypothesis 2: Cell lines carrying transgenic telomerase have modified expression of growth-promoting genes.

Specific Aim 2: Confirm that the genes identified by Smith, et al. are indeed modulated by telomerase, and identify new genes by repeating gene expression microarray analysis with a larger range of oligomers.

The MGH Microarray Core Facility uses Qiagen human v3.0 (34,580 oligos plus 12 controls) arrays. MGH offers support for analysis and interpretation of microarray data. Analysis will be performed according to the criteria set in Smith, et al. (2003). Results will be verified by RT-PCR and Western blot. Antibodies are available for genes identified by Smith et al. from AbCam. It is expected that the microarray data will confirm data shown previously and should include downregulated genes PTHLH, TRAIL, IL-1ra (IL-1 receptor agonist), and IAP (integrin associated protein) as well as upregulated genes ATF3, XBP-1, FGF2, and EGFR (Smith, et al 2003).

Potential pitfalls: Microarray analysis may not show a greater number of genes than that discovered by Smith, et al (2003). If this is the case, the study can be continued using those previously identified genes in Aim 3.

P16^{INK4a}-positive hTERT-HMECs may not modulate expression of growth-controlling genes. If so, this implicates a role for p16^{INK4a} cell cycle control in the regulation of growth genes, and should be examined in another study.

Hypothesis 3: Telomerase acts as a transcription factor, directly modifying expression of growth-related genes.

Specific Aim 3: Analyze DNA binding activity of telomerase at promoters of genes identified in the microarray studies.

Chromatin immunoprecipitation assay (ChIP) will be used evaluate whether TERT is bound to distinct proximal-promoter DNA sequences in hTERT-HMECs. Specificity of transcription-factor binding will be achieved by immunoprecipitation of formaldehyde crosslinked protein-DNA complexes. The sequences will be verified by primer-specific PCR of DNA purified from chromatin precipitates (Natesampillai, et al. 2005). Complexes will be precipitated using antibodies to hTERT (AbCam). Regulatory regions of genes such as EGFR, its transcription factor SP1, FGF2, ATF3 and TRAIL (Smith, et al. 2003) will be examined for TERT binding, as well as any other genes identified in Aim 2. For a positive control for DNA binding, primers for telomeric DNA will be used.

Potential pitfall: It is likely that telomerase does not act as a transcription factor. If telomerase binds only to telomeres, other possible telomerase interactions will be examined in Aim 4.

Hypothesis 4: Telomerase modifies the expression of growth-related genes by binding to transcription factors or signal transduction pathway members.

Specific Aim 4: Identify proteins that interact with telomerase, searching for transcription factors or enzymes involved in growth signal transduction.

This experiment will be carried out according to the procedure used in Crockett et al (2004). Protein lysates from hTERT-HMECs will be incubated with anti-hTERT (AbCam) primary antibody, followed by incubation with Protein G agarose. The antibody-selected proteins will be eluted from the agarose beads, resolved by SDS-PAGE and visualized by coomassie staining. After staining, bands of interest will be excised according to Crockett et al (2004). Tandem MS will be carried out at the Harvard Medical School Taplin Biological Mass Spectrometry Facility. The amino acid sequence of the peptides will be determined by tandem mass spectrometry (MS/MS) and database searching. Provided the hTERT antibody can bind to DN hTERT we will perform the same experiment on cells expressing DN hTERT as a control. Any proteins bound to both DN hTERT and TERT could be eliminated from our analysis since DN hTERT does not have an effect on gene expression.

Potential pitfalls: It is possible that telomerase binds a myriad of proteins. If so, the scope can be narrowed by looking for specific transcription factors of genes identified in Aim 2 by coimmunoprecipitation and Western blot. A positive control would be the protein DKC1 (dyskeratosis congenita 1) which is known to associate with TERT (Blasco Nat. Rev. Genet. 2005).

It is also possible that gene expression could be mediated by a byproduct of the telomerase reaction or that telomerase regulates gene expression indirectly. Perhaps there exist transcription factors that recognize and are activated by freshly lengthened telomeric repeats. This class of proteins falls outside the scope of this study.

Methods

Cells:

Cells will be derived from reductive mammaplasty (BioWhittaker). Two donor cell populations each of p16^{INK4a}-expressing and p16^{INK4a}-deficient cells will be used. Expression or lack of expression of p16^{INK4a} will be confirmed by Western blot. Cells will be propagated in MEGM (BioWhittaker) serum-free medium containing EGF and bovine pituitary extract. Cells will be grown at 37C in 5% CO₂ (Smith, et al, 2003).

Generation of cell lines:

Phoenix cells (American Type Culture Collection) will be transfected with pBabe-puromycin-hTERT, pBabe-puromycin-DNhTERT and pBabe-puromycin using Fugene (Roche). The viral supernatants from transduced phoenix cells will be collected after 48 hours and used to infect HMECs. Two days after infection cells will be selected in 2µg/ml puromycin for 7 days. Stable control, hTERT and DN hTERT lines will be made from two donor cell populations for p16^{INK4a}-positive and -negative cells (Smith, et al. 2003).

Telomerase activity:

Telomerase activity will be measured using the Telomerase PCR ELISA kit (Roche) according to the manufacturer's instructions.

Western blotting:

Protein lysates will be prepared from hTERT-HMECs using RIPA lysate buffer with protease inhibitors. Protein concentrations will be estimated using the Bradford colorimetric method against known concentrations of BSA. Antibodies against p16^{INK4a} and TERT (AbCam) will be used with the WesternBreeze immunodetection system (Invitrogen) according to the manufacturer's instructions.

Cell growth assay:

hTERT-HMECs and uninfected HMECs will be plated in MEGM at 1000 cells/well. The following day, cells will be washed to remove the medium and replaced with MEGM or MM (which lacks EGF and bovine pituitary extract). To harvest plates and assay for growth, the wells will be washed and fixed with 10% formalin, washed again and stained with 0.1% crystal violet solution. Excess crystal violet will be removed by washing, eluted, and the absorbance measured at 590 nm (Smith, et al. 2003).

Gene expression analysis:

RNA will be isolated using the Totally RNA isolation kit (Ambion) according to manufacturer's instructions. Purified total RNA will be subjected to quality-control analysis using an Agilent BioAnalyzer. Samples must have an rRNA ratio of 1.3 or greater. cDNA will be synthesized with Superscript RT (Invitrogen) using an oligoDT primer, labeled using the Atlas Powerscript Fluorescent Labeling Kit (Clontech), and purified using the Cyscribe GFX Purification Kit (Amersham) according to manufacturer's instructions. Gene expression analysis will be carried out at the MGH Microarray Core Facility, using Qiagen human v3.0 (34,580 oligos plus 12 controls)

arrays. This oligo set is based off the ENSEMBL database. Hybridization will be carried out using the Genomic Solutions GeneTAC Hybridization Station (Perkin Elmer) according to manufacturer's instructions. Fluorescence will be measured on an Axon (Molecular Devices) 4000B 2-color single-slide scanner or a Perkin-Elmer 3-color, 20-slide scanner. Each chip will be scaled such that the average intensity is 1,000 while masking outliers, and only genes with average difference values in the first control sample between 10 and 6,700 will be considered for analysis (Smith, et al 2003).

Data analysis:

A "template matching" approach as described by Smith, et al (2003) will be used to identify genes that are consistently upregulated or downregulated in samples expressing hTERT. The Pearson correlation coefficient will be used to compare the pattern of expression levels of a specific gene with an idealized template. Genes whose expression changed by more than 2-fold in all experiments compared to the average of all controls will be considered candidate genes for Aim 2 (Smith, et al, 2003).

Chromatin immunoprecipitation:

Protein-DNA complexes in hTERT-HMECs will be crosslinked by addition of 1% formaldehyde, quenched by adding glycine, and harvested by scraping in ice-cold PBS with protease inhibitors. Samples will then be pelleted and resuspended in SDS lysis buffer containing inhibitors, sonicated on ice to release 200-800 bp DNA fragments, microfuged and resuspended in dilution buffer, precleared with DNA- and albumin-blocked Protein A-agarose, and incubated overnight with antibody to TERT (AbCam). Samples will be precipitated with salmon sperm DNA-Protein A agarose and washed in buffer (Upstate Cell Signaling Solutions). Crosslinked DNA complexes will be dissociated by incubation in high-salt buffer, then purified by alcohol extraction and reprecipitated (Natesampillai, et al. 2005). PCR will be carried out with primers specific for proximal promoter sequences of genes identified in Aim 1, including those already identified by Smith, et al, including EGFR, its transcription factor SP1, FGF2, ATF3 and TRAIL (2003).

Immunoprecipitation:

Protein lysates will be prepared from hTERT-HMECs using RIPA lysate buffer with protease inhibitors. Protein concentrations will be estimated using the Bradford colorimetric method against known concentrations of BSA. For immunoprecipitation, protein lysates will be precleared using the appropriate isotype IgG antibody, mixed with 2 µg of TERT primary antibody, then incubated overnight. Protein G agarose will be added to each tube and the samples incubated again. The immunocomplex will be washed with cold RIPA buffer and the antibody-selected proteins eluted from the agarose beads by boiling in SDS-loading buffer. Samples will be resolved by SDS-PAGE and visualized with coomassie staining. Similar conditions with appropriate IgG antibody will be used as a control. After staining, bands of interest will be excised, destained, digested and extracted according to Crockett et al (2004), who use an Invitrogen kit.

Tandem Mass Spectrometry and Data Analysis:

An aliquot of each sample will be analyzed by using the LCQ DECA ion-trap mass spectrometer (ThermoFinnigan) at the Harvard Medical School Taplin Biological Mass Spectrometry Facility. Peptide analysis will be performed using data-dependent acquisition of one MS scan followed by MS/MS scans of the three most abundant ions in each MS scan. Protein matches will be determined according to the procedure used by Crockett, et al. (2004). The MS/MS acquired data will be searched with TurboSequest against amino-acid sequences in the National Center for Biotechnology Information nonredundant (NCBI nr) protein database (Crockett, et al. 2004).

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