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Mutant-template human telomerase RNA inhibits tumor cell proliferation and identification of a hypomorphic human telomerase mutant

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Mutant-template human telomerase RNA inhibits tumor cell proliferation and identification of a hypomorphic human telomerase mutant

by

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DISSERTATION

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Degree Conferred.....

This dissertation is dedicated to

God, who taught me there are more important things than this.

Linda H. Chung, M.D., my lovely wife, without whom this would not have been possible.

Mother and father, who supported me with unending prayer and encouragement.

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Acknowledgements (Published Materials)

The text of Chapter Two of this dissertation is a reprint of the material as it appears in *PNAS*. I contributed most of the work involving LNCaP cells. This included constructing the AU5 & U11-hTer mutants, LNCaP portion of figure 2, figures 3, 4a, 5a, 5b, 6a, 6b, 6c, and LNCaP portion of figure 8a.

E. H. Blackburn, the last co-author listed on each publication, directed and supervised the research that forms the basis for the dissertation.

Dissertation Abstract

The ribonucleoprotein telomerase synthesizes telomeric DNA at the ends of chromosomes by copying an intrinsic RNA template. We expressed mutant-template human telomerase RNAs in prostate (LNCaP) and breast (MCF-7) cancer cell lines. Even a low threshold level of expression of telomerase RNA gene constructs containing various mutant templates, but not the control wild-type template, decreased cellular viability and increased apoptosis. This occurred despite the retention of normal levels of the endogenous wild-type telomerase RNA and endogenous wild-type telomerase activity, and unaltered, stable telomere lengths. Therefore, mutant-template telomerase RNAs exert a strongly dominant-negative effect on cell proliferation and tumor growth. We cloned and sequenced terminal telomeres from LNCaP cells expressing WT-hTER/parental, U11-hTer, and AU5-hTer and discovered non-TTAGGG repeats. More significantly, telomeres from U11-hTer expressing cells had the highest frequency of variant repeats, which suggests that this mutant template perturbs telomeres. Telomerase activation induced lifespan extension is generally accompanied by net telomere lengthening, suggesting that lifespan extension is dependent upon telomere length. We showed that hTERT+C, a telomerase mutant with ten additional residues at the C-terminus, is a hypomorph and confers limited lifespan extension to IMR90, a human primary lung fibroblast, without net telomere lengthening. Telomeres erode to very short levels, shorter than what is normally seen at senescence or crisis. These results all show the importance of telomere functionality in cancer and ageing.

Elizabeth H. Blackburn, thesis advisor

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Chapter One

Introduction

End of the chromosome

Telomeres are specialized structures at the end of chromosomes. Thus, it is fitting that the introduction of this work starts at the beginning of the end. The term, "telomere" was coined by *Drosophila* geneticist Herman J. Muller in the 1930's. He postulated that telomeres had a "special function, that of sealing the end of the chromosome." His experiments with X-ray induced chromosomal breaks in *Drosophila* led him to conclude that "at least two breakages are necessary (for chromosomal rearrangements) ... (and) there must be reattachment, and this must occur between the broken ends, not between originally free ends, or between a broken and a free end, or between any end and a free end" (Muller, 1938).

Around the same time, Barbara McClintock was studying the breakage-fusion-bridge cycle of chromosomes in maize. She induced the formation of dicentric chromosomes (a chromosome with two centromeres) and noted that when the centromeres were pulled to opposite poles of the mitotic spindle, a bridge formed between the two poles. As the cell progressed through mitosis, the chromosomal bridge would break, creating two monocentric chromosomes with a "new" end. This "new" end was sticky and would join to other "new" ends at a high frequency, leading to the formation of more dicentric chromosomes. These would then undergo another round of breakage and fusion in the next cell cycle. The breakage-fusion-bridge cycle would continue throughout the development of the plant (McClintock, 1941). Thus, Muller and McClintock showed that a newly formed end of a chromosome is "sticky" and prone to annealing with other newly formed ends, whereas, normal ends of normal chromosomes do not behave this

way. In other words, broken ends of chromosomes are prone to end to end fusions because they lack telomeres, which normally provide protection from these events, preventing anaphase bridges and chromosomal rearrangements.

The end replication problem

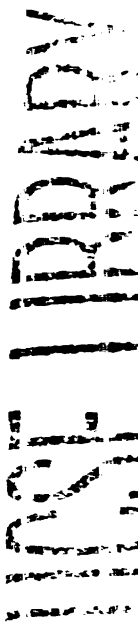
The discovery that chromosomes are made up of DNA was a momentous occasion in modern molecular biology, but as with most things in science, led to more questions. One such question concerning telomeres came to light as the mechanism of DNA replication was worked out. In eukaryotes, DNA synthesis is initiated at multiple origins of replication simultaneously. At these specific points along the chromosome, the double stranded DNA is first unwound by a helicase. This creates two replication forks, which travel in opposite directions as the DNA is replicated. At a replication fork, a helicase continuously unwinds the DNA, a primase synthesizes a small strand of RNA "primer" complementary to the DNA, and DNA polymerase starts to synthesize DNA off the 3' end of the RNA primer. Since DNA polymerase synthesizes in only the 5' to 3' direction, the leading strand is replicated continuously, but the lagging strand is replicated discontinuously. Short DNA pieces (Okazaki fragments) are generated serially along the lagging strand as the replication fork travels downstream. As the DNA polymerase reaches the previous Okazaki fragment, the RNA primer is removed and replaced by DNA. These short pieces of DNA are then ligated together to form a continuous strand of DNA.

DNA replication by DNA polymerase is extraordinarily accurate and is sufficient for most of the genome. However, successful copying of the very tips of the chromosome is not possible due to the need of the RNA primer for DNA synthesis. Once the final RNA primer is removed from the tip of the lagging strand, replication of that region is not possible. Therefore, chromosomes of eukaryotic cells were predicted to shorten after each round of synthesis, whereas circular chromosomes of prokaryotes and viruses would not have this "end replication problem." Watson (1972) was the first to recognize this and proposed that the presence of "concatemers" at the ends of linear chromosomes would avoid this problem.

Telomeres

Telomeres, the very tips of chromosomes, are not abundant in most cells - *S. cerevisiae* has only 32, *C. elegans* 12, and humans 92 chromosome ends. Thus, the low concentration of telomeres made sequencing a difficult task. Fortuitously, ciliated protozoans have a different nuclear organization. These organisms have a micronucleus, which undergoes "normal" mitosis, and contain chromosomes detectable by microscopy. In addition, they have a macronucleus, which contain many small pieces of gene encoding DNA (Prescott and Murti, 1974). After conjugation, the macronucleus is formed from the fragmentation of the germ-line chromosomes. During the formation of the macronucleus, telomeres are added onto the ends of each small piece of DNA. Therefore, the macronucleus of ciliated protozoans has a high number of telomeres and this relative abundance made sequencing feasible.

Blackburn and Gall (1978) first successfully sequenced the telomeres of the ciliated protozoan *Tetrahymena thermophila*, and as predicted by Watson, they were found to be concatemers, tandem repeats of GGGGTT. Soon afterwards, *Oxytricha* and *Stylonychia* macronuclear telomeres were shown to be repeats of GGGGTTTT (Klobutcher et al., 1981; Oka et al., 1980). The telomeres of vertebrates, including humans, are GGGTTA, which, surprisingly, are very similar to the *Tetrahymena* telomeres (Moyzis et al., 1988). Telomeric sequences of many organisms are known to date, and they seem to share some common features. The 3' end is usually G rich and is called the G strand, and conversely, the 5' end is generally C rich and called the C strand. Also, there is a 3' single stranded overhang of two telomere repeats in *Tetrahymena* (Henderson and Blackburn, 1989) or 200 +/- 75 nucleotides in human cells (Wright et al., 1997), which is believed to be important for telomere length regulation.



Telomerase, a terminal transferase

DNA polymerase is unable to fully copy the ends of linear DNA, leading to the loss of telomeres with each successive round of replication. Thus, telomere lengths are expected to gradually decrease over many cellular divisions. However, Southern blots probed with telomeric oligonucleotides revealed that in most cells, telomere lengths remained range bound and under special circumstances, even lengthened over many cellular divisions. For instance, trypanosomes continuously passaged in animal hosts (Bernards et al., 1983) and *Tetrahymena* kept in log-phase growth in culture (Larson et al., 1987) increased their net telomere lengths. These results implied that telomeres were somehow being replenished. Two models were proposed.

The first hypothesized that either DNA polymerase slippage or recombination (Walmsley et al., 1984), both of which require pre-existing telomeric DNA, was the primary mode of telomere maintenance. And in support of this, Dunn and Szostak (1984) showed that recombination can cause addition of telomeric ends to a linear plasmid. The second model proposed that telomerase, a yet unidentified polymerase, catalyzed the de novo synthesis of telomeres (Shampay et al., 1984). This model was supported by the observation that when a linear plasmid with *Tetrahymena* telomeres was introduced into yeast, only yeast telomeres were added onto it. The pre-existing cis telomeres did not "code" for the newly synthesized telomeres, arguing against recombination or DNA polymerase slippage. We now know that both recombination and telomerase can maintain telomeres, but telomerase seems to be preferred method in most organisms.

Greider and Blackburn first showed the presence of telomerase activity in *Tetrahymena* extracts (Greider and Blackburn, 1985). Telomerase in these extracts was able to elongate a telomeric primer, $(TTGGGG)_4$, in the presence of dTTP and ^{32}P-dGTP, producing a distinctive ladder pattern on polyacrylamide sequencing gels. This laddering pattern is now known to be characteristic of a positive telomerase activity assay. dCTP and dATP were not required for this activity. The banding pattern revealed a six base periodicity, with two dark then four light bands, suggesting a six base concatemer. Furthermore, the addition of ddGTP shifted the banding pattern to two light and four dark. Conversely, ddTTP "accentuated the relative intensities of the two originally darkest bands." (Greider and Blackburn, 1985) Thus, the products were generated by the

addition of six base repeats consisting of four G's and two T's. Telomerase was adding GGGGTT repeats to the telomeric primer.

Characterization of telomerase revealed that it was not a typical DNA polymerase copying from a DNA template, but instead, a reverse transcriptase, using an RNA template. The first hint that RNA was an important component of telomerase was seen when the *Tetrahymena* extracts were treated with RNAse A. This destroyed the laddering pattern, whereas, pretreatment with both RNAse A and RNasin (RNAse inhibitor) had no effect on telomerase activity. In essence, these results suggested that telomerase was a ribonucleoprotein (Greider and Blackburn, 1987). And this was confirmed when the telomerase RNA was identified. Telomerase activity was purified through a series of five different chromatographic steps and RNA of approximately 159 nucleotides was purified. This 159 RNA was derived from a gene in the macronuclear genome and named TER for telomerase RNA. Strikingly, the sequence at positions 43 to 51 coded for one and a half telomeric repeats [5'CAACCCCAA], which could act as a template (Greider and Blackburn, 1989). Overexpression of several mutants of this "template" region resulted in the incorporation of the corresponding telomeric repeats *in vivo* and altered telomere lengths (Yu et al., 1990). These series of experiments conclusively showed that telomerase was a ribonucleoprotein with reverse transcriptase activity, and that it was the primary mode of telomere maintenance in *Tetrahymena*.

Human Telomerase RNA

The telomerase RNA functions as the template for the reverse transcriptase activity of telomerase. And the template region usually consists of about one and a half telomeric repeats. Thus, once the telomere sequence is known for a given species, TER can be isolated by screening for RNAs containing this sequence. And this has been accomplished for many species, including *homo sapiens*. The human telomerase RNA (hTER) is 451 nucleotides long, with the template at positions 43-51 [5'CTAACCCTA] (Feng et al., 1995). Mutations within the template region leads to the synthesis of corresponding repeats and antisense to hTER inhibits telomerase activity, causing telomere erosion and eventual cessation of cell division (Feng et al., 1995).

Further study of the mature hTER transcript revealed that it is a RNA polymerase II transcript, is not poly-adenylated, and a member of the box H/ACA small nucleolar RNA family (Feng et al., 1995; Mitchell et al., 1999a). hTER's H box and characteristic ACA trinucleotide, three bases upstream from the 3' end are predicted to form a hairpin-hinge-hairpin-tail secondary structure. Disruption through mutation of the H box or the ACA causes instability of hTER (Mitchell et al., 1999a). Interestingly, box H/ACA small nucleolar RNAs (snoRNA) interact with pseudouridine synthases and participate in pseudouridine modification. Dyskerin, a pseudouridine synthase, interacts with hTER and affects its stability. Patients with one form of inherited dyskeratosis congenita have mutations in the gene encoding for dyskerin and suffer from defects in regenerating tissues such as skin and bone marrow. These patients have lower levels of hTER and telomerase activity, and shorter telomeres (Mitchell et al., 1999b). In essence, hTER's

box H/ACA structure is important in mediating its interaction with dyskerin and dyskerin is critical in maintaining healthy telomere length regulation.

Telomerase, a reverse transcriptase

With the identification of an RNA template, the protein component of telomerase was expected to be a reverse transcriptase. Initially, two telomerase associated proteins (p80, p95) were identified in *Tetrahymena* (Collins et al., 1995), but neither turned out to have reverse transcriptase activity. Then, in *Euplotes aediculatus*, two additional telomerase associate proteins, p123 and p43, were isolated (Lingner et al., 1997). p123 was homologous to the yeast gene, Est2 (Ever shorter telomeres), which was identified in a genetic screen for short telomeres and senescence (Lendvay et al., 1996). These two homologous proteins contained a set of motifs found in reverse transcriptases; and amongst a subset of these, three invariant aspartates in the active site were critical for catalytic function, since single amino acid changes to any of them abolished telomerase activity (reviewed in Nugent and Lundblad, 1998). Soon afterwards, several groups identified the human homolog, human telomerase reverse transcriptase (hTERT) (Harrington et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997).

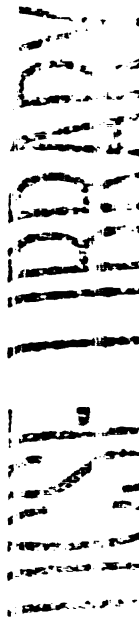
The telomerase RNA and the telomerase reverse transcriptase are the only components required for telomerase activity in *in vitro* reconstitution experiments (Weinrich et al., 1997). Thus, hTER and hTERT are both essential subunits of telomerase. However, expressions profiles of both subunits add another level of complexity. For instance, hTER is expressed in most cell types, but the expression levels do not strictly correlate

with telomerase activity. Low levels of hTER are readily detectable in normal primary human cells even though they lack telomerase activity (Feng et al., 1995). In contrast, hTERT mRNA is seen only in cells with detectable telomerase activity (Meyerson et al., 1997). And ectopic expression of only hTERT in telomerase negative fibroblasts induces telomerase activity (Nakayama et al., 1998). Thus, hTER and hTERT are both essential components of telomerase, but hTERT is the key regulated subunit.

Telomeres are DNA-protein complexes

Telomeres are nucleoprotein complexes, consisting not only of tandem DNA repeats, but also proteins bound to it in a sequence specific manner. These proteins play a crucial role in telomere function and length regulation. For instance, in budding yeast, Cdc13, a single stranded telomeric DNA binding protein functions as a "loading platform" for telomerase and telomere end protection proteins. Mutations in CDC13 cause telomere shortening and expression of Cdc13-Est 2 (telomerase reverse transcriptase) fusion results in telomere lengthening (Evans and Lundblad, 1999). And fusion of the DNA binding domain of Cdc13 to Stn1 (involved in chromosome end protection) rescues cdc13 null strains (Pennock et al., 2001). Rap1, a double-stranded telomeric DNA binding protein, is also essential for proper telomere function. Altering the telomeric DNA sequence essential for Rap1 binding disrupts the interaction and leads to telomere length dysregulation and senescence (Krauskopf and Blackburn, 1996). Cdc13 and Rap1 are two examples of telomeric biding proteins and they illustrate the importance of the DNA-protein interaction of telomeres.

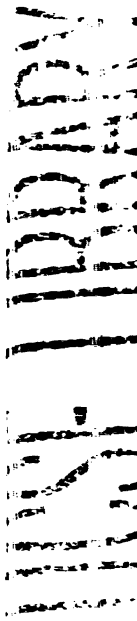
In humans, the double-stranded telomeric DNA binding proteins, TRF1 and TRF2, are important for telomere structure and function. TRF1 negatively regulates telomere lengths since overexpression of wildtype TRF1 causes telomere shortening, whereas overexpression of the dominant negative leads to telomere elongation (van Steensel and de Lange, 1997). TRF2, on the other hand, helps maintain telomeric structure and integrity. It can facilitate the formation of t-loops at the ends of linear DNA *in vitro* and more specifically, is localized to the D-loop (displacement loop) where the single stranded 3' G strand invades the duplex telomeric DNA (Griffith et al., 1999). Expression of dominant negative TRF2 results in end to end chromosomal fusions and growth arrest (van Steensel et al., 1998), and can also cause an ATM and p53 dependent apoptosis (Karlseder et al., 1999). Thus, TRF1 and TRF2 help "cap" the ends of chromosomes to protect them from degradation, end to end fusions, and inappropriate recombination. Humans have a protein, Pot1p, which is homologous to the single-stranded telomeric DNA-binding protein Pot1p of *S. pombe*. In that species, Pot1 is essential for stable telomere maintenance. The DNA binding domain of Pot1, as well as that of the related single-stranded telomeric DNA-binding alpha protein of ciliates, is structurally similar to the DNA binding domain of Cdc13p (Mitton-Fry et al., 2002).



Telomerase is essential for immortalization

Normal somatic cells have a limited proliferative capacity in culture, as first described by Hayflick (1965; 1961). He noted that normal fibroblasts grown in culture ceased to divide after a finite number of population doublings. They do not die, but instead exit the cell cycle and remain metabolically active. This state of replicative quiescence is called

senescence. Senescent cells are large, flat, and have low nucleocytoplasmic ratios (Bowman et al., 1975; Sherwood et al., 1988). In a given population of cells, there exists a fraction which stochastically senesces. This fraction increases as a function of population doublings, until the entire population is senescent (reviewed in Blackburn, 1995; pgs 247-263). Most normal somatic cell types, including epithelial cells, endothelial cells, myoblasts, astrocytes, and lymphocytes undergo a similar process (Harley, 1995).



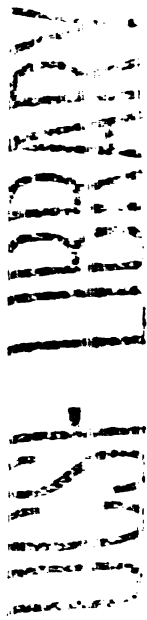
The telomere hypothesis of aging states that as cells divide, their telomeres shorten to some critically short length, at which point, the cells senesce. This occurs in most somatic cells since they do not have active telomerase, and are unable to replenish their telomeres. During the lifespan of a cell, the probability of it senescing increases as telomeres shorten. The point at which the entire population is senescent is called Mortality Stage 1 (M1). It is hypothesized that at M1, a checkpoint mechanism detects the critically short telomeres as damaged DNA and signals the cells to exit the cell cycle. Presumably, this sensing mechanism signals through the p53 and pRB pathways. And, in support of this, abrogation of both pathways by the SV40 large T antigen bypasses M1 and confers an extended lifespan phenotype. And as these cells continue to divide past M1, their telomeres continue to shorten until it reaches a second checkpoint, Mortality Stage 2 (M2), at which point the cells enter crisis and die. At M2, telomeres are very short, on average about 1-4Kb in length (Counter et al., 1992), and very few cells (10^{-7}) survive crisis (Zhu et al., 1999). Those that survive can proliferate indefinitely and are immortal. As expected, most cells that survive crisis have managed to maintain stable

telomere lengths, either through telomerase activation or recombination (Counter et al., 1992; Klingelhutz et al., 1994).

Immortalization of human primary somatic cells requires stabilization of telomeres. For instance, in primary fibroblasts, ectopic expression of hTERT alone can restore telomerase activity, lengthen telomeres, and extend their lifespan indefinitely (Bodnar et al., 1998). In epithelial cells, telomerase activation alone is not sufficient for immortalization, but also requires the viral oncoprotein E7 (Kiyono et al., 1998). Presumably, in these cells, culturing conditions promote the accumulation of $p16^{INK4a}$, a potent inhibitor of CDK4/CDK6. This in turn prevents CDK4/CDK6 from phosphorylating pRb; and hypophosphorylated pRb sequesters E2Fs, transcription factors responsible for activating mitogenic genes, thus, preventing entrance into S phase (reviewed in Sherr, 2000). E7 blocks this pathway by binding to pRb, causing the release of E2F. Senescence of epithelial cells is directly attributable to the accumulation of $p16^{INK4a}$ and most human mammary epithelial cells (HMEC) that spontaneously bypass senescence fail to express $p16^{INK4a}$ (Romanov et al., 2001). Thus, inactivation of $p16^{INK4a}$/pRb pathway, in addition to telomerase activation, is essential in the immortalization of epithelial cells. All of these findings support the telomere hypothesis of aging and suggests that maintenance of telomeres is an important factor of cellular aging *in vitro*.

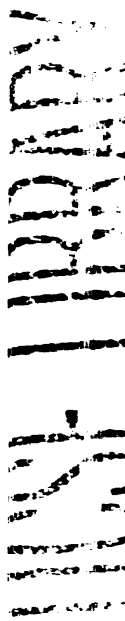
Telomerase, a double edged sword in tumor progression

Just as in immortalization, telomere stabilization is required for tumor progression and growth. This is not surprising since telomeres have to be continuously replenished in dividing cells. Most somatic cells do not have active telomerase, but 90% of all tumors do (Kim et al., 1994). Thus, most tumors must activate telomerase to maintain telomeres and disruption of telomerase activity inhibits their growth. For instance, inhibition of telomerase by small molecules or antisense, causes telomere erosion, and apoptosis in tumor cells (Kondo et al., 1998; Naasani et al., 1999). Overexpression of an aspartate to alanine mutant of hTERT, which inhibits telomerase activity in a dominant negative fashion, also causes telomere shortening, apoptosis, and reduced tumor growth in mouse xenograft models (Hahn et al., 1999a; Zhang et al., 1999). Furthermore, hTERT expression, combined with H-rasV12, SV40 large T antigen, and small T antigen, are sufficient to transform fibroblasts and epithelial cells (Elenbaas et al., 2001; Hahn et al., 1999b). H-rasV12, an oncogenic allele of H-ras, provides a continuous mitogenic signal and SV40 large T antigen disrupts both the p53 and pRb pathways. The role of small T antigen is unclear. Thus, telomere maintenance by telomerase is essential for tumor growth and progression.



Conversely, telomerase deficiency can increase genomic instability and promote tumorigenesis. In the telomerase null mouse model, splenocytes from sixth generation mice have increased aneuploidy (Lee et al., 1998). In addition, these mice have higher rates of spontaneous tumor formation originating from highly proliferative cell types, and more specifically teratocarcinomas, lymphomas, and squamous cell carcinomas (Rudolph et al., 1999). This most likely is due to the absence of telomerase activity leading to

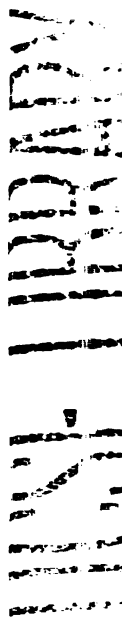
telomere erosion and dysfunction. Dysfunctional telomeres are unable to protect chromosomes from end to end fusions, resulting in chromosomal breakage and rampant genomic instability. And as in most instances, genomic instability gives rise to higher rates of tumorigenesis. Removing p53, a tumor suppressor involved in the DNA damage response pathway, has an additive effect. For instance, telomerase deficiency in p53 null background increases the total rate of tumorigenesis and also the rate of epithelial derived tumors (Artandi et al., 2000). Most likely, dysfunctional telomeres trigger a p53 dependent apoptosis and/or cell cycle arrest (Karlseder et al., 1999). In the absence of p53, cells with dysfunctional telomeres and rampant genomic instability are not culled and progress to cancer.



This is in direct contrast to another set of experiments which suggest the inability to maintain functional telomeres prevent tumorigenesis. In normal mice, initiation with 7,12-dimethylbenz (a) anthracene (DMBA) and promotion with 12-O-tetradecanoylphorbol 13-acetate (TPA) result in well-differentiated skin papillomas. In fifth generation telomerase null mice, however, these agents fail to induce papillomas (Gonzalez-Suarez et al., 2000). And in telomerase and INK4a double null mice, oncogenic potential was reduced when compared to INK4a only null mice (Greenberg et al., 1999). So, telomere dysfunction can promote tumorigenesis by contributing to genomic instability, but subsequent telomere maintenance is most likely required for continual tumor growth and progression.

Project rationale and thesis overview

I was initially interested in investigating the role of telomerase in tumorigenesis. And mutant template telomerase RNA (MT-hTer) provided a means to not only study its effects on cancer, but a way to test the feasibility of this reagent as a novel antitumor agent. I reasoned that expressing MT-hTer in cancer cells with high telomerase activity would cause synthesis of the corresponding mutant repeats. And since altering even just one repeat in budding yeast was sufficient to cause cell cycle arrest, incorporation of a single mutant telomere may be sufficient to have an equivalent effect in human cancer cells. Early on, it became clear that the hTER processing was going to limit the level of exogenous MT-hTer expression. Fortuitously, this did not hamper the experiments, but instead provided a valuable insight into the sensitivity of telomere perturbation. In an attempt to better understand the effects of MT-hTer on telomeres, I sequenced telomeres from cancer cells transfected with two of the mutant templates, looking for MT-hTer's corresponding mutant repeat. The results were not what I had expected, but informative nevertheless.



Chapter four summarizes a very interesting set of experiments involving a C-terminal hTERT mutant. These studies clearly show the dissociation between lifespan extension and *in vivo* telomere length. This is in direct contrast to the concept of telomere length being the sole determinant of lifespan. A common, recurring theme amongst all the experiments is that telomere end protection or capping is the most important factor for a dividing cell, not telomere length nor telomerase activity.

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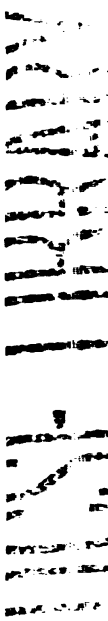

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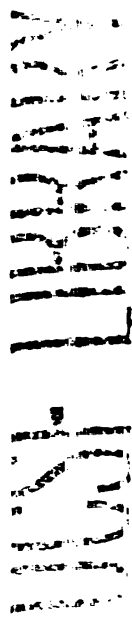

Chapter Two

A low threshold level of expression of mutant-template telomerase RNA is sufficient to inhibit human tumor cell proliferation

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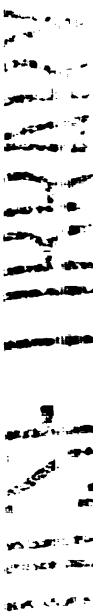
Abstract

The ribonucleoprotein telomerase synthesizes telomeric DNA at the ends of chromosomes by copying an intrinsic RNA template. In most cancer cells, telomerase is highly activated. Here we report a novel telomerase-based anti-tumor strategy: expression of mutant-template telomerase RNAs in human cancer cells. We expressed mutant-template human telomerase RNAs in prostate (LNCaP) and breast (MCF-7) cancer cell lines. Even a low threshold level of expression of telomerase RNA gene constructs containing various mutant templates, but not the control wild-type template, decreased cellular viability and increased apoptosis. This occurred despite the retention of normal levels of the endogenous wild-type telomerase RNA and endogenous wild-type telomerase activity, and unaltered, stable telomere lengths. *In vivo* tumor xenografts of a breast cancer cell line expressing a mutant-template telomerase RNA also had decreased proliferation rates. Therefore, mutant-template telomerase RNAs exert a strongly dominant-negative effect on cell proliferation and tumor growth. These results support the potential use of mutant-template telomerase RNA expression as a novel anti-neoplastic strategy.



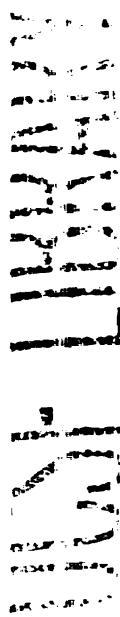
Introduction

Telomeres are specialized functional complexes at the ends of chromosomes, consisting of the terminal stretch of chromosomal DNA and associated proteins. The telomeric DNA in most eukaryotes consists of tandem repeats of a simple sequence unit, which is TTAGGG in humans and other vertebrates (reviewed in Zakian, 1995). DNA-sequence-specific binding and other proteins associate with telomeric DNA repeats to create a complex that "caps" the telomere, preserving its physical integrity so the cell can continue to divide. Recent evidence suggests that the telomeric DNA-protein complexes are dynamic and normally can switch stochastically between a nonfunctional, uncapped state and a functional, capped state (reviewed in Blackburn, 2000). Unless recapped in a timely fashion, an uncapped telomere can suffer degradation, inappropriate recombination, and end-to-end fusions manifested as anaphase bridges (Blackburn, 2000; Kirk et al., 1997; van Steensel et al., 1998). Uncapped telomeres appear to act similarly to DNA damage in eliciting cell cycle exit arrest or apoptosis (Karlseder et al., 1999; Sandell and Zakian, 1993). In yeast, only one chromosome break is sufficient to elicit cell cycle arrest in cells with intact checkpoint controls (Bennett et al., 1993), and loss of a single telomere can cause a *RAD9* dependent temporary cell cycle arrest (Sandell and Zakian, 1993). Therefore, it has been suggested that the uncapping of even one telomere may be sufficient to cause cell cycle arrest in human cells (Levy et al., 1992).

The ribonucleoprotein enzyme telomerase helps maintain capped telomeres, adding tandem telomeric DNA repeats to chromosome ends by copying a short template sequence within its RNA moiety TER (Yu et al., 1990). Such elongation of the telomeric

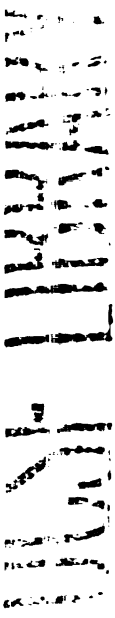
DNA compensates for the inability of the DNA replication machinery to completely copy the tips of the chromosomal DNA. The higher-order complex nucleated on the telomeric DNA by sequence-specific binding proteins is also critical for maintaining functional, capped telomeres. Mutating the telomeric DNA or its proteins can disrupt this complex, thereby uncapping the telomere. In a ciliate and two budding yeasts, such disruption has been achieved by mutating the sequences of only the terminal few telomeric repeats, generating "toxic" telomeres (i.e., telomeres containing deleterious terminal telomeric DNA sequences) (Gilley et al., 1995; Kirk et al., 1997; Krauskopf and Blackburn, 1996; McEachern and Blackburn, 1995; Smith and Blackburn, 1999; Yu et al., 1990). These mutant telomeric DNA repeats were created by mutating the template sequence in TER. In these systems, rapid telomere fusions, massive failure of chromosome segregation and decreased cellular proliferation resulted. For some template mutants in yeasts, the effects correlated with loss of binding affinity to the yeast telomeric DNA-sequence-specific protein Rap1p (Krauskopf and Blackburn, 1996). That these effects were caused by disruption of the telomeric DNA-protein complex is supported by other experiments involving mutant telomere binding proteins. For instance, mutating the DNA binding domain of the yeast protein Cdc13p, which binds the terminal telomeric single-stranded DNA overhang, led to telomeric DNA degradation and cell cycle arrest (Hughes et al., 2000). In human cells, overexpression of a truncated form of TRF2 (a human telomeric DNA-binding protein postulated to bind telomeric end regions) (Griffith et al., 1999) caused telomeric fusions (van Steensel et al., 1998) and ATM- and p53-dependent apoptosis (Karlseder et al., 1999).

Telomerase is inactive in many adult human cell types, but is highly activated in most human cancers. Telomerase activation promotes proliferation of cultured pre-cancerous human cells (Bodnar et al., 1998; Kiyono et al., 1998; Yang et al., 1999; Zhu et al., 1999) and reviewed in (Blackburn, 2000). Experimental activation of telomerase (by ectopic over-expression of the core subunit hTERT), coupled with the expression of multiple oncogenes, induced malignant transformation of primary human diploid cells (Elenbaas et al., 2001; Hahn et al., 1999b). Thus, telomerase provides a potential anti-neoplastic therapeutic target, and inhibition of telomerase activity by various means inhibits the proliferation of tumor cells in culture and in mouse xenograft models (Hahn et al., 1999a; Kondo et al., 1998; Naasani et al., 1999; Zhang et al., 1999). However, in all these previous reports of perturbations of human telomerase, inhibition of cell proliferation required essentially complete loss of telomerase activity, via either an inhibitor, or expression of a great excess of a mutated telomerase component(s) to swamp out the endogenous wild-type telomerase (Hahn et al., 1999a; Zhang et al., 1999).



Previous work on cancer cells has involved inhibiting telomerase activity, and thus shortening telomeres, as a means to uncap telomeres (Hahn et al., 1999a; Kondo et al., 1998; Naasani et al., 1999; Zhang et al., 1999). Here we report a novel way of uncapping telomeres in human cancer cells without significant telomere shortening or loss of telomerase activity. We demonstrate that expressing various mutant-template human telomerase RNAs (MT-hTers) decreases cancer cell proliferation both *in vitro* and *in vivo*. We report that expression of even very low levels of MT-hTers, in either human breast or prostate cancer cells, decreases cellular viability and significantly increases

apoptosis rates. Tumors in mice xenografted with human breast cancer cells expressing an MT-hTer were smaller than those generated from cells expressing the control wild-type template gene. Most importantly, a low threshold level of mutant-template telomerase expression was sufficient to cause these effects on cell and tumor growth. Thus, it was unnecessary to block or overwhelm the expression of the endogenous telomerase RNA. MT-hTers therefore display true dominance over the wild-type telomerase RNA. To account for these novel findings we propose that uncapping of as few as one telomere by the action of these mutant-template telomerases elicits cell cycle arrest and apoptosis in human cancer cells.



Methods

Plasmid construction:

hTER was PCR cloned from human genomic DNA (Promega, Madison, Wisconsin) using 5'-GTGGAATTCGGGTTGCGGAGGGTGGGC-3' and 5'-GCTGGATCCCGACTTTGGAGGTGCCTTCA-3' (Bryan et al., 1997). Products were completely sequenced and subcloned into pTRE (Clontech, Palo Alto, California) to generate pTRE-hTER. MT-hTer constructs were generated by site-directed mutagenesis (Clontech mutagenesis kit) using the following hTER mutagenesis oligonucleotides: 49A-hTer (5'-GGTGGCCATTTTTTGTCTAAAACCCTAACTGAGAAGGGCG-3'), AU5-hTer (5'-GGGGTGGTGGCCATTTTTTGTTATATATATAATGAGAAGGGCGTAGGCGCCG-3'), U11-hTer (5'-GGGGTGGTGGCCATTTTTTGTTTTTTTTTTTTTGAGAAGGGCGTAGGCGCCG-

3'). Additional mutant template sequences were: 51G (5'-GGAAGGCUAAC-3'), 53G (5'-GGAAGGGGAAC-3'), 50G (5'-CAAAGCCUAAC-3'), and 53A (5'-CAAAGCCAAAC-3'). hTERT was cloned into pBabe-puro and stable lines were generated by retroviral mediated transfection and subsequent selection with puromycin (Zhu et al., 1999).

Cell lines:

MCF-7 Tet-off cell line was obtained from Clontech (Palo Alto, California), and LNCaP from the University of California, San Francisco cell culture facility. Tetracyline inducible clones of LNCaP were generated by stable transfection of pTet-On. Clonal isolates were assayed for tetracycline inducibility by transient transfection of a luciferase reporter construct. LNCaP-tet-on c.3 had a 1,044 fold induction in the presence of doxycycline (2μg/ml) and was used for subsequent experiments. The tetracycline inducible MCF-7 and LNCaP cell lines were transfected with pTRE-hTER and selected with hygromycin (300μg/ml for MCF-7 and 250μg/ml for LNCaP). Doxycycline (2μg/ml) was added to either induce (LNCaP) or uninduce (MCF-7) the hTER expression.

RNA Analysis:

RNA was isolated from cells using RNEasy (Qiagen, Valencia, California) columns and the samples were treated with RQ1 RNAse free DNAse (Promega, Madison, Wisconsin) prior to analysis. RT-PCR was performed by using an hTER specific primer, 5'-CATGTGTGAGCCGAGTCCTGGGTGCA-3', for the RT step (Superscript II, Life

Technologies, Gaithersperg, Maryland). The PCR step amplified the cDNA by using the MT-hTer specific oligos: RT-WT (5'-GGGAGGGGTGGTGGCCATTTTTTGTCTAAC-3'), RT-49A (5'-GGGAGGGGTGGTGGCCATTTTTTGTCTAAAA-3'), RT-AU5 (5'-GGGAGGGGTGGTGGCCATTTTTTGTTAT-3'), and RT-U11 (5'-GGGAGGGGTGGTGGCCATTTTTTGTTTT-3'). Northern: total RNA was denatured by glyoxal/DMSO and resolved by electrophoresis in 1.5% agarose gel. The full length hTER gene was random primed labelled to generate the probe.

Cell viability assays:

Expression of exogenous hTER was induced for minimum of 2 weeks before analysis. For ^{3}H-thymidine assays, cells were plated on 12-well plates at three indicated densities in triplicates. One (MCF-7) or two (LNCaP) days later, cells were incubated with 10uCi (MCF-7) or 2.5uCi (LNCaP) of ^{3}H-thymidine for 18hrs. Subsequently, the cells were lysed and analyzed by scintillation counter. For colony forming ability assays, 10^{3} (MCF-7) or 10^{5} (LNCaP) cells were seeded onto 10cm dishes. They were maintained in continuous culture for 21 days, then fixed and stained with 1% crystal violet in 50% methanol. Percent confluency was determined using an Alpha Innotech Corp. digital photo-documentation system with Alphaimager 2200 software, calculated as the total area on the plates covered by the cell colonies, which combined contributions from both the number of colonies and sizes of the colonies. For long term growth analysis, the cells were grown continuously in culture for 150-200 days. The dilution factor at each passage was used to estimate the cumulative dilutions (rough estimate of population doublings).

Apoptosis and cell cycle assays:

Apoptosis was assessed by flow cytometric DNA analysis. Both floating and attached cells were collected and fixed with 70% ethanol for 30mins on ice. The cells were exposed to a solution of RNAse A and propidium iodide. The cellular debris was gated out and the fraction of cells in sub-G1 was determined. As a control for LNCaP, cells were cultured for 3 days in the presence of 10μM of mifepristone or 5μM of 4-hydroxytamoxifen (apoptosis inducing agents for LNCaP) before flow cytometric DNA analysis. MCF-7 cells were pulse labelled with BrdU for four hours, trypsinized, fixed and analyzed by flow cytometry (Kastan et al., 1991).

Telomere length and telomerase activity:

Telomere length was measured by hybridizing a ^{32}P-labeled telomeric probe (CCCTAA)3 to genomic DNA digested with RsaI and HinfI (Counter et al., 1992). Telomerase activity was assessed by a PCR-based telomeric repeat amplification protocol (TRAP) assay (Weinrich et al., 1997).

Mouse xenograft tumor growth analyses

Five to six week old female NCR *nu/nu* mice were purchased from Taconic (Germantown, NY) and were implanted subcutaneously with 72 mg of 60-day release 17-beta-estradiol pellets one to two days prior to injections of human tumor cells. MCF-7 cells stably expressing WT-hTER (clonal line K3) or 49A-MT-hTer (clonal line C1) were suspended in medium, mixed with an equal volume of Matrigel, and injected subcutaneously at the base of the right scapula at 5 million cells per mouse (12 animals

for WT-hTER and 8 or 9 animals for 49A-MT-hTer). Tumors were measured twice weekly until day 38 post injection, at which point, all animals were euthanized. Tumor xenografts were processed for histological examination and cells undergoing mitosis were scored to determine the mitotic index. Immunohistochemistry with TUNEL was performed to determine the apoptotic index.

Results

Expression of MT-hTer constructs occurs at low levels

The choice of MT-hTers tested was based upon corresponding mutations in the *T. thermophila* telomerase RNA (Kirk et al., 1997; Ware et al., 2000) and/or on the predicted loss of DNA sequence-specific binding by the human telomere binding proteins TRF1 and TRF2 (Broccoli et al., 1997; Chong et al., 1995). The templating domain of the human telomerase RNA (hTER, also known as hTR) is an 11-nucleotide (nt) telomeric sequence in the 451-nt RNA (Fig. 1a). Here we report in full the results obtained with three of these MT-hTers: AU5-hTer, U11-hTer, and 49A-hTer (Fig. 1b). As will be described below, similar results were also obtained with four other mutant template hTers. The MT-hTers were expressed under the control of either a tetracycline-inducible system (AU5 and U11) or a tetracycline-repressible system (49A). The AU5-hTer and U11-hTer mutants were transfected into LNCaP, a prostate cancer cell line, and the 49A-hTer mutant into MCF-7, a breast cancer cell line. Both tumor cell lines are telomerase-positive. The wild-type (WT-hTER) gene was also transfected in parallel in both cell types. Four WT, seven U11, and five AU5 clonal LNCaP lines, and seven WT

and twelve 49A clonal MCF-7 lines, were analyzed in depth, and their proliferative and other properties were compared with the respective parental cell lines.

We analyzed the levels and molecular forms of the transcripts of the introduced MT-hTer and WT-hTER genes, and of the endogenous wild-type hTER gene (endo-hTER), using Northern blotting analysis. The mature telomerase RNA species from the introduced gene construct (Fig. 2a, RNA species marked with arrow) was the expected ~80 bases longer than the endogenous hTER, due to the difference in the transcription initiation site of the minimal CMV promoter and the endogenous hTER promoter (Fig. 1a). The 3' terminus of the mature WT-hTER or MT-hTer species was processed at the normal position (Zaug et al., 1996) (the 5' terminus is not processed (Mitchell et al., 1999a). Notably, all stably selected cell lines induced to express the AU5-hTer or 49A-hTer gene had lower levels of mature MT-hTer RNA (Fig. 2a, arrow) than endogenous hTER (Fig. 2b, bar), with the MT-hTer level typically being several-fold lower. This was also the case for the 49A-hTer RNA levels in cells re-cultured after growth as tumors in mouse xenograft experiments (data not shown). The endogenous hTER levels remained relatively constant in nearly all cell lines (Fig. 2b and data not shown).

As reported previously (Mitchell et al., 1999a), expression of the introduced telomerase RNA gene constructs produced two other RNA species besides the mature processed telomerase RNA (Fig. 2a). An ~1kb poly-adenylated species detected by Northern blotting resulted from use of the SV40 poly-A signal in the expression construct (Fig. 1a The poly-adenylated status of this species was verified by a Northern blot analysis of a

poly-A specific RNA preparation and by RT-PCR analysis with oligo dT and hTER-specific primers (data not shown). A ~300 nucleotide degradation fragment (which like the polyA species was detected only when the introduced hTER/hTer construct was expressed) is a 3' fragment lacking the templating sequence (Mitchell et al., 1999a) and data not shown). The 1kb species and ~300 bp degradation product did not adversely affect cell proliferation despite accumulating to high levels in some lines, since proliferation rates of high expressor WT-hTER controls were indistinguishable from that of the parental line. Only the mature processed telomerase RNA appears to associate with active telomerase. It had previously been shown that the poly-A^+ and degradation products do not co-immunoprecipitate with hTERT protein in the telomerase RNP, which contains only the mature processed telomerase RNA (Mitchell et al., 1999a). In addition, we stably transfected the hTERT gene (on a retroviral construct) into several WT-hTER and MT-hTer expressing LNCaP lines. In different clonal lines stably expressing various steady-state levels of hTERT mRNA, the higher the level of hTERT mRNA, the lower was the ratio of polyA^+ to mature processed telomerase RNA (Fig. 2b).

Decreased cell proliferation and viability of cells expressing low levels of mutant telomerase RNAs

Expression of each of the seven MT-hTer genes tested caused decreased cell proliferation rates, usually preventing these cell populations from reaching confluence as compared to the parental lines or the control WT-hTER-expression lines, despite continuous passaging for 6 months. Monitoring population growth rates (recording the dilution factor used at each passage) during prolonged continuous serial passaging showed that, over 150-200

days, the average inferred population doubling times of the LNCaP clonal lines were 3.16 +/- 0.10 days for the WT clonal lines, 4.20 +/- 0.43 days for the U11-hTer lines and 5.75 +/- 1.05 days for the AU5-hTer lines (Fig. 3). However, cells are in log growth phase only at optimal densities, and while the WT cell populations reached confluence at each passage, the AU5 and U11 cell cultures only rarely reached confluence before passaging. Therefore these values most likely underestimate both the true population doubling rates and the difference between WT and the MT-hTer lines. Over a 60-day period of continuous passaging, the inferred average doubling times for MCF-7 cells were 4.20 +/- 1.19 days for 49A-hTer expressing cells, and 1.82 +/- 0.24 days for the control WT-hTER expressing cells. Notably, even after 200 days of culturing, no fast-growing subpopulations that evaded the effects of the MT-hTer expression developed in either LNCaP or MCF-7 cells. Similar results were also obtained in MCF-7 cells expressing four additional, different MT-hTer's (51G, 53G, 50G and 53A; see Methods for their template sequences; data not shown). Expression levels of these four MT-hTers were also low (data not shown). In summary, nearly all WT-hTER clonal lines maintained rapid cell proliferation upon long term culturing and reached confluence at each passage. In contrast, expressing the mutant telomerase RNAs caused significant proliferative defects.

Relative ^{3}H-thymidine incorporation rates (DNA synthesis) were measured to quantify the cellular proliferation of the MT-hTer clonal lines. Cells from each line were seeded at three different cell concentrations and pulsed for 18 hours with ^{3}H-thymidine. On average, the AU5 clonal lines incorporated three fold less, and the 49A clonal lines eight

fold less, ^{3}H-thymidine than their respective WT-hTER controls or the parental cell line (Fig. 4a & b). Although the differences between ^{3}H-thymidine incorporation rates of the U11 and WT clonal lines were not statistically significant (Fig. 4a), the U11 lines did show decreased long term proliferation rates and increased apoptosis (Fig. 3).

These findings were corroborated by colony plating assays. The AU5 and 49A cells produced fewer and, on average, smaller colonies than the WT-hTER controls or parental lines (Fig. 5a & c). Based on the measurements of percent of each plate surface covered by colonies (reflecting both colony number and sizes), the average colony forming efficiencies of AU5 and 49A lines were, respectively, 30- and 12-fold lower than those of their control WT lines (Fig. 5b & d). The difference between the U11 and WT lines was not statistically significant. Microscopic examination of the 49A colonies, but not WT colonies, showed frequent gaps between the cells in a colony (data not shown), consistent with cell death.

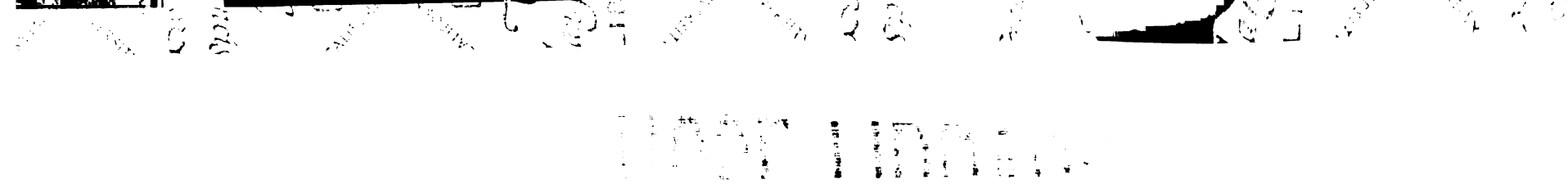
Cell cycle effects and apoptosis of cells expressing low levels of mutant telomerase RNAs

Flow cytometry showed that the fraction of cells with sub-G1 levels of DNA, indicative of apoptosis, was significantly increased in AU5 and U11 compared with the WT-hTER or parental control LNCaP lines (Fig. 6a & b). The sub-G1 peak levels observed were similar to those of LNCaP cell cultures treated with tamoxifen or mifepristone, chemotherapeutic agents known to induce apoptosis in LNCaP cells (El Etreby et al., 2000). Similarly, apoptosis was significantly increased in the 49A clonal lines compared

with WT-hTER and parental MCF-7 controls (Fig. 6c). In addition, cell cycle analysis using BrdU incorporation showed that the fraction of 49A cells in G1 (or G0) was significantly increased, and the fraction in S-phase was correspondingly decreased, compared to the WT control lines (Fig. 6d). Similar cell cycle effects and increased apoptosis were also seen with multiple independent clonal MCF-7 lines expressing either of two other MT-hTer genes (53G and 51G; data not shown). We conclude that both apoptosis and G1 (or G0) cell cycle arrest contribute to the decreased cell proliferation caused by MT-hTer expression.

Tumor growth of breast cancer cells expressing low levels of mutant-template telomerase RNA

Stable transfectant MCF-7 breast cancer cell lines expressing either the 49A-MT-hTer (clonal line C1) or the control WT-hTER gene (clonal line K3) were xenografted into nude mice and assessed for tumor growth over 38 days. In two independent experiments, the average growth rate of the 49A-hTer tumors was 7-fold less than that of the WT-hTER control tumors (Fig. 7). Some regression in size was observed at late time points for the 49A-hTer, but not the WT-hTER, tumors. By immunohistological analysis, in the 49A compared with the WT-hTER tumors, the mitotic index was decreased (average mitotic index 6.3 versus 13.7 per 1000 cells), and the apoptotic index increased (average apoptotic index 49.0 versus 14.4 per 1000 cells). In summary, MT-hTer expression increased apoptosis and decreased tumor growth rates in this *in vivo* system, as well as in the cells cultured *in vitro*.



Bulk telomere length and telomerase activity are maintained despite loss of cell viability

Southern blotting analysis revealed no significant difference in average telomere lengths between the MT-hTER versus the WT-hTER-expressing LNCaP and MCF-7 cells. Although telomere lengths and MT-hTer levels varied among clonal lines within each group (Fig. 8a), for the individual clonal lines we found no correlation between mean telomere length, ^{3}H-thymidine incorporation rate or expression level of the introduced MT-hTer or WT-hTER. Wild-type telomerase activity, measured in standard TRAP assays, was retained in all cell lines regardless of whether WT-hTER or MT-hTer was introduced (Fig. 8b). This was consistent with the retention of normal levels of the endogenous wild-type hTER (Fig. 2a).

Discussion

Here we report the surprising finding that expressing even low levels of mutant-template telomerase RNA decreases cancer cell viability and breast tumor growth in a model xenograft animal system. Apoptosis rates and cell cycle arrest were both increased. Most notably, the mutant-template telomerase RNA showed strong dominance over wild-type with respect to cellular phenotype, despite much higher levels of the wild-type RNA and no abrogation of wild-type telomerase activity. These novel dominant-negative effects were exerted without apparently changing bulk telomere length.

These findings contrast with previous reports of inhibiting cancer cell proliferation by inhibiting telomerase, which was associated with net telomere shortening (Hahn et al.,

1999a; Kondo et al., 1998; Zhang et al., 1999). Overexpressing catalytically inactive mutant hTERT caused telomere shortening and senescence or apoptosis (Hahn et al., 1999a; Zhang et al., 1999), but the inactive hTERT protein was always present in large excess over the endogenously expressed wild-type hTERT, which was therefore likely out-competed for limiting components of the telomerase complex. Similarly, in previous experiments using mutant-template telomerase RNAs in the ciliate *T. thermophila* and in budding yeasts, deleterious effects on cell proliferation also required complete or near-complete replacement of the wild-type RNA by mutant-template RNA (Gilley et al., 1995; Kirk et al., 1997; Krauskopf and Blackburn, 1996; McEachern and Blackburn, 1995; McEachern et al., 2000; Smith and Blackburn, 1999; Yu et al., 1990). We propose that these previously-described "dominant-negative" phenotypic effects, which to date have involved experimentally "swamping out" the wild-type components in the telomerase RNP macromolecular complex by mutant components, be termed "over-expression negative" effects. In contrast, except for the U11 lines, all the independently transfected clonal lines analyzed (over 60 lines) had steady-state levels of processed MT-hTer RNA (and often the poly-A species as well; see Fig. 2a, lanes 5, 11, 12, and 20),) which were consistently low and never exceeded that of wild-type endogenous telomerase RNA, even following induction of gene expression. Hence the MT-hTer RNA was unlikely to have out-competed the endogenous wild-type telomerase RNA. Thus, the human cancer cells tested here appear to be more sensitive to MT-telomerase RNAs than ciliate or yeast cells. Although different mutant hTer sequences were tested in the two cancer cell lines used here, the results were very similar between the two lines, suggesting a common mechanistic basis for the observed effects. Previously, Marusic et

al (Marusic et al., 1997) studied a single-base substitution template mutant in HT-1080 cells. Although it was expressed at similar levels to the endogenous RNA, and mutant repeats were detectably incorporated into telomeres, cell viability was only slightly decreased. The contrastingly potent and similar effects caused by low expression of all seven template mutants tested in our work may be attributable to our use of different template mutations, cell types, and/or expression vector.

A striking and consistent finding with the large number of clonal MT-hTer expressing lines analyzed (total >60) was that a positive RT-PCR scoring for MT-hTer expression always correlated with cell proliferation inhibition. Conversely, MT-hTer lines that scored negative by RT-PCR had growth rates similar to parental and WT-hTER lines (data not shown). The Northern blotting results (Fig. 2a) confirmed that, in contrast to approaches that depend on "over-expression negative" effects, only a low threshold level of MT-hTer expression was necessary for strong cell proliferation and apoptosis effects in the two epithelial cancer cell lines investigated. Interestingly, in the WT and U11 lines, which had, respectively, no and relatively little change in cell proliferation rates, average steady state RNA levels were higher than in the other six MT-hTer lines (Fig. 2a and data not shown). Although the initial stable selection of the clonal lines with geneticin was performed in a supposedly uninduced state, in all the RT-PCR positive lines we found a low level of "leaky" expression even without induction (data not shown), like that reported previously for other genes with these Tet-Off and Tet-On systems (Rossi et al., 1998). Therefore, we suggest that the negative effects of MT-hTers

(excepting U11) combined with the low leakage level of these systems led to the preferential selection of low expressors of these MT-hTers.

The negative effects on cell proliferation caused by MT-hTer expression are unlikely to be caused by non-specific toxic effects related to the construct, or to any effects of MT-hTers on processes other than telomerase action. We found no significant differences in the rates of proliferation, ^{3}H-thymidine incorporation or apoptosis between cells expressing the WT-hTER gene construct and the parental cell line controls, ruling out the possibility of non-specific toxic effects of he WT-hTER construct. hTER is not known to have any function besides that in telomerase. The only difference between the WT-hTER and MT-hTer constructs was the mutated template sequence — 5' CUAACCCUAAC 3' for the WT template, and 5' UUUUUUUUUU 3', 5' UAUAUAUAUAA 3', 5' CUAAAACCCUAAC 3', 5' GGAAGGCUAAC 3', 5' GGAAGGGGAAC 3', 5' CAAAGCCUAAC 3' and 5' CAAAGCCAAAC 3' for the seven different MT-hTers. Since each is very different from the others, it is unlikely that chance similarity to a cellular RNA species caused the observed apoptotic and proliferative effects of all seven MT-hTers.

We propose that uncapping of even one telomere per cell accounts for the low threshold of MT-hTer expression required to cause cellular effects in human cells. This hypothesis is consistent with findings in yeast showing that only one double-stranded DNA break, or loss of only one functional telomere per cell, elicits cell cycle arrest (Bennett et al., 1993; Levy et al., 1992; McEachern and Blackburn, 1996; Sandell and Zakian, 1993). In this

model, the MT-hTer-containing telomerase perturbs a low fraction of the telomeres, or even only one telomere per cell, triggering a DNA damage response. High sensitivity to MT-hTers, which was observed despite the relaxed checkpoint pathways and resistance to apoptosis likely to exist in these cancer cells, can account for the strong dominance and low expression threshold needed to cause cell cycle arrest and apoptosis. In *T. thermophila* telomerase, the template substitutions equivalent to AU5 and U11 caused the expected sequences to be synthesized in vitro (Ware et al., 2000), and the equivalent of 49A caused the corresponding repeats to be added in vivo (Kirk et al., 1997). In two budding yeasts and *T. thermophila*, addition of only a few mutant repeats by a mutant-template TER telomerase is sufficient to interfere with cell proliferation (Kirk et al., 1997; Krauskopf and Blackburn, 1996; Krauskopf and Blackburn, 1998; McEachern and Blackburn, 1995; McEachern et al., 2000; Smith and Blackburn, 1999). In the human cancer cells analyzed here, such mutant repeats added to a telomere are predicted to perturb binding of the DNA sequence-specific proteins TRF1 and/or the capping protein TRF2, and hence to become uncapped. As the cells containing such telomeres ceased to proliferate or underwent apoptosis, mutant telomeres would remain greatly underrepresented, accounting for the unchanged bulk telomere length.

The inhibition of proliferation by the telomerase template mutants reported here exploits the activation of telomerase that characterizes most human tumor cells. It converts the active telomerase pathway, normally advantageous for tumor cell proliferation, into a process detrimental to cancer cells. Tumors expressing an MT-hTer grew more slowly,

with higher apoptotic rates, than controls. Therefore, use of MT-hTer genes, or agents that mimic their effects, may be useful in anti-tumor therapy.

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a

b

Mutant Template hTER	Template Sequence	Predicted Telomere Sequence
WT-hTER	5' CUAACCCUAAC	GGGTTA
AU5-hTer	5' AUAUAUAUAUA	TATATA
U11-hTer	5' UUUUUUUUUUU	AAAAAA
49A-hTer	5' CUA(AA)ACCCUAAC	GGGT(TT)TA

Figure 1: Design of hTER construct and mutant templates

A - The 11-nucleotide template region is located near the 5'-end of the human telomerase RNA. Ectopic expression of the hTER construct leads to the production of three detectable species (green bars), in addition to the endogenous hTER (blue bar). The exogenous hTER is ~70 nucleotides longer than endogenous hTER due to the location of the P_{CMV} transcription initiation site. **B** - The template sequences of WT, AU5, U11, and 49A telomerase RNAs and their predicted telomere sequences.

Chapter2 - Figure2

Figure 2: Expression of mutant-template telomerase RNA

A - Northern blotting analysis of various WT-hTER- and MT-hTer-expressing clonal lines. The 451-nucleotide endogenous hTER is present in every cell line examined (arrow). Parental lines express only this endogenous species. Broad band at ~1 kb, polyadenylated species transcribed from the introduced hTER/hTer construct. Lower band at ~300 nucleotides, degradation product from the introduced hTER/hTer construct. LNCaP clonal lines: Lanes 1-4, WT-hTER expressing lines R10, R11, R12, and R19; lanes 5-8, U11-hTer expressing lines 11.1, 11.3, 11.6, and 11.19; lanes 9-12, AU5-hTer expressing lines 5.4, 5.5, 5.14, and 5.15. MCF-7 clonal lines: Lanes 13-16, WT-hTER expressing lines K2, K3, P1, and A4; lanes 17-20, 49A-hTer expressing lines D2, G4, K6, and P1. **B** - hTERT is limiting for telomerase RNA levels. Northern blotting analysis of LNCaP clonal lines R10 (WT, lanes 1-3), 11.6 (U11, lanes 4-5), and 5.4 (AU5, lanes 6-8) after transfection with hTERT. Lane 1: clonal line R10-c (vector control); lane 2: clonal line R10-6 (low level of hTERT mRNA expression); lane 3: clonal line R10-3 (high level of hTERT mRNA expression); Lane 4: clonal line 11.6-2 (hTERT expression not detected); lane 5: clonal line 11.6-1 (high level of hTERT mRNA); lane 6: clonal line 5.4-c (vector control); lane 7: 5.4-19 (hTERT expression not detected); lane 8: clonal line 5.4-5 (high level of hTERT mRNA).

Chapter2 - Figure3

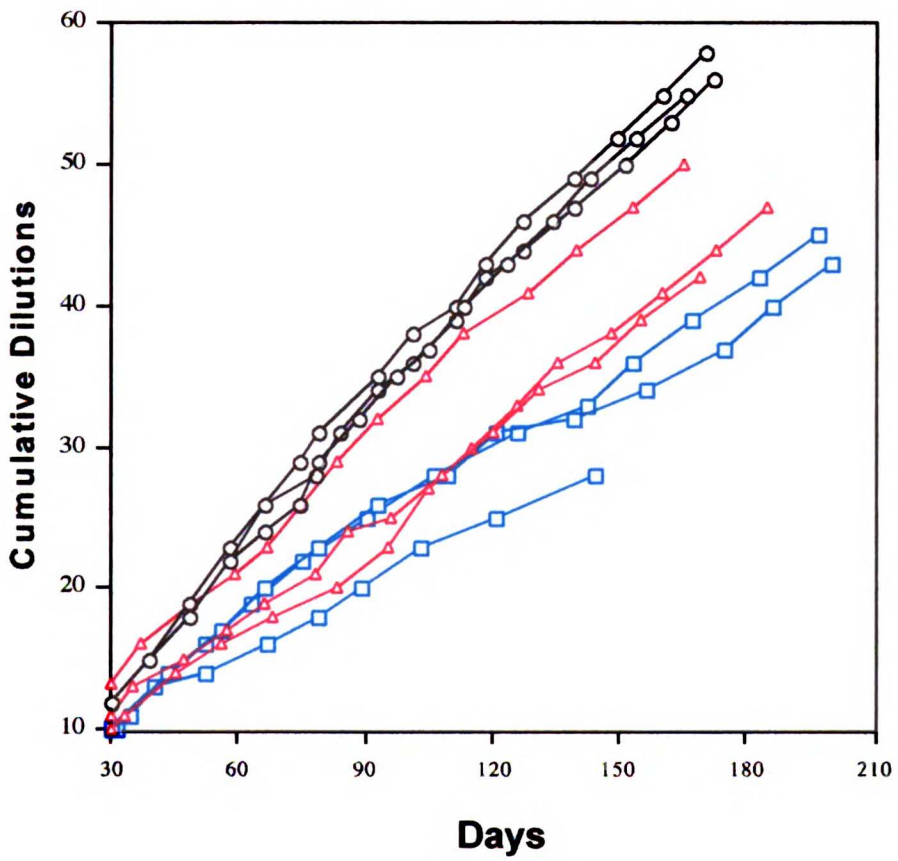


Figure 3: MT-hTer expression decreases growth rate – long term culturing

Long term growth of individual LNCaP clonal lines serially passaged for up to 200 days. The cumulative dilution is the sum of the dilution factors from each passage. Black – WT-hTER, red – U11-hTer, blue – AU5-hTer.

Chapter2 - Figure4

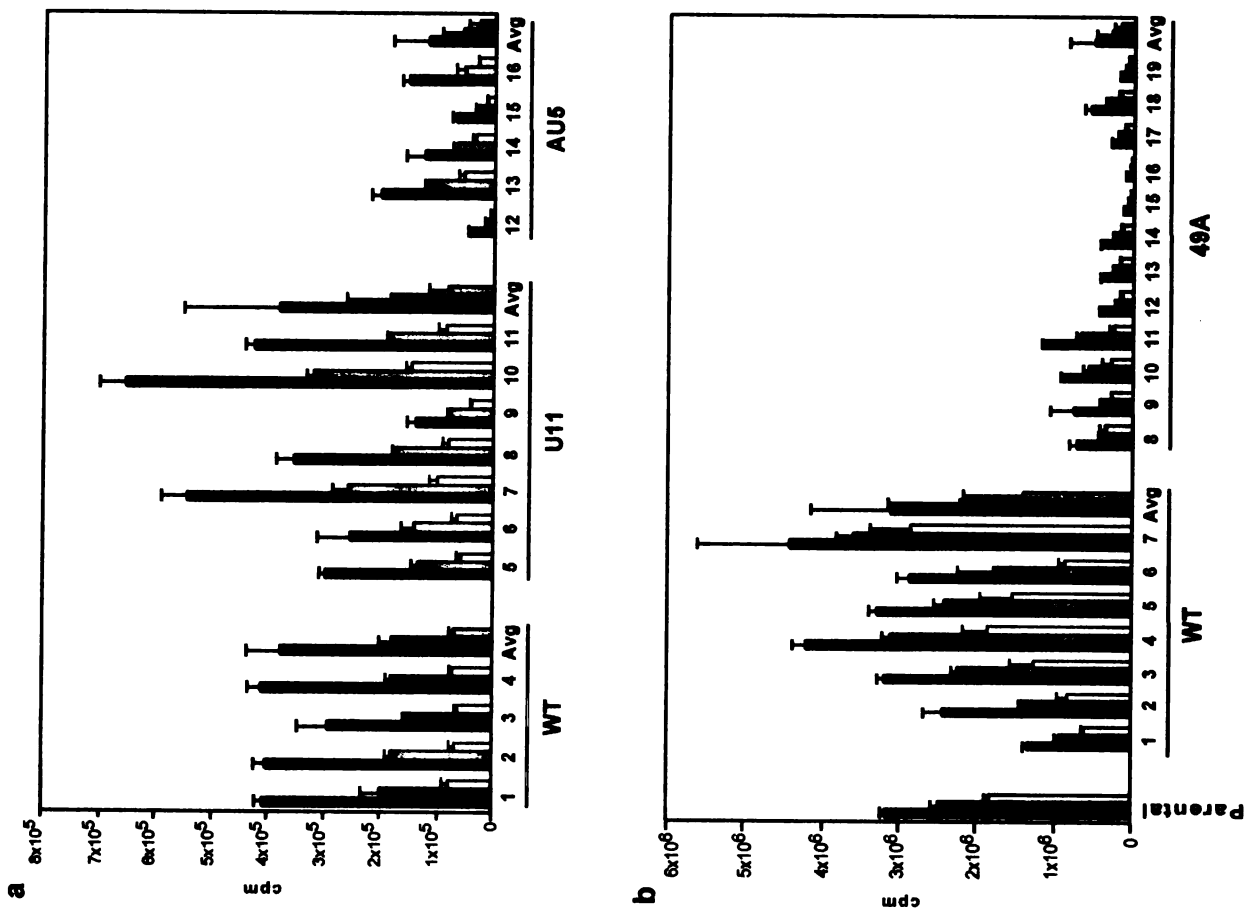
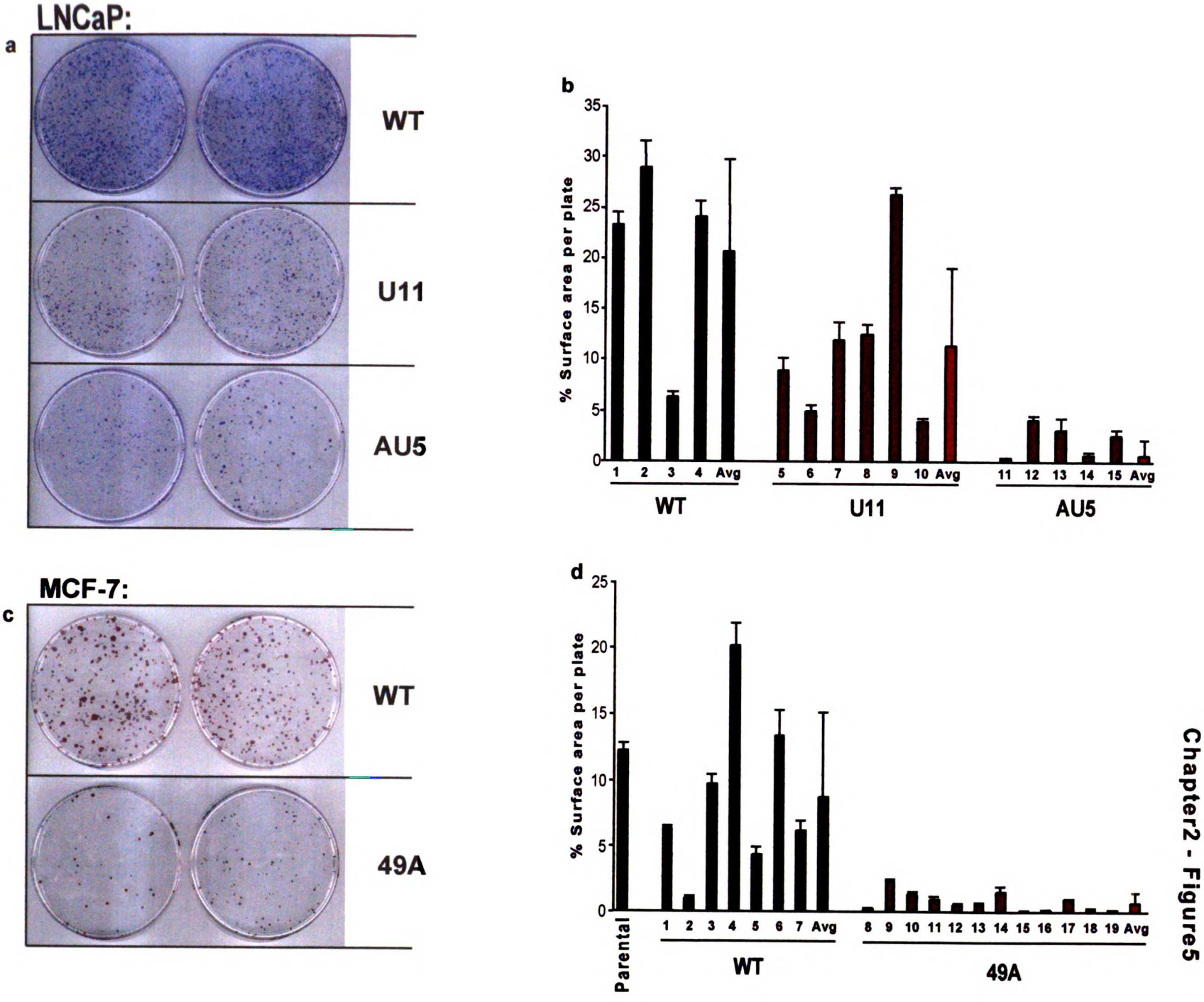


Figure 4: MT-hTer expression decreases cell proliferation and viability - ^{3}H-thymidine incorporation.

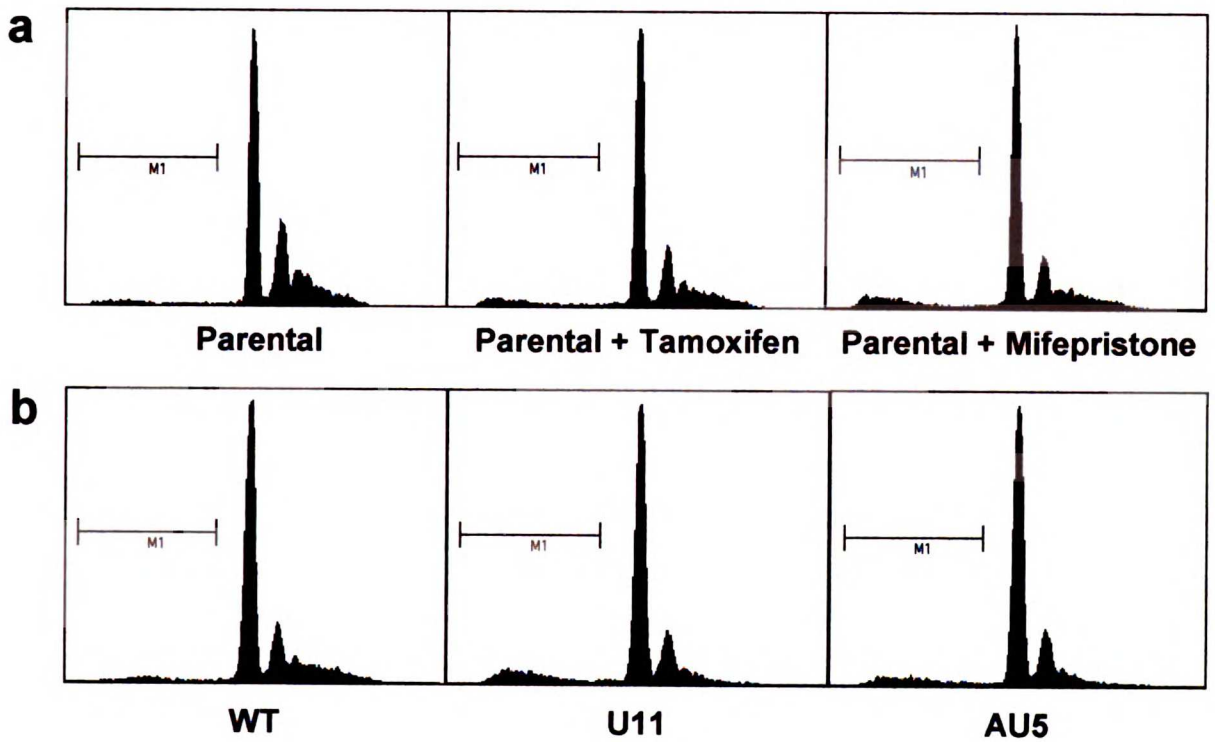
A - LNCaP clonal lines: $2.5x10^{5}$, $1.25x10^{5}$, and $6.25x10^{4}$ cells were plated in triplicates and pulsed with ^{3}H-thymidine. Columns 1-4, WT-hTER expressing lines R10, R11, R12, and R19; columns 5-11, U11-hTer expressing lines 11.1, 11,3, 11.6, 11.10, 11.12, 11.14, and 11.19; columns 12-16, AU5-hTer expressing lines 5.4, 5.5, 5.10, 5.14, and 5.15. **B** - MCF-7 clonal lines: $3.0x10^{5}$, $1.5x10^{5}$, and $7.5x10^{4}$ cells were plated in triplicate. Columns 1-7, WT-hTER expressing lines F4, A4, P1, K3, K2, K1, and H6; columns 8-19, 49A-hTer expressing lines C1, P1, M2, O1, K6, K5, K4, J2, G4, G3, D2, and D1. The averages (avg) for each group of clonal lines are also shown.

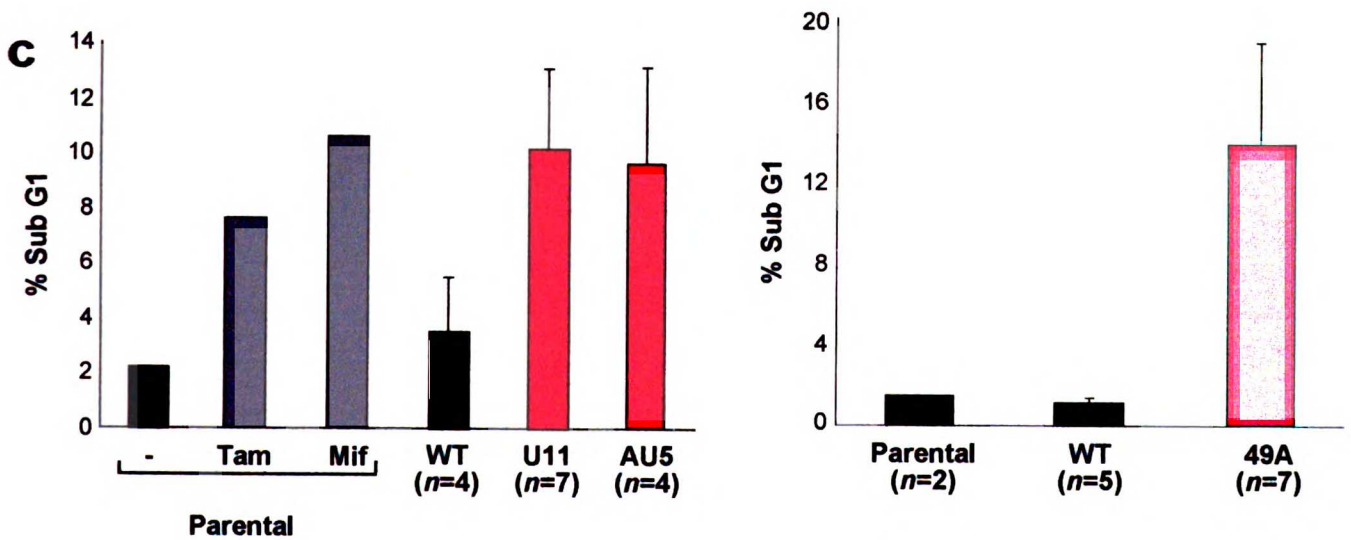


Chapter2 - Figure5

Figure 5: Colony forming ability assays for LNCaP and MCF-7 clonal lines.

For individual lines, the percent of the plate surface area covered by the cells is plotted. **A, C** - Representative plates are shown below. **B** - LNCaP clonal lines: columns 1-4, WT-hTER expressing lines R10, R11, R12, and R19; columns 5-10, U11-hTer expressing lines 11.1, 11.3, 11.6, 11.10, 11.14, and 11.19; columns 11-15, AU5-hTer expressing lines 5.4, 5.5, 5.10, 5.14, and 5.15. **D** - MCF-7 clonal lines: columns 1-7, WT-hTER expressing lines F4, A4, P1, K3, K2, K1, and H6; columns 8-19, 49A-hTer expressing lines C1, P1, M2, O1, K6, K5, K4, J2, G4, G3, D2, and D1. The averages (avg) for each group of clonal lines are also shown.


a
M1
M1
M1
Parental
Parental + Tamoxifen
Parental + Mifepristone
b
M1
M1
M1
WT
U11
AU5


c
% Sub G1
14
12
10
8
6
4
2
0
-
Tam
Mif
Parental
WT (n=4)
U11 (n=7)
AU5 (n=4)
% Sub G1
20
16
12
8
4
0
Parental (n=2)
WT (n=5)
49A (n=7)

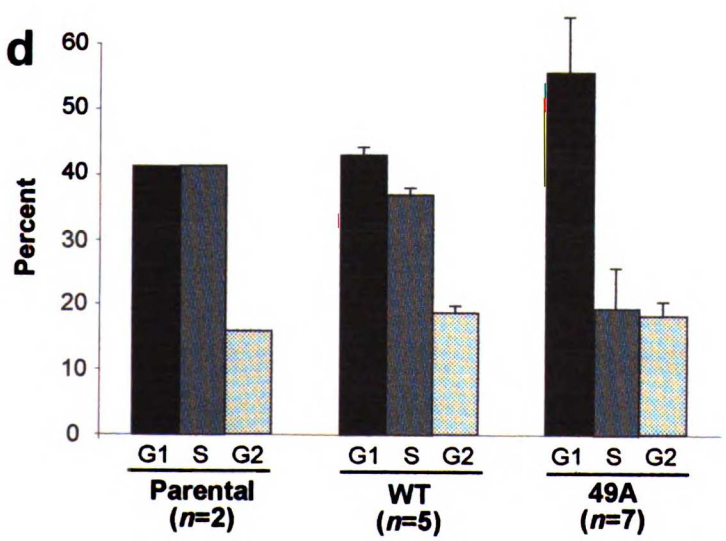
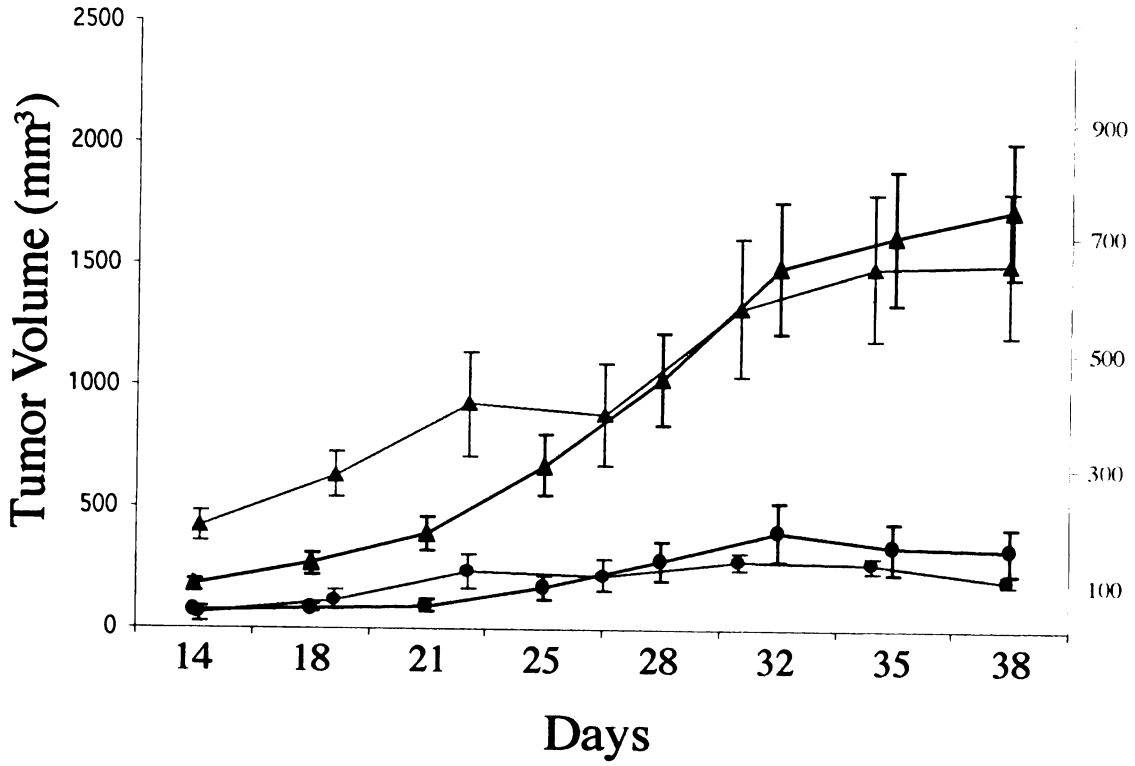

d
Percent
60
50
40
30
20
10
0
G1 S G2
Parental (n=2)
G1 S G2
WT (n=5)
G1 S G2
49A (n=7)

Figure 6: MT-hTer expression increase apoptosis and induces a G1 arrest

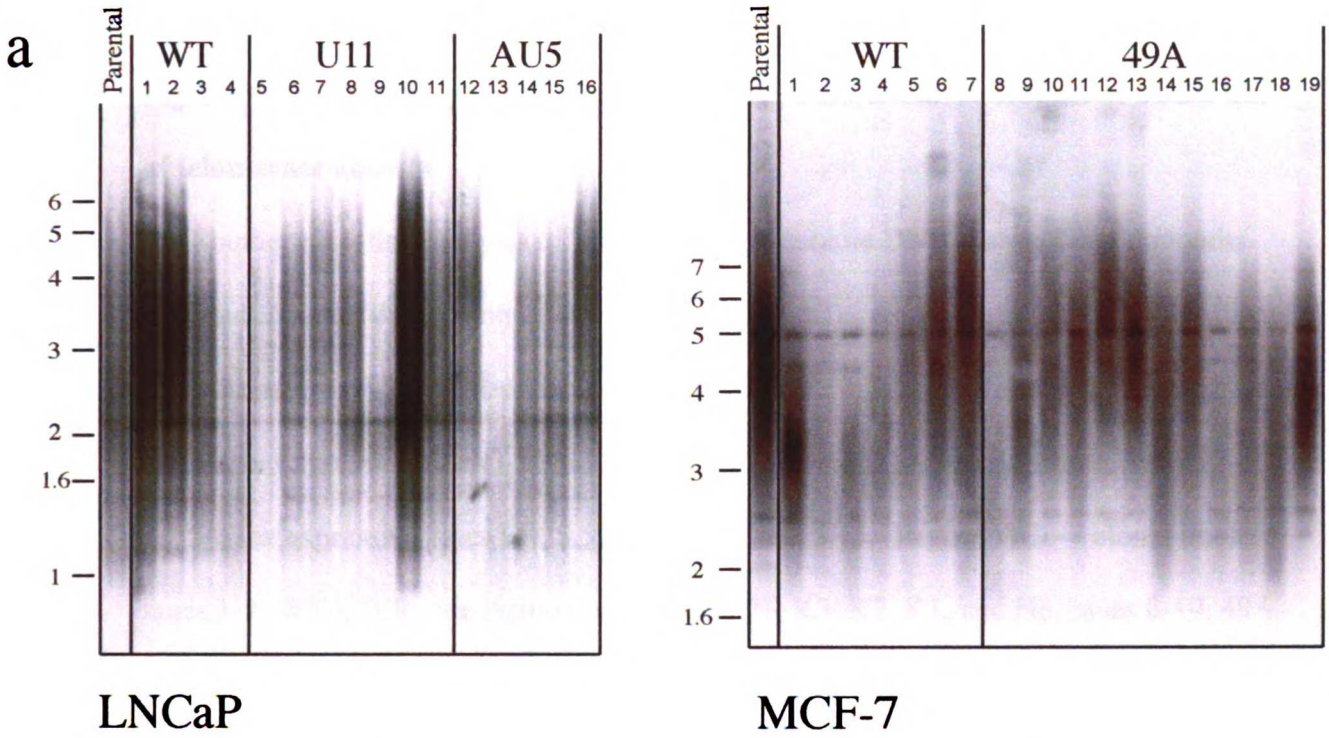
A - Flow cytometry analysis of LNCaP parental (no treatment), parental + tamoxifen, and parental + mifepristone. Representative clonal lines of LNCaP expressing WT, U11, or AU5-hTer are also shown. Sub-G1 was determined by measuring the area under the curve in M1. **B** - LNCaP: parental (-), no treatment; tam, tamoxifen treated; mif, mifepristone treated. The average percent sub-G1 of WT, U11, or AU5-hTer expressing LNCaP clonal lines are also shown (*n* = number of clonal lines). **C** - Flow cytometry analysis of parental, WT, or 49A-hTer expressing MCF-7 clonal lines. **D** - Cell cycle analysis by flow cytometry after BrdU labelling of MCF-7 cells. There is a higher percentage of cells in G1 and less in S of 49A-hTer expressing clonal lines when compared with the parental or WT-hTER expressing clonal lines.



	WT-hTer	49A-hTer
Mitotic index (per 1000)	13.7	6.3
Apoptotic index (per 1000)	14.4	49.0

Figure 7: 49A-hTer expressing cells form smaller tumors in mice

Nude mice were injected with MCF-7 cells expressing either WT-hTER (clonal line K3; triangles) or 49A-hTer (clonal line C1; circles) and tumor volume was measured twice weekly for 38 days. Results from two independent experiments are shown (first: black; second: red).


a
Parental
WT
U11
AU5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
6
5
4
3
2
1.6
1
Parental
WT
49A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
7
6
5
4
3
2
1.6
LNCaP
MCF-7

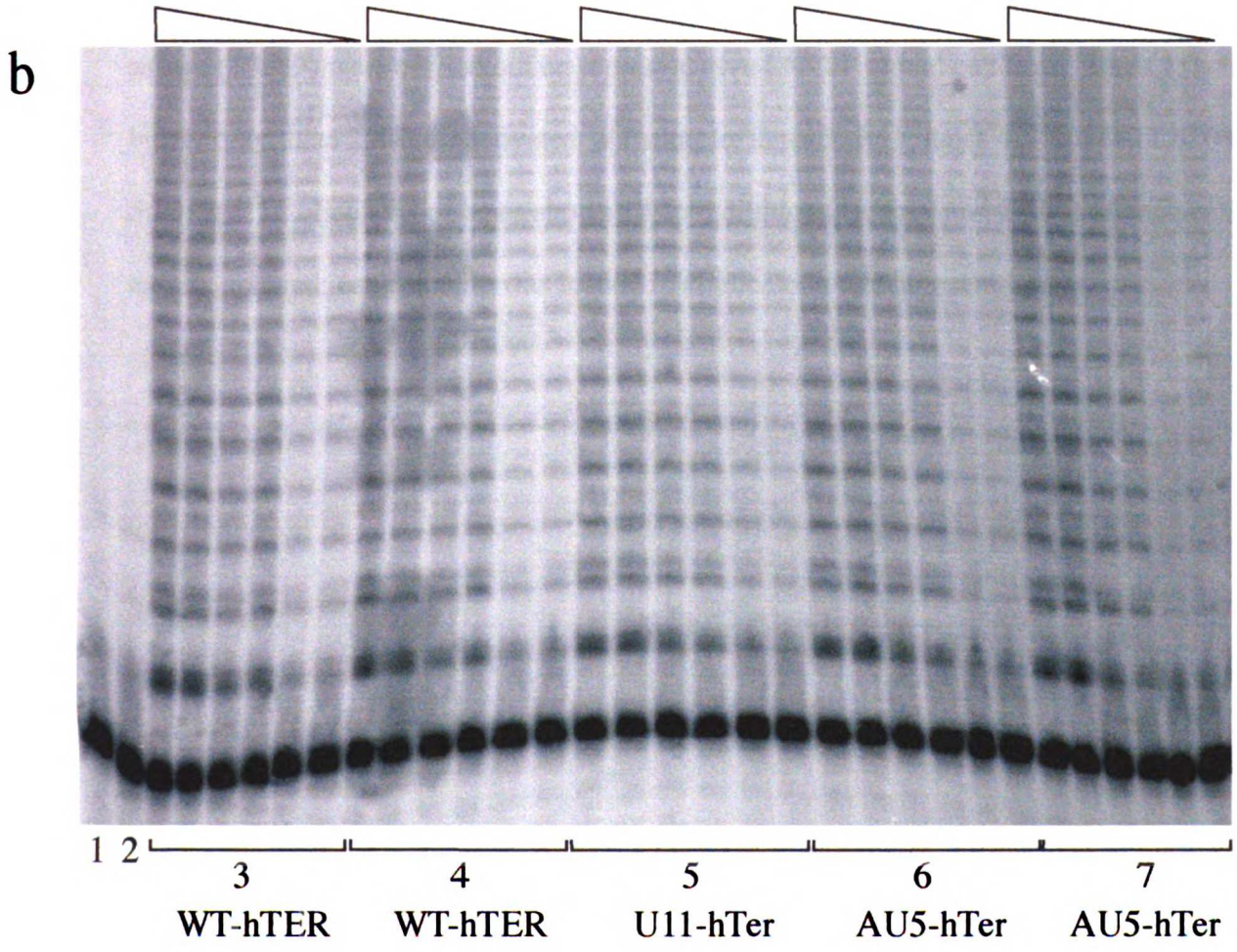
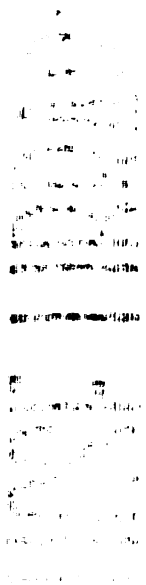

b
1 2
3
4
5
6
7
WT-hTER
WT-hTER
U11-hTer
AU5-hTer
AU5-hTer

Figure 8: MT-hTer expression does not change bulk telomere lengths or cause loss of telomerase activity

A - Southern blotting analysis of TRF lengths. Genomic DNA was isolated 4 months after induction of expression of WT- hTER or MT-hTer expression. **Left:** LNCaP clonal lines: lanes 1-4, WT-hTER expressing clonal lines R10, R11, R12, and R19; lanes 5-11, U11-hTer expressing lines 11.1, 11.3, 11.6, 11.10, 11.12, 11.14, and 11.19; lanes 12-16, AU5-hTer expressing lines 5.4, 5.5, 5.10, 5.14, and 5.15. **Right:** MCF-7 clonal lines: lanes 1-7, WT-hTER expressing lines F4, A4, P1, K3, K2, K1, and H6; lanes 8-19, 49A-hTer expressing lines C1, P1, M2, O1, K6, K5, K4, J2, G4, G3, D2, and D1. There was no correlation between TRF lengths and proliferation rates. **B** - Telomerase activity assays (TRAP) were carried out on representative LNCaP clonal cell lines. Lane 1: lysis buffer only; lane 2: heat inactivated WT sample; lanes 3: R11; lanes 4: R19; 5: 11.6; 6: 5.14; 7: 5.15. Each set of lanes shows 10-fold dilutions (1, 1:10 and 1:100) of each cell extract assayed; each sample was loaded in duplicate in two adjacent lanes.

Chapter Three

High sequence variation in telomeres of U11-hTer expressing LNCaP cells



Abstract:

Telomeres are complex DNA-protein structures that "cap" the tips of chromosomes and prevent them from eliciting the DNA damage cascade. They also protect chromosomes from degradation, inappropriate recombination, and end-to-end fusions. Telomeric DNA is comprised of tandem repeats of TTAGGG. Expression of mutant template human telomerase RNAs (MT-hTers) inhibit growth of LNCaP, a prostate cancer derived cell line. We cloned and sequenced terminal telomeres from LNCaP cells expressing WT-hTER/parental, U11-hTer, and AU5-hTer and discovered non-TTAGGG repeats. More significantly, telomeres from U11-hTer expressing cells had the highest frequency of variant repeats, which suggests that this mutant template perturbs telomeres. Better understanding of MT-hTer's effects on telomeres may improve utilization of this potential anti-tumor agent.

Introduction:

Complex telomeric DNA-protein structures "cap" the tips of chromosomes and protect them from being recognized as damaged DNA (reviewed in Blackburn, 2000). Uncapped telomeres appear to act similarly to DNA damage in eliciting cell cycle arrest or apoptosis (Karlseder et al., 1999; Sandell and Zakian, 1993). Telomeres also protect chromosomes from degradation, inappropriate recombination, and end-to-end fusions (Blackburn, 2000; Kirk et al., 1997; van Steensel et al., 1998). Due to the end replication problem, the very terminal telomere repeats are unable to be replenished during DNA synthesis and gradually erode over many cell divisions. A unique enzyme, telomerase, through utilization of an RNA template, synthesizes telomeres by a reverse transcriptase mechanism. There are two distinct essential components of telomerase: telomerase reverse transcriptase and telomerase RNA (reviewed in Nugent and Lundblad, 1998).

The higher-order telomeric complexes are dynamic and can switch stochastically between a nonfunctional, uncapped state and a functional, capped state (reviewed in Blackburn, 2000). Telomeric DNA, its associated proteins, and telomerase all function in overlapping ways to cooperatively cap the telomere (reviewed in Blackburn, 2001). Mutating the telomeric DNA, its proteins, or telomerase itself can uncap the telomere. In a ciliate and two budding yeasts, mutating the template sequence of the telomerase RNA resulted in the synthesis of the corresponding mutant telomere repeats, leading to telomere fusions and decreased cellular viability (Gilley et al., 1995; Kirk et al., 1997; Krauskopf and Blackburn, 1996; McEachern and Blackburn, 1995; Smith and Blackburn,

1999; Yu et al., 1990). And in human cells, overexpression of a truncated form of TRF2 (a human telomeric DNA-binding protein) caused telomeric fusions (van Steensel et al., 1998) and ATM- and p53-dependent apoptosis (Karlseder et al., 1999). Similarly, overexpression of an alanine mutant of the human telomerase reverse transcriptase (hTERT) behaved as a dominant negative and inhibited telomerase activity and rapidly induced apoptosis (Hahn et al., 1999a; Zhang et al., 1999).

Mammalian telomeric DNA is normally comprised of tandem repeats of TTAGGG. However, non-TTAGGG sequence variants are quite numerous and are located mostly in the proximal regions of the telomere (closer to the centromere). In one study (Brown et al., 1990), the variants TTAGGGG, TTGGGG, TGAGGG, and TAGGG appeared frequently in the proximal telomere. Other variants which appeared less frequently were TTGCGG, TTTGGG, TTAGGGG, TAGGGG, TGGGG, TTCGGG, TTAAGGG, and AGGG. And telomeric variants tended to be clustered together in tandem repeats. These findings were consistent with another study which used hybridization and restriction analysis to map the positions of TTAGGG, TGAGGG, and TTGGGG variants (Allshire et al., 1989). Thus, the proximal regions of the telomere are populated with telomeric variants, while the distal regions are mostly TTAGGG.

Mutating the template region of the human telomerase RNA (hTER) can reduce the cellular viability of cancer cells. Expression of AU5-hTer (substitution of the 11 base template region with 5'TATATATATAA) and U11-hTer (replaced with 5'TTTTTTTTTTT) template mutants decreased the viability of LNCaP, a prostate cancer

derived cell line. AU5-hTer had a greater inhibition of cellular viability than U11-hTer, with the growth inhibitory effects of the latter exhibiting itself only in long term growth experiments. These mutants appeared to uncap telomeres and thereby, increase G1 cell cycle arrest and apoptosis without change in *in vitro* telomerase activity and net telomere lengths (Kim et al., 2001). To better understand the effects of the mutant template telomere RNA on telomeric DNA, we cloned and sequenced telomeres from these cells. We report here evidence of telomere perturbation in U11-hTer expressing LNCaP cells.

Methods:

Tissue Culture

LNCaP was obtained from the University of California, San Francisco cell culture facility. Tetracyline inducible clones of LNCaP were generated by stable transfection of pTet-On. Clonal isolates were assayed for tetracycline inducibility by transient transfection of a luciferase reporter construct. LNCaP-tet-on c.3 had a 1,044 fold induction in the presence of doxycycline (2μg/ml) and was used for subsequent experiments. The tetracycline inducible LNCaP cell lines were transfected with pTRE-hTER and selected with hygromycin (250μg/ml for LNCaP). Doxycycline (2μg/ml) was added to induce hTER expression (Kim et al., 2001).

Genomic DNA

Doxycycline (2ug/ml) was added to parental LNCaP (tet-on c.3), LNCaP expressing WT-hTER, AU5-hTer, or U11-hTer to induce expression of the telomerase RNA. One P-100

plate of cells was harvested and genomic DNA purified using the GenomicPrep Cells and Tissue DNA Isolation Kit from Amersham Biosciences (Piscataway, NJ). Products were analyzed on 1% agarose gel to verify intactness of the genomic DNA.

Telomere Cloning and Sequencing

The anchor primer, RA20 - 5'TTAGTGAGGGTTAATAAGCGGCCGCGTCGTGACTGGGAGCGC, has a seven carbon chain with a terminal amino group 3' cap. RNA ligase was used to ligate RA20 to the ends of the genomic DNA due to its ability to ligate single stranded DNA: 10µl of 2X RNA ligation buffer, 1.2 µl of RA20 (25µM), 1µl RNA ligase, 1µg of genomic DNA in 20µl total volume; 2hours at 37°C, heat inactivate for 15minutes at 70°C. 10X RNA ligation buffer: 100mM Tris-HCl pH8.0, 25% PEG 8,000, 20mM MgCl, 2mM Hexamine Cobalt Chloride, 40uM ATP, and 20µg/ml BSA. Then, EcoRI restriction digest: 20µl genomic DNA sample, 10µl 10X EcoRI buffer, 3.0µl EcoRI, and 67.0µl of dH_20 for 100µl total volume; 2hours at 37°C. Clean sample with Qiaprep (Qiagen, Valencia, California) and resuspend in 50µl. Use 2µl for polymerase chain reaction: 2µl of Qiaprep eluate, 5µl of 10X PC2 buffer, 0.5µl Klntaq, 1µl of dNTP's , 1µl of RA24 - 5'CTCCCAGTCACGACGCGGCCGC (25µM), 1µl of Pst-telo5 - 5'GGGGCTGCAG$(TTAGGG)_5$ (25µM), dH_20 to 50µl total. PCR conditions: 95°C x 2minutes; 2 cycles of 95°C x 30seconds, 60°C x 90seconds, 72°C x 90seconds; 28 cycles of 95°C x 30seconds, 66°C x 45seconds, 72°C x 90seconds; final step at 72°C x 5minutes. Gel purify PCR product and cut band in the 0.5 - 1kb range. Restriction digest with PstI and EagI for minimum of 2 hours. Again, clean sample with with Qiaprep

(Qiagen, Valencia, California). Clone into pBluescript and sequence. The telomere cloning protocol was modified from Tzfati (2000).

Results:

Average lengths of cloned telomeres are the same

Telomeres were cloned and sequenced from untransfected LNCaP cells, and from those transfected with WT-hTER, AU5-hTer, or U11-hTer. A non-telomeric anchor primer with a 3' cap (seven carbon chain with a terminal amino group) was ligated onto the genomic DNA. After an EcoRI restriction digest, PCR was performed using primers complementary to the telomere and to the anchor primer. A restriction site was placed on each primer and these were utilized to clone the PCR product into pBluescript, and sequenced. In this manner, the terminal 3' telomeres were cloned and sequenced. Also, due to the use of a telomeric primer during PCR, telomeres of various lengths were isolated. For WT-hTER transfected or parental, AU5-hTer transfected, and U11-hTer transfected, 11, 19, and 32 telomeres were isolated respectively (Table 1). And the average number of telomeric repeats isolated was 12.18 +/- 3.78 for WT-hTER or parental; 11.32 +/- 2.68 for AU5-hTer; and 12.03 +/- 1.76 for U11-hTer (Table 1). There was no difference in the average length of cloned telomeres between the samples

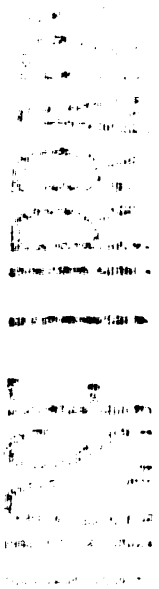
Variations in the telomeric sequences

Surprisingly, the telomeres did not have uniform sequences, but instead, contained many variations. Wildtype telomere sequence on the G-strand is TTAGGG. But in WT-hTER

transfected or parental cells, TTTAGGG was isolated six times and TTTAGGG three times (Fig. 1). And in AU5-hTer, three copies of CTAGGG (same as WT-hTER) and TTAGGGG, and two copies of TTGGGG or TCAGGG (Fig.1) were isolated. And in U11-hTer, there were again three repeats of CTAGGG (same as WT-hTER and AU5-hTer), but in addition, there were 22 repeats of TGAGGG, 18 repeats of TTGGG, 14 repeats of TTCGGG, 13 repeats of TTAGGGG, and a smaller number of repeats of many other variations (Fig. 1). In total, there were two telomere sequence variants for WT-hTER/Parental, four for AU5-hTer, and 24 for U11-hTer.

Telomeres from U11-hTer expressing cells have high frequency of variants

Strikingly, there were high numbers of telomere variants in U11-hTer expressing cells. In addition, they contained a higher frequency of variants. Dividing the number of variant bases by the number of telomeres gives the number of variant bases per cloned telomere. And there were 1.27 +/- 0.9 variant bases per telomere for WT-hTER/parental; 0.58 +/- 0.26 variant bases per telomere for AU5-hTer; and 4.56 +/- 1.58 variant bases per telomere for U11-hTer (Fig. 2). Also, there were 134 total telomere repeats in the 11 telomeres cloned for WT-hTER/parental; and 9 repeats were variants or one out of every 14.9 repeats was a variant. And, there were 215 total telomere repeats in the 19 telomeres cloned for AU5-hTer and 10 repeats were variants or one out of every 21.5 repeats was a variant. And, there were 385 total telomere repeats in the 32 telomeres cloned for U11-hTer and 118 repeats were variants or one out of every 3.2 repeats was a variant (Table 2). Telomeres from U11-hTer expressing cells have about three to six fold



higher frequency of variants when compared to telomeres from WT-hTER/Parental and AU5-hTer expressing cells.

Long telomeres from U11-hTer expressing cells contain many variants

Examination of the relationship between cloned telomere lengths and number of variant bases (Fig. 3) revealed that longer telomeres had more variants, which seemed to be exaggerated in telomeres form U11-hTer expressing cells. Most WT-hTER/parental and AU5-hTer telomeres contained less than two variants per cloned telomere. And the ones with more than two variants (one WT-hTER/parental telomere had ten variants and two of the AU5-hTer telomeres had three and four variants) had greater than 20 telomeric repeats. However, 8 of the 32 U11-hTer telomeres had greater than two variants per telomere and most of them were in telomeres with more than ten repeats. Strikingly, three of the U11-hTer telomeres longer than 30 repeats had over 25 variants. There was a trend in which the longer the telomere, the greater the number of variants. Not infrequently, variant repeats were tandemly clustered, with as many as seven consecutive variant repeats (data not shown). In essence, most of the telomeres longer than ten telomeric repeats from U11-hTer expressing cells had a high number of variant repeats, unlike the WT-hTER/parental and AU5-hTer telomeres.

Discussion:

In the previous chapter, it was reported that expression of AU5-hTer and U11-hTer decreased the viability of LNCaP, a prostate cancer derived cell line (Kim et al., 2001).

However, the effects of MT-hTer (mutant template human telomerase RNA) on telomeres seemed to be subtle since G1 cell cycle arrest and apoptosis were not accompanied by changes in net telomere length nor telomerase activity, as measured by an *in vitro* PCR based method. Thus, sequencing the telomeres was predicted to provide insight in determining MT-hTer's effects on telomeres. And examination of the 3'terminal telomeric sequences showed that U11-hTer perturbs telomeres and that different MT-hTers can have differing effects on telomeres.

Cloned telomeres from WT-hTER/parental cells contained the telomeric variants TTTAGGG and CTAGGG (Fig. 1). This suggested that telomeric variants occur normally at some frequency, which may be due to errors introduced during telomerase action. And in one experiment, sequencing analysis of human telomeres revealed that telomeric variants were quite common, especially in the proximal regions (Brown et al., 1990). So the presence of telomeric variants in these "normal" cells is consistent with previous findings.

Comparing telomere sequences against WT-hTER/parental telomeres is feasible since all the samples were processed in parallel, using identical reagents and primers. Thus, we can conclude that telomeres from U11-hTer expressing cells had much greater frequency of variants when compared to the frequency of variants in WT-hTER/parental telomeres (Table 2, Fig. 2). U11-hTer had greater frequency of variant bases per cloned telomere and per cloned telomere repeat. And comparison of the long telomeres revealed that only U11-hTer telomeres had very high numbers of variant repeats (Fig. 3). The increase in

the frequency of variants in U11-hTer telomeres may be due to: 1) direct effects on telomerase - U11-hTer decreases the fidelity of telomerase, causing synthesis of variant repeats at the terminus; or 2) effects on the telomere - proximal variant repeats are moved to the terminus through an unknown mechanism.

Interestingly, AU5-hTer telomeres did not have an increased frequency of variants, even though it had greater growth inhibition than U11-hTer. This suggests that these two mutant templates may inhibit growth through two different mechanisms. AU5-hTer's effects may be more immediate, with disruption of the telomere structure and rapid onset of cell cycle arrest or apoptosis. This may explain the lack of detectable telomere sequence alteration since this cell would quickly drop out of the population. On the other hand, U11-hTer generated variant telomeric repeats at the terminus, which may not consistently disrupt the telomeric structure, leading to less growth inhibition and more readily detectable telomere sequence changes.

In conclusion, telomeres from U11-hTer expressing cells have a high number of variant repeats at the terminus, which suggest that U11-hTer can perturb telomeres. In contrast, telomeres from AU5-hTer cells do not have increased telomere sequence variation, which indicate that its mechanism of growth inhibition may be different from that of U11-hTer. Further understanding of MT-hTer's effects on telomeres will be helpful in realizing its potential as an anti-tumor agent.

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	Number of Telomeres	Avg. number of Telomeric repeats
WT-hTER/Parental	11	12.18 +/- 3.78
AU5-hTer	19	11.32 +/- 2.68
U11-hTer	32	12.03 +/- 1.76

Table 1: Average number of telomeric repeats are the same for all cell types.

Telomere Variants	WT-hTer/ Parental	AU5-hTer	U11-hTer
1) TTTAGGG	6		4
2) CTAGGG	3	3	3
3) TTAGGGG		3	13
4) TTGGGG		2	18
5) TCAGGG		2	4
6) TGAGGG			22
7) TTCGGG			14
8) TAGGG			7
9) GTAGGG			6
10) TTTGGG			4
11) TTGGGG			3
12) TCAAGGG			2
13) TTAAGGG			2
14) TAGGGG			2
15) TTAGGC			2
16) GTGGGG			2
17) TTATGG			2
18) TTCAGG			2
19) GTGAGGG			1
20) TAAGGG			1
21) TTAAC			1
22) TTACGG			1
23) TTCGGA			1
24) CTTAGGG			1

Figure 1: Frequency of telomere sequence variants - WT-hTER/Parental and AU5-hTer telomeres have only two and four sequence variants respectively. 24 variants found in telomeres from U11-hTer. Table indicates number of times the variant was detected.

	Number of telomere repeats	Number of variant repeats	Number of telomere repeats per variant repeat
WT-hTER/Parental	134	9	14.9
AU5-hTer	215	10	21.5
U11-hTer	385	118	3.2

Table 2: U11-hTer has the highest frequency of variants per telomere repeat - one out of every 3.2 telomere repeats is a variant; one out of every 14.9 and 21.5 telomere repeats is a variant for WT-hTER/Parental and AU5-hTer respectively.

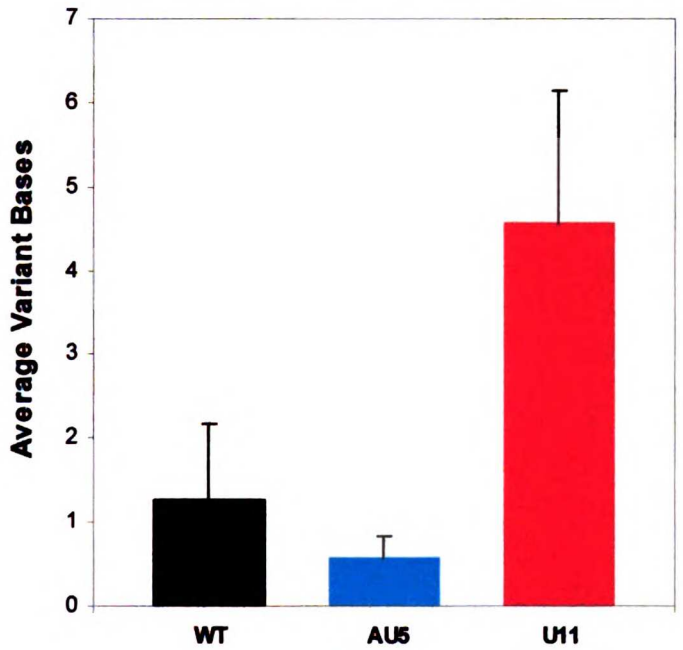
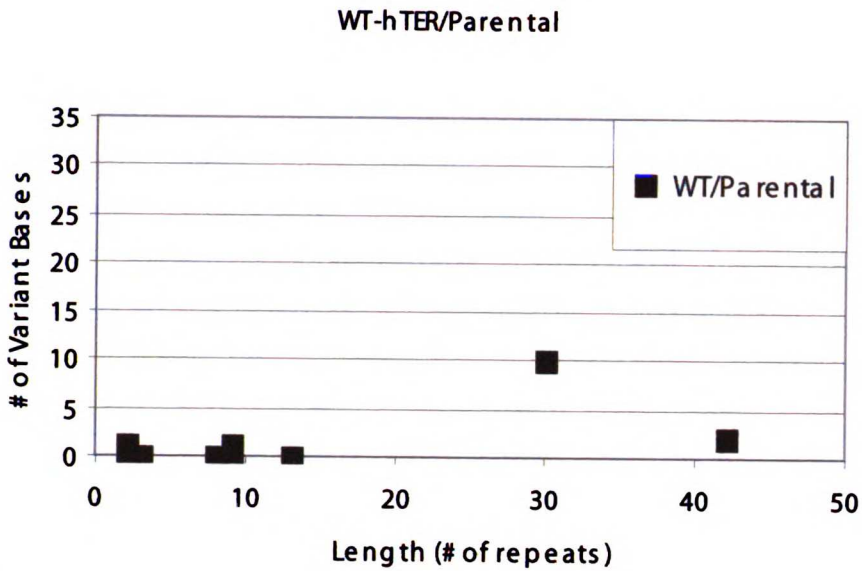
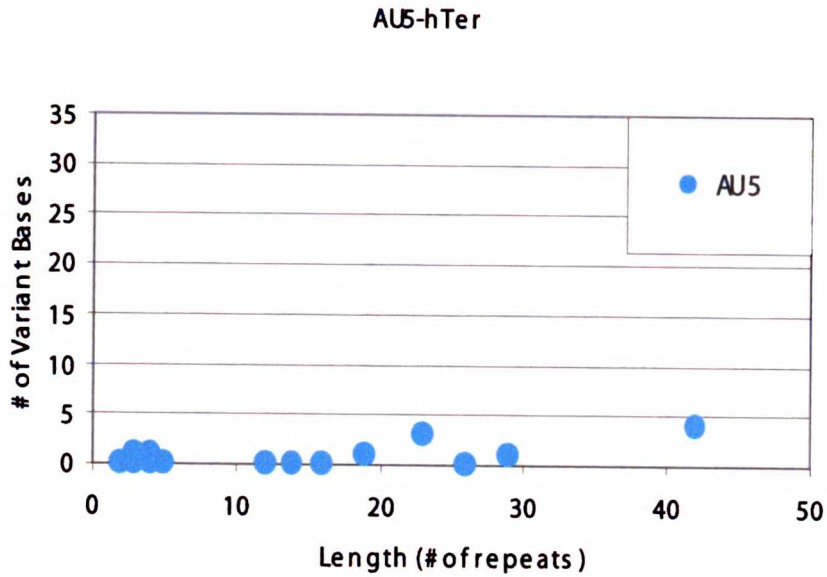
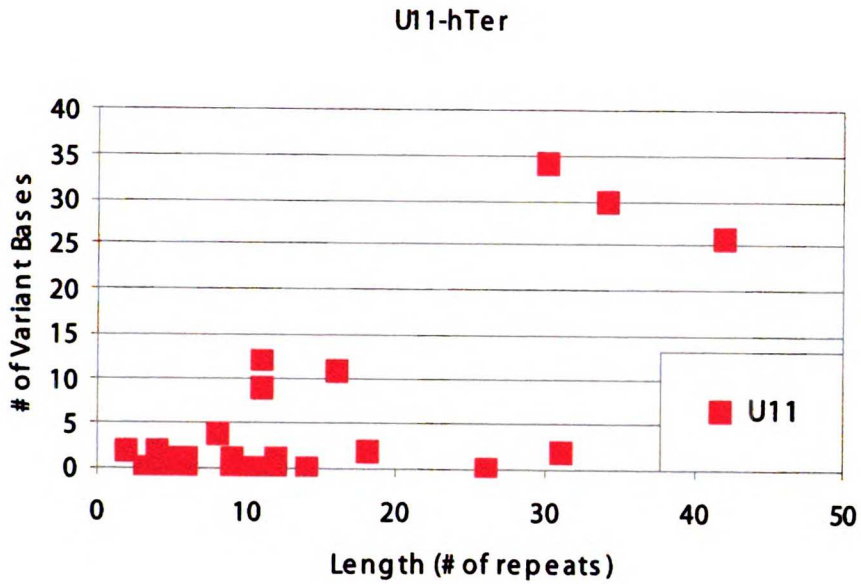
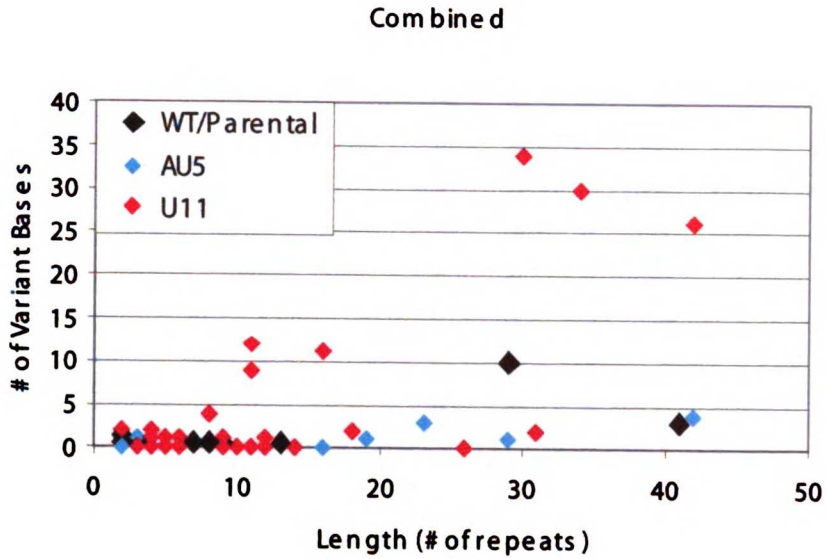


Figure 2: U11-hTer telomeres have significantly greater number of variant bases per telomere - the number of variant bases was divided by the number of telomeres cloned. WT-hTer/Parental: 1.27 +/- 0.9; AU5-hTer: 0.58 +/- 0.26; U11-hTer: 4.56 +/- 1.58.









Chapter3 - Figure3

Figure 3: Relationship between number of variant bases and telomere lengths -

For WT-hTER/parental (black) and AU5-hTer (blue), there are only a few variations per telomere, irrespective of length. But for U11-hTer (red), there are many more variations per telomere, and the longer the telomere, greater the number of variants. The differences are obvious in the combined graph.

Chapter Four

A hypomorphic telomerase mutant

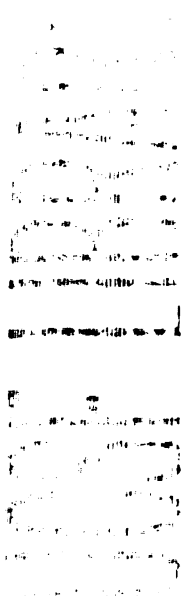
Abstract

Expression of hTERT, the telomerase reverse transcriptase, leads to indefinite lifespan extension of human primary fibroblasts (Bodnar et al., 1998) and is critical for tumorigenesis (Hahn et al., 1999b). Telomerase activation induced lifespan extension is generally accompanied by net telomere lengthening, suggesting that lifespan extension is dependent upon telomere length. Here we show that hTERT+C, a telomerase mutant with ten additional residues at the C-terminus, is a hypomorph and confers limited lifespan extension to IMR90, a human primary lung fibroblast, without net telomere lengthening. Telomeres erode to very short levels, shorter than what is normally seen at senescence or crisis. These results show that lifespan extension and net telomere lengthening can be functionally separated.

Introduction

Primary human fibroblasts have a limited proliferative capacity in culture, as first described by Hayflick (1965; 1961). Further study revealed that there are two mortality stages (reviewed in Blackburn, 1995). At Mortality Stage 1 (M1), which usually occur at about 50-80 doublings for human fibroblasts (Hayflick, 1961), cells irreversibly exit the cell cycle and senesce, becoming large and flat with a low nucleocytoplasmic ratio (Bowman et al., 1975; Sherwood et al., 1988). Expression of oncogenes such as simian virus 40 (SV40) large T antigen or loss of tumor suppressors p53 and pRb can bypass M1, and add about 20-40 more doublings. At this point, cells enter Mortality Stage 2 (M2), where they undergo crisis and die (Hara et al., 1991; Shay et al., 1991). Fibroblasts spontaneously survive M2 and become immortal at a frequency of 10^{-7} (Shay and Wright, 1989).

The telomere hypothesis of aging states that the inability to replenish telomeres leads to their erosion, and at some minimum threshold length, causes cessation of cellular division. Thus, telomere maintenance is required for continual cellular division. This is supported by experiments that overexpress telomerase in fibroblasts, which maintains telomeres, and bypasses M1, extending lifespan indefinitely (Bodnar et al., 1998). Bypassing M1 through any means without activating telomerase (through inactivation of p53 and pRb tumor suppressor pathways) leads to telomere shortening to critical lengths. As the telomeres become critically short, there is a dramatic increase in genomic instability as evidenced by an increase in end to end chromosomal fusions (Zhu et al.,



1999). Fibroblasts that survive crisis are considered immortal and are able to maintain telomeres through telomerase activation or recombination.

There are two competing models explaining the role of telomeres in determining cellular lifespan. One is that as telomeres shorten, silencing of a lifespan regulatory loci in subtelomeric regions may become hampered. And luciferase reporter constructs in subtelomeric regions are more inhibited when telomerase is overexpressed and telomeres are lengthened (Baur et al., 2001). An alternative model suggests that telomere shortening may lead to unraveling of a telomeric tertiary structure (uncapping), which then may be sensed as DNA damage and activate the DNA damage pathway, causing cell cycle arrest or apoptosis. Perturbation of telomeres by dominant negative TRF2, a telomere binding protein, causes activation of ATM and p53 dependent apoptosis, showing that the DNA damage sensing pathway is involved in detecting telomere dysregulation (Karlseder et al., 1999). And a recent model goes one step further in suggesting that telomeric capping is dependent upon multiple overlapping factors: "(1) a higher order DNA protein complex structure along the entire telomeric tract, which is dependent upon telomere length," (2) active telomerase at the telomere, and (3) terminal repeat complexes (both single and double stranded) (reviewed in Blackburn, 2001). Their overlapping functions can compensate for the reduction of one of the factors. For instance, in the presence of very long telomeres, active telomerase is not required for continued proliferation. And in the presence of active telomerase, telomeres shorter than normally tolerated do not cause senescence or crisis (Ouellette et al., 2000; Yang et al., 1999; Zhu et al., 1999).

In Zhu et al (1999), the "wildtype" telomerase was not a true functional wildtype, in that in the presence of telomerase activity and lifespan extension, telomeres did not lengthen, but instead became shorter. Upon characterization of the "wildtype" telomerase from Zhu et al, it was discovered that an additional ten residues were inadvertently added to the c-terminus. We present evidence that this c-terminal extension mutant (hTERT+C) is a hypomorph in that it can extend the lifespan of fibroblasts, which is a marker of *in vivo* telomerase activity, but allows telomere to erode to very short levels.

Methods

Plasmid Construction

hTERT+C contains an unintentional frameshift mutation at the stop codon, which results in the addition of 10 amino acids at the most C-terminus of hTERT. The sequence of the extra amino acids is as follows: LSRPCGMCVS(end). hTERT and hTERT+C were expressed from pBabe-puro.

Cell culture

IMR90 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Culturing conditions: minimal Eagle's media with Earl's BSS, 10% FBS, 0.1mM nonessential amino acids, 10mM Na pyruvate. Retroviral infections were carried out by transiently transfecting pBabe puro based plasmids into phoenix amphotropic cells (Fujita et al., 1992). The supernatant was collected 48hours after transfection and centrifuged

1000RPM for 5 minutes. IMR90 cells were incubated with the supernatant and DEAE-dextran (20μg/ml) for 24 hours. Cells were selected with puromycin at 0.5μg/ml for one week and G418 at 800μg/ml for two weeks. Cells were passaged at 90% - 100% confluency and the dilution factor was followed. A dilution factor of 1:4 increased the cumulative dilution by 2, dilution of 1:6 by 2.5, dilution of 1:8 by 3, dilution of 1:12 by 3.5, and dilution of 1:16 by 4.

Telomere length

Genomic DNA was isolated using the GenomicPrep Cells and Tissue DNA Isolation Kit from Amersham Biosciences. Products were analyzed on 1% agarose gel to verify intactness of the genomic DNA. 1ug of genomic DNA was digested with RsaI and HinfI and then electrophoresed on 0.65% agarose gel. DNA was transferred to a nylon membrane and hybridized by a ^{32}P-labeled telomeric probe (CCCTAA) (Counter et al., 1992). Membrane was exposed to a phosphorimager and the signal quantitated using Imagequant software. The signal intensity was plotted and the area under the curve calculated. The point on the curve where 50% of the area was on one side and 50% on the other was the median telomere length.

Results

hTERT+C: ten additional residues at the C-terminus

In Zhu et al (1999), expression of "wildtype" hTERT in post-M1 fibroblasts resulted in robust telomerase activity and lifespan extension beyond M2. However, these effects

were accompanied by continual telomere erosion, the exact opposite of what was expected. The "wildtype" hTERT used in these experments was constructed by removing the HA tag from the C-terminus of hTERT-HA. And during removal of the tag, an extra nucleotide was inadvertently added to the stop codon, resulting in a frameshift mutation, which added ten extra residues (LSRPCGMCVS) to the C-terminus. Thus, the "wildtype" hTERT was not a true wildtype, but instead a C-terminal extension mutant (hTERT+C).

hTERT+C confers limited lifespan extension to fibroblasts

IMR90, a primary human lung fibroblast, divides approximately 60-70 times before M1 or senescence (Kim & Blackburn, unpublished results). IMR90 cells were infected with retrovirus containing hTERT+C or pBabe-puro vector control at cumulative dilution (CD) 32. Cumulative dilution is a rough approximation of population doublings and is calculated from the dilution factor of each passage. At early passages, CD and PD are similar, but with each additional passage, the difference becomes greater. After drug selection with puromycin, the entire population of cells was continuously passaged serially. The untransfected (IMR90) and the vector control senesced at CD 65-70 (Fig. 1); they appeared large and flat with a low nucleocytoplasmic ratio (data not shown). The population of cells transfected with hTERT+C (hTERT+C-1), however, continued to proliferate well beyond both predicted M1 and M2 mortality stages (Fig. 1). Interestingly, at CD 115, most of the plate was populated with senescent cells with a few areas of rapidly growing cells, which suggests that most of the cells were senescing at this point, but a few cells were able to escape senescence and overgrow the population.

The growth curve shows a slight slowing at this point before accelerating once again. Even so, the entire population eventually senesced at about CD 150. Thus, hTERT+C confers lifespan extension of fibroblasts, but does not immortalize.

hTERT+C is unable to increase net telomere length *in vivo*

Exogenous telomerase activation in human primary cells generally leads to net telomere lengthening (Bodnar et al., 1998, Kiyono, 1998 #25). In contrast, hTERT+C, which can extend the lifespan of fibroblasts and is active *in vitro* (Zhu et al., 1999), cannot elongate net telomere lengths. Untransfected IMR90's net telomere length shortened from about 9-11kb at CD40, to about 5-6kb at CD65 (Fig. 2a), at which point, they senesced. Initially, telomeres of hTERT+C transfected IMR90 behaved similarly and shortened at the same rate (Fig. 2a, 2b). But then, as the cells continued to divide past M1, these telomeres continued to erode, well below lengths normally seen at M1, and finally stabilized at very short lengths (Fig. 2a). The telomere shortening rate gradually decreased as the telomeres shortened, suggesting that hTERT+C was able to slow the rate of telomere shortening as telomeres became shorter and shorter. Despite this, hTERT+C was unable to maintain net telomere lengths above 2-3kb.

hTERT immortalized fibroblasts grow faster and maintain longer telomeres than hTERT+C cells

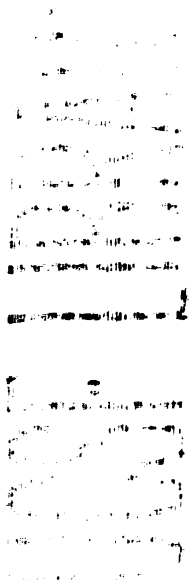
A direct comparison between hTERT+C and hTERT transfected cells revealed that the extension telomerase mutant cells grow slower than the wildtype immortalized cells. One plate of cells was transfected in parallel with hTERT or hTERT+C at CD32 and long

term growth rates monitored by cumulative dilutions. Initially, they were indistinguishable with similar morphology and growth rates. However, starting at about day 100, the hTERT+C cells' growth rates gradually slowed with eventual cessation (Fig. 3a). hTERT transfected cells showed no sign of slowing down and continued to grow at a constant rate. By day 350, hTERT+C transfected cells were large and flat, characteristic of senescent cells, but hTERT transfected cells were similar in appearance to pre-M1 fibroblasts (data not shown). Even at an early time point, hTERT+C cells grew slower than hTERT cells, suggesting that hTERT+C is not as effective as hTERT in promoting cellular growth. Additionally, hTERT was able to significantly lengthen telomeres, while hTERT+C was again unable to maintain telomere lengths (Fig. 3b). The long term culturing experiments of hTERT+C was performed twice, approximately a year apart. Culturing of hTERT+C-1 was started about a year before hTERT+C-2 and hTERT and the latter two cultures were started and passaged in parallel. The dilution ratio at each passage of hTERT+C-1 was consistently less than the ratio of hTERT+C-2, so it is difficult to compare the growth rates of hTERT+C-1 to hTERT+C-2. Despite this, both experiments independently revealed that hTERT+C confers a limited lifespan extension and cannot maintain telomeres above 2-3kb.

.

<u>E6, E7, and SV40 Large T antigen, together with hTERT+C, can immortalize fibroblasts</u>

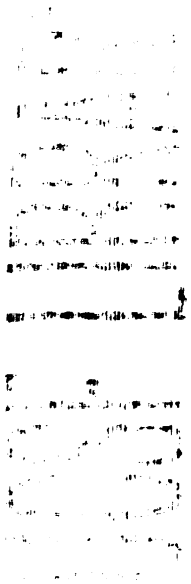
The oncoproteins E6, E7, and SV40 Large T antigen (LT) can extend the lifespan of primary fibroblasts beyond M1 (Shay et al., 1991). This is believed to be through their ability to abrogate the tumor suppressors p53 (E6) or pRb (E7) or both p53 and pRb (LT). However, E6 or E7 alone adds only about 5-10 doublings and LT transfected cells can



divide an additional 20 times (Fig. 4) before M2. So these oncoproteins can extend lifespan, but not immortalize. Similarly, hTERT+C alone can extend lifespan, but not immortalize. When hTERT+C is coexpressed with one of the oncoproteins, E6, E7 or LT, fibroblasts are immortalized (Fig. 4). However, similar to the results above, hTERT+C plus an oncoprotein grew slower than hTERT plus the equivalent oncoprotein transfected cells. In addition, telomeres from cells transfected with hTERT+C and an oncoprotein shortened to about 2-3kb before stabilizing (Fig. 5). And as expected, telomeres from cells transfected with hTERT and an oncoprotein become longer (Fig. 5). These results again show that hTERT+C is able to maintain telomeres only when they become very short. In essence, coexpression of E6, E7, or SV40 large T antigen with hTERT+C is sufficient to immortalize human primary fibroblasts.

Discussion

We have presented evidence that the c-terminal extension mutant (hTERT+C) is hypomorphic, in that it is able to extend the lifespan of human primary fibroblasts, but unable to maintain telomere length. hTERT-HA is catalytically active *in vitro*, but is unable to extend lifespan (Counter et al., 1998; Zhu et al., 1999). hTERT+C contains ten additional residues at the C-terminus and HA tag contains nine residues (Wilson et al., 1984); they both have almost identical molecular weights. It is intriguing that two very similar peptides at the C-terminus of hTERT can have such different effects. An explanation may involve telomere access. In budding yeast, enhancing telomere access by directly tethering the reverse transcriptase to CDC13, a telomere binding protein,



elongates telomeres and obviates the need for the telomerase recruitment factor Est1 (Evans and Lundblad, 1999). Similarly, hTERT-HA may not be recruited to the telomere, while hTERT+C may be recruited in a limited fashion. Thus, hTERT+C may provide limited functional telomerase activity at the telomere, making it functionally hypomorphic. And limiting amounts of telomerase activity can lead to telomere shortening to subsenescent lengths (Ouellette et al., 2000). Additionally, our results show that the C-terminus of telomerase may be important in the recruitment of telomerase to the telomere.

Overexpresison of hTERT+C extends the lifespan of fibroblasts, but does not immortalize. Telomeres are not maintained, but shorten well beyond lengths normally found in M1 and M2, stabilizing at 2-3kb, at which point the cells senesce. The rate of shortening decreases as the net telomere length shortens. These results show that hTERT+C preferentially acts on short telomeres. And in the presence of an oncoprotein, telomeres are indefinitely maintained at 2-3kb, further supporting that hTERT+C can actively synthesize telomeres on the short telomeres. It has been proposed that limiting amounts of telomerase is preferentially recruited to the shortest telomeres (Sprung et al., 1999); and long telomeres do shorten while short telomeres remain stable in low levels of telomerase activity (Ouellette et al., 2000). Our results provide further evidence that shorter telomeres may be more accessible to telomerase action.

An alternative hypothesis may be that as telomeres shorten, there is an increase in the likelihood of T-loops unraveling, making telomeres more telomerase accessible. These

"naked" or "uncapped" telomeres may need to recruit telomerase in order to avoid being recognized as DNA damage, causing p53 dependent cell cycle arrest or apoptosis. hTERT+C may be inefficient in being recruited to the uncapped telomeres, leading to a portion of the population being lost, which may explain the slower growth rates of hTERT+C transfected cells. This also suggests that the presence of active telomerase at an uncapped telomere may protect it from being recognized as DNA damage. Coexpression of hTERT+C with any one of the oncoproteins E6, E7, or SV40 large T-antigen immortalizes cells in the presence of shortening telomeres, even though hTERT+C alone immortalize. This result suggests that once telomeres shorten to about 2-3kb, telomerase may not be able to cap telomeres. Removal of a growth inhibitory signal or addition of a growth promoting signal may be sufficient in blocking the uncapped telomere signal, allowing immortalization of cells even at such short lengths.

We have shown that hTERT+C is a hypomorphic mutant of hTERT. It can provide limited lifespan extension to fibroblasts, while maintaining stably short telomeres. These results also show that lifespan extension and net telomere lengthening can be functionally separated. Thus, the quantity of telomerase activity is important in the regulation of lifespan extension, which can be separated from its regulation of net telomere length. It also suggests that telomere capping, not absolute telomere length, is the most important factor in promoting growth. Better understanding of chromosomal end protection can only add to our understanding of ageing and cancer development.

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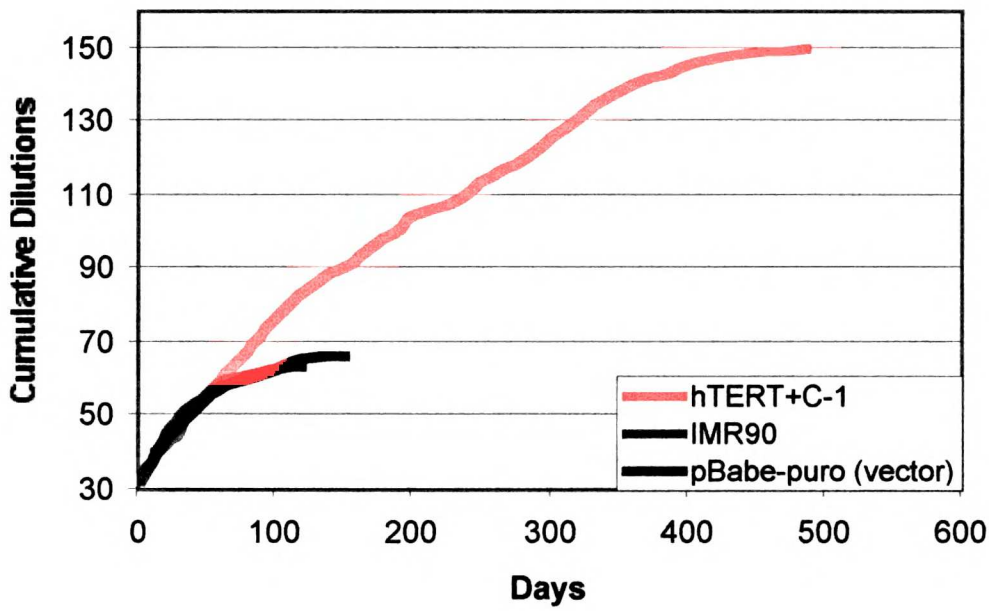


Figure 1: hTERT+C confers limited lifespan extension to IMR90 - untransfected control (blue) and vector control (black) divides approximately 65-70 times before M1. M2 is predicted to be at CD 90-100. hTERT+C (red) undergoes about 150 cumulative dilutions before senescence.

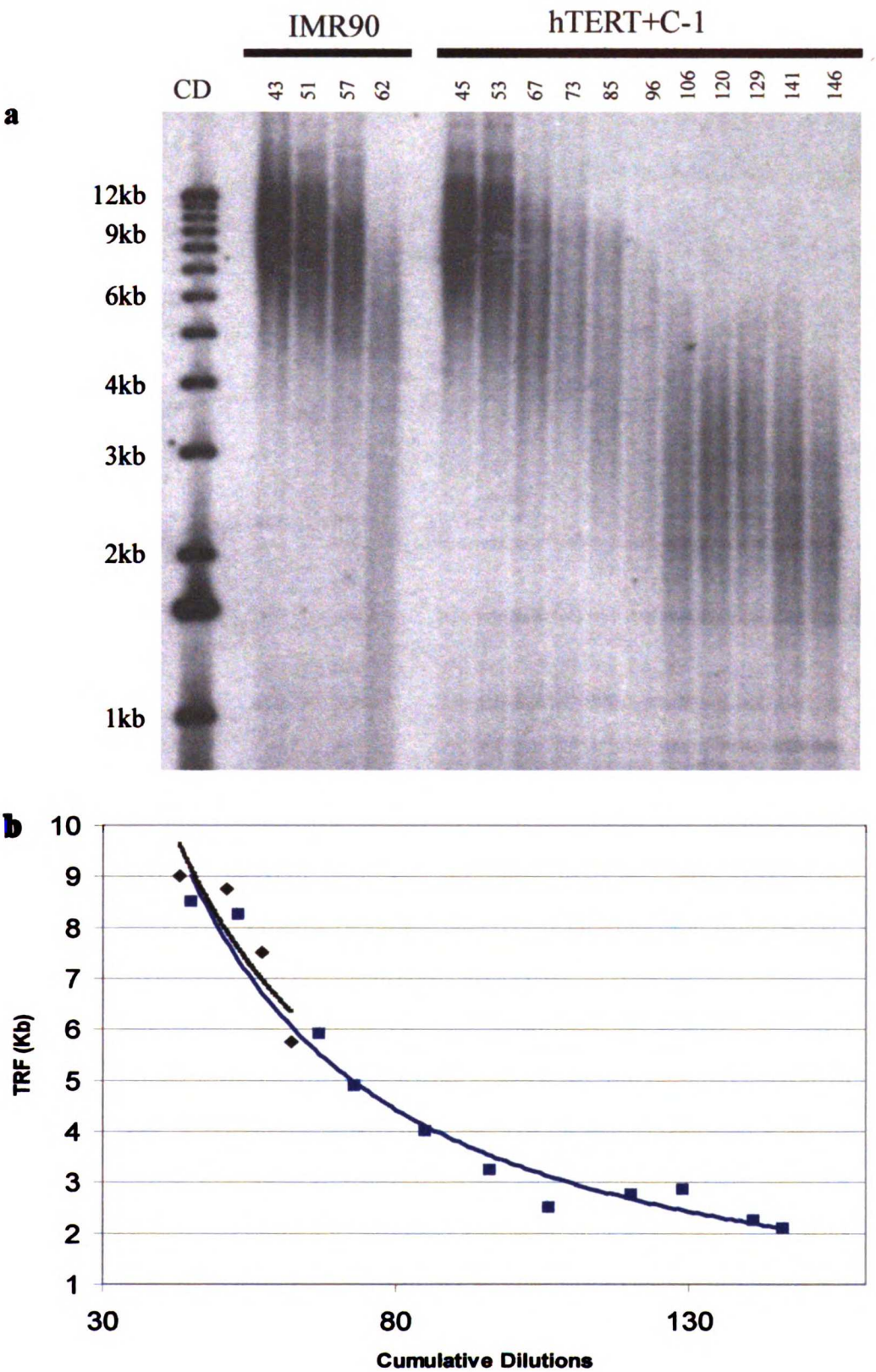
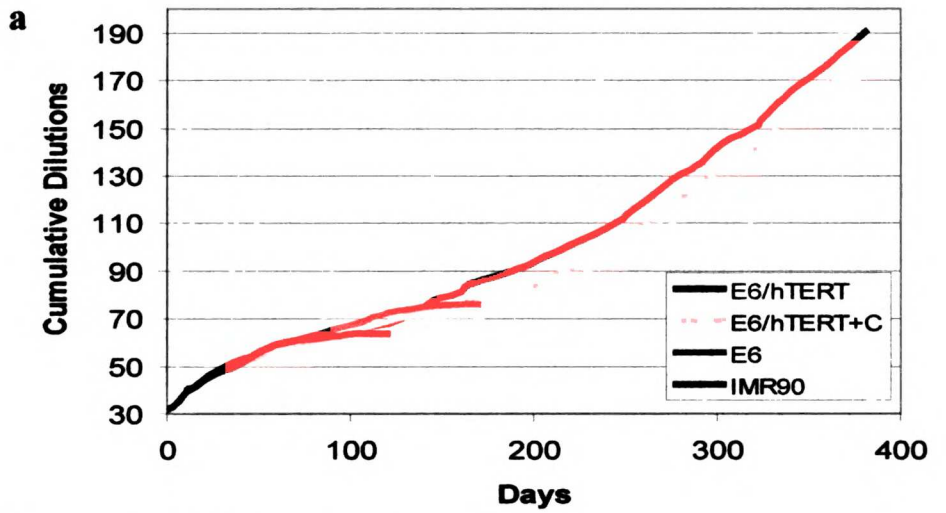
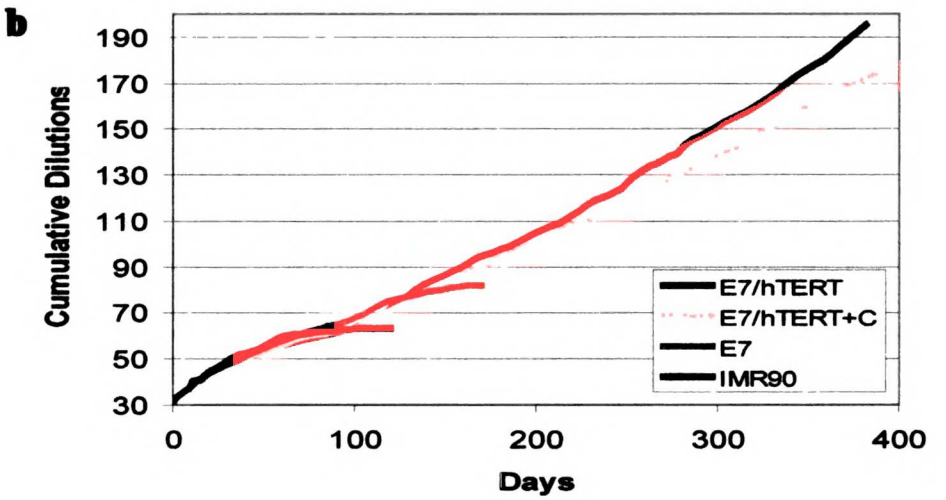

a
IMR90
hTERT+C-1
CD
43
51
57
62
45
53
67
73
85
96
106
120
129
141
146
12kb
9kb
6kb
4kb
3kb
2kb
1kb
b
TRF (Kb)
10
9
8
7
6
5
4
3
2
1
30
80
130
Cumulative Dilutions

Figure 2: Telomeres shorten in the presence of hTERT+C - Telomere lengths were analyzed at the indicated cumulative dilutions (CD). a) IMR90 untransfected control cells shortened their telomeres to 6-7kb before senescing. hTERT+C allowed telomeres to shorten to 2-3kb before stabilizing. b) The rate of telomere erosion is similar for both IMR90 (black) and hTERT+C (blue). The rate of telomere shortening decreases as telomeres shorten.

a
Cumulative Dilutions
130
110
90
70
50
30
0
100
200
300
400
500
Days
hTERT
hTERT+C-2
IMR90
b
CD
hTERT+C-2
53
67
88
113
hTERT
53
67
83
102
124
12kb
9kb
6kb
4kb
3kb
2kb
1kb

Figure 3: hTERT immortalizes IMR90 and lengthens telomeres - a) hTERT+C (red) transfected cells grow slower than hTERT (black) cells starting from about day 100. hTERT+C does not immortalize IMR90. b) Again, telomeres shorten in the presence of hTERT+C. hTERT lengthens net telomere length.


a
Cumulative Dilutions
190
170
150
130
110
90
70
50
30
0
100
200
300
400
Days
E6/hTERT
E6/hTERT+C
E6
IMR90


b
Cumulative Dilutions
190
170
150
130
110
90
70
50
30
0
100
200
300
400
Days
E7/hTERT
E7/hTERT+C
E7
IMR90

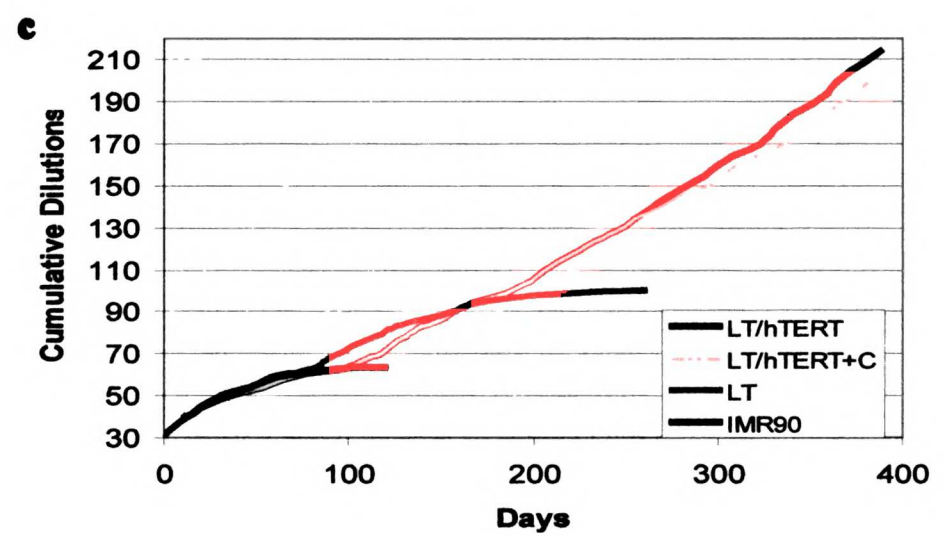

c
Cumulative Dilutions
210
190
170
150
130
110
90
70
50
30
0
100
200
300
400
Days
LT/hTERT
LT/hTERT+C
LT
IMR90

Figure 4: Coexpression of hTERT+C with E6, E7, and SV40 large T-antigen immortalizes IMR90 - a) E6/hTERT cells grow slightly faster than E6/hTERT+C cells. b) E7/hTERT cells grow slightly faster than E7/hTERT+C cells. c) SV40 large T-antigen/hTERT cells grow slightly faster than LT/hTERT+C cells. Co-expressing one of these oncogenes with either form of hTERT immoralizes IMR90.

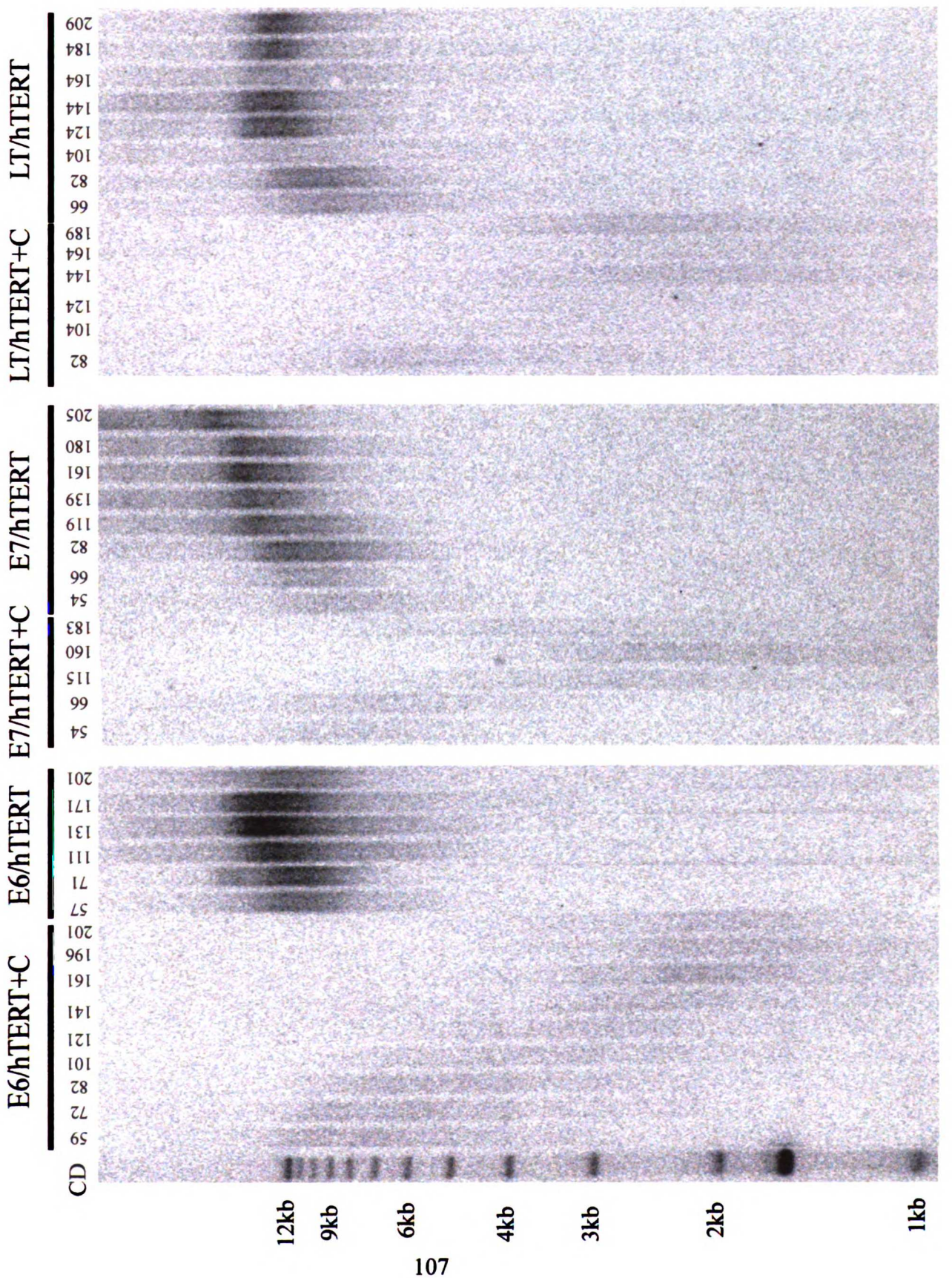

LT/hTERT
209
184
164
144
124
104
82
66
LT/hTERT+C
189
164
144
124
104
82
E7/hTERT
205
180
161
139
119
82
66
54
E7/hTERT+C
183
160
115
66
54
E6/hTERT
201
171
131
111
71
57
E6/hTERT+C
201
196
161
141
121
101
82
72
59
CD
12kb
9kb
6kb
4kb
3kb
2kb
1kb

Figure 5: Telomere length analysis of immortalized IMR90 cells - E6/hTERT+C, E7/hTERT+C, and SV40 large T-antigen (LT)/hTERT+C immortalized IMR90 continues to have telomere erosion. Conversely, co-expression of hTERT with the oncoproteins lengthens telomeres.

Chapter Five

Conclusions and future directions

When we began our work on mutant template human telomerase RNA (MT-hTer), much work had already been done in *T. thermophila* and *S. cerevisiae*. They had shown that gross overexpression of mutant templates in those organisms had devastating effects on cellular viability and chromosomal maintenance by creating so called "toxic telomeres" (Gilley et al., 1995; Kirk et al., 1997; Krauskopf and Blackburn, 1996; McEachern and Blackburn, 1995). These "toxic telomeres" contained the corresponding sequences of the mutant templates and they were thought to work by "uncapping" telomeres. And it was seen that in one instance, pRap1, a telomeric binding protein in budding yeast, was unable to bind a "toxic telomere" (Krauskopf and Blackburn, 1996). Also at that time, work on human telomere binding proteins was advancing rapidly. TRF2, a human telomere binding protein, was shown to be important in telomere capping. It facilitated the formation of T-loops (Griffith et al., 1999), which was predicted to be the protective structure for human telomeres. And overexpression of a dominant negative mutant of TRF2 quickly induced telomeric fusions (van Steensel et al., 1998) and ATM and p53 dependent apoptosis (Karlseder et al., 1999). So, we predicted that expression of MT-hTer would cause incorporation of the corresponding repeats, as in the single cell organisms, and cause disruption of telomere binding proteins such as TRF2, thereby uncapping the telomeres.

What we soon discovered was that unlike the situation in the single cell organisms, overexpression of the telomerase RNA was inherently difficult to achieve. We observed that a large amount of poly-adenylated MT-hTer was piling up without being processed to its final form. Fortuitously, even though overexpression was never achieved, low

levels of the final processed species was sufficient to cause G1 cell cycle arrest and apoptosis in LNCaP, a prostate cancer derived cell line, and MCF-7, a breast cancer derived cell line. (We chose these two cell lines because they were cancer derived and we were interested in using MT-hTer as an anti-tumor agent; and these cell lines had high levels of endogenous telomerase activity.) We learned that human cells were very sensitive to telomere perturbation since very low levels of MT-hTer was sufficient to produce a phenotype. And we predicted that as few as one uncapped telomere per cell would be sufficient to cause activation of the DNA damage sensing pathway.

Increasing the relative expression of MT-hTer over the endogenous hTER is the next logical step in producing a clinical useful agent. This may be attacked from several directions. First, reduce the endogenous levels of hTER, which can be achieved by antisense or ribozyme or RNAi. Second, increase the copy number of MT-hTer through the use of better vectors such as adenovirus. Third, increase the processing efficiency of MT-hTer by placing appropriate 3' processing signals on the construct. The third strategy is limited by the fact that the mechanism of hTER processing has not been completely solved.

Dudy Tzfati's development of a methodology to sequence terminal telomeres was a great boon (Tzfati et al., 2000). By modifying his protocol, I was able to clone and sequence multiple terminal telomeres from AU5 and U11-hTer expressing LNCaP. The results showed that telomeric sequence variants were dramatically increased in U11-hTer cells, but not in AU5-hTer. What was puzzling was that I was expecting to find the

corresponding mutant repeats, not variants. A quick literature search revealed that telomeric sequence variants were quite common (Allshire et al., 1989; Brown et al., 1990). However, the variants were more commonly located in the proximal regions, but in the U11-hTer telomeres, the variants were terminally located. This suggested that either the U11-hTer was decreasing telomerase's fidelity or the proximal variants were translocated to the terminal regions by an unknown mechanism. The absence of variants in AU5-hTer was not that surprising since this mutant had a more severe growth inhibition phenotype than U11-hTer, suggesting that cells that were incorporating AU5 were quickly dropping out of the population. It would be very interesting to see these experiments repeated using an optimized form of MT-hTer expression.

A previous post-doc in our lab (He Wang) had made the mistake of inserting a nucleotide into the stop codon of hTERT as he was trying to remove an HA tag from the C-terminus. He believed this was "wildtype" and proceeded to do several experiments, which were published (Zhu et al., 1999). In that paper, they observed telomere shortening in the presence of lifespan extension when they introduced this "wildtype" hTERT. I had initially tried to use hTERT to induce MT-hTer's phenotype in IMR90, primary human lung fibroblast. What I noticed was that the mutant template had minimal effects on their growth, but the telomeres continued to shorten. Lifeng Xu proceeded to sequence the "wildtype" hTERT and discovered that it contained an extra ten residues at the C-terminus. This extension mutant (hTERT+C) caused limited lifespan extension even in the absence of telomere lengthening. Instead, the telomeres became very short, to approximately 2-3kb. These results clearly demonstrated that hTERT+C was a

hypomorphic mutant of hTERT and that lifespan extension and net telomere lengthening was functionally separable. And it was another piece of evidence supporting that catalytically active telomerase helps cap telomeres.

The experiments involving hTERT+C suggested that telomerase may have an additional function other than telomere lengthening, possibly a mitogenic signalling function. High levels of hTERT cause faster growth of cells than hTERT+C overexpression. And telomerase immortalized HMECs become TGF-ß insensitive (Stampfer et al., 2001). Thus, inhibiting various parts of the TGF-B signalling cascade may provide valuable insight into telomerase's possible mitogenic function. Another interesting line of investigation would be the role of the C-terminus of hTERT in regulating telomere access. Varying the size and the amino acids of the C-terminal extension would provide insights into the mechanism behind telomerase recruitment. Telomeres and telomerase are involved in ageing and cancer. Further elucidation of the mechanisms of telomere capping, telomerase regulation, and telomerase's possible signalling function may help improve the quality of life for our ageing population.

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