AD_____

Award Number: DAMD17-01-1-0419

TITLE: Telomere Length and Genomic Stability as Indicators of Breast Cancer Risk

PRINCIPAL INVESTIGATOR: Joseph A. Baur

CONTRACTING ORGANIZATION: The University of Texas Southwestern Medical Center at Dallas
Dallas, Texas 75390-9105

REPORT DATE: May 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Annual Summary (1 May 02 - 30 Apr 03)

4. TITLE AND SUBTITLE
Telomere Length and Genomic Stability as Indicators of Breast Cancer Risk

5. FUNDING NUMBERS
DAMD17-01-1-0419

6. AUTHOR(S)
Joseph A. Baur

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas Southwestern
Medical Center at Dallas
Dallas, Texas 75390-9105
E-Mail: Joseph.Baur@UTSouthwestern.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Telomeres are repetitive sequences that protect the ends of linear chromosomes and shorten during each cell division. Very short telomeres have been associated with changes in gene expression (in yeast) and decreased genomic stability. In the first year we published the first proof that silencing effects can occur at human telomeres. In the second year we have shown that expression of telomeric genes spontaneously switches on and off. We have begun an experiment to test the effect of telomere length and time in culture on immortalization frequency and we have developed several in situ techniques to examine human subtelomeres. Only 5-10% of breast cancers are hereditary and very little is known about the factors influencing sporadic cases. Further study of gene expression near telomeres will help determine whether telomere length could play a role in the progression of breast cancer.

14. SUBJECT TERMS
telomere/telomerase, genome stability, subtelomere, gene expression, age-related

15. NUMBER OF PAGES
24

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Telomeres are repetitive sequences that protect the ends of linear chromosomes. Telomeres shorten with each cell division, eventually reaching a critically short length that causes the cell to permanently cease dividing after reaching number of divisions known as the Hayflick limit. It has been shown in our lab and others that this telomere initiated growth arrest (termed M1) is mediated through the tumor suppressors p53 and pRb. Inhibiting these proteins with SV40 T antigen or HPV E6/E7 leads to further division until a second limit (M2) is reached. This proliferation in the presence of short telomeres is associated with end to end chromosome fusions and genomic instability [1, 2]. This genomic instability occasionally results in the production of a rare clone that can continue to divide due to the activation of telomerase, an enzyme capable of adding new DNA to the telomeres. This situation may parallel the formation of cancerous cells *in vivo*, which in many cases show evidence of genomic rearrangements near telomeres and sometimes centromeres, losses of entire chromosomes, and activation of telomerase [1-4]. Dicentric chromosomes have been shown to have little or no telomeric DNA present at the junction in humans [5] and mice [1]. Note that telomere shortening is correlated with aging, and aging is one of the most important risk factors for sporadic breast cancer. Evidence for telomere length as an important regulator of the intracellular environment beyond its role as a "mitotic clock" originally came from *Drosophila* [6] and has been further characterized in yeast [7]. In Saccharomyces cerevisiae, it has been shown that a transgene integrated near a telomere will be transcriptionally silenced by an active mechanism involving SIR proteins that depends both on the length of the telomere and on the distance to the transgene [8]. More recently, an endogenous gene has been characterized that exhibits this silencing, or telomere position effect (TPE), in yeast [9]. Our work during the first year of this grant extended these observations from yeast to human cancer cells. In the second year, we have further characterized human TPE, resulting in a second publication, and we have made significant progress in our efforts to understand the molecular changes that occur in telomeric and subtelomeric regions during breast cancer progression.

Body

"Task 1" from my statement of work (months 1-12) was to establish the existence of telomere position effect (TPE) in human cancer cells through studies on the behavior of a luciferase reporter gene inserted either at a telomere or at an internal (control) site. As outlined in my previous report, this task was completed ahead of schedule and significantly extended in scope. During the second year, the involvement of hRap1 in human telomere position effect was confirmed through overexpression studies (Appendix A1). Using a fluorescent reporter integrated into human subtelomeres, I have shown that expression switches on and off, in contrast to a previous report and in good agreement with the yeast [7] and *Drosophila* [6] model systems (Appendix A2). I have also demonstrated using these cells that silencing can be relieved by treatment with 5-bromodeoxyuridine (Appendix A2).

"Task 2" from my statement of work (months 12-30) was to investigate the effect of transient telomerase expression on the frequency of immortalization of human mammary epithelial cells. We had originally planned to use a retroviral vector, followed by excision with Cre recombinase and selection of clones to achieve transient expression. However, an adenoviral vector has since become available that makes it possible to transiently express telomerase in a population of cells without additional complications since this virus does not integrate into the genome and consequently does not need to be excised. The efficacy of adenoviral infection of these cells was shown using a green fluorescent protein (GFP) vector (Appendix A3). Telomerase expression after adenoviral infection was assessed using the telomerase repeat amplification protocol (TRAP) assay and shown to be comparable to the level exhibited by the human tumor cell line H1299 (Appendix A4). Unfortunately, the population of human mammary epithelial cells that had been infected with hTERT (the telomerase catalytic subunit) underwent a gradual morphology change and eventually growth arrested during subsequent passaging. This type of effect has been observed previously when using high doses of adenoviral vectors and was not observed in cells infected with a control virus, leading us to suspect that an error was made by the supplier in calculating viral titer. We have now received a fresh supply of hTERT adenovirus and repeated the infection of human mammary epithelial cells. At present, the cells appear to be doing well and we are optimistic that it will be possible to passage these cells until senescence. This task should be completed on, or nearly on schedule despite a major setback.

"Task 3" from my statement of work (months 18-36) was to investigate the features of malignant and pre-malignant breast cancer cells by fluorescence in situ hybridization. A quantitative protocol has been developed for *in situ* hybridization to telomere repeats (Appendix A5). Work on characterizing sub-telomeric genes known to be involved in breast cancer, including hTERT (Appendix A6) has begun. The breast cancer-related regulator of TP53 (BCRP) is mentioned in the original description of this task. It has not been possible to pursue this gene because it has not, as anticipated, been positively identified. Although its behavior has been well-documented and it is known to reside in chromosome band 17p13.3 [10], a large number of open reading frames remain candidates for this gene at present. In addition to the *in situ* studies, we are also in the process of examining the state (presence, absence, or amplification) of subtelomeric genes using a 70-mer array. By this method, we will be able to generate data on a much larger sample of subtelomeric genes than originally anticipated. This task is proceeding on schedule.

Key Research Accomplishments

Extension of Statement Task 1:

- Involvement of hRap1 in human telomere position effect, previously suggested by RNAi, was confirmed by overexpression.
- Telomeric clones bearing a telomeric reporter were fully characterized and it was demonstrated that expression switches on and off (as opposed to low uniform expression) at telomeres.
- 5-bromodeoxyuridine was found to relieve silencing of both telomeric and internal clones.

Original Statment Task 2. Show that elongation of telomeres through transient expression of telomerase leads to a reduction in the frequency of immortalization of human mammary epithelial cells (months 12-30):

- Adenoviral vectors were obtained and shown, using a GFP control vector, to efficiently infect human mammary epithelial cells (HMEs).
- Infection of HMEs with adenoviral hTERT was shown to effectively reconstitute telomerase activity.
- After one unsuccessful experiment, HMEs have now been infected with either hTERT or a control vector and are being passaged in order to determine immortalization frequency.

Original Statment Task 3. Investigate the features of malignant and pre-malignant breast cancer cells by fluorescence *in situ* hybridization (months 18-30):

- A quantitative protocol has been developed for *in situ* hybridization to telomere repeats.
- A protocol for analysis of the hTERT gene by *in situ* hybridization has been developed and used on immortal and pre-immortal human mammary epithelial cells.
- A 70-mer array is in development for the comprehensive analysis of subtelomeric gene status.

Reportable Outcomes

Publications

Baur, J.A., Shay, J.W., and W.E. Wright. Spontaneous reactivation of a silent telomeric transgene. *In submission.*

Abstracts

Baur, J.A., Zou, Y., Li, B., de Lange, T., Shay, J.W., Wright, W.E. Characterization of telomere position effect in human cells. Presented at the Molecular Genetics of Aging meeting, Cold Spring Harbor, October, 2002.

Baur, J.A., Zou, Y., Li, B., de Lange, T., Shay, J.W., Wright, W.E. Characterization of telomere position effect in human cells. Presented at the UT Southwestern GSO Poster Session. November, 2002.

Baur, J.A., Zou, Y., Li, B., de Lange, T., Shay, J.W., Wright, W.E. Characterization of telomere position effect in human cells. Presented at The Role of Telomeres and Telomerase in Cancer, San Francisco, December, 2002.

Reagents Under Developement

A 70-mer array containing most of the sub-telomeric genes in the human genome is being developed. This array includes many genes obtained through a collaboration that are not currently in the public genome assembly. This array should prove a valuable tool in the field for assessing the role of subtelomeric rearrangements and misregulation in breast cancer.

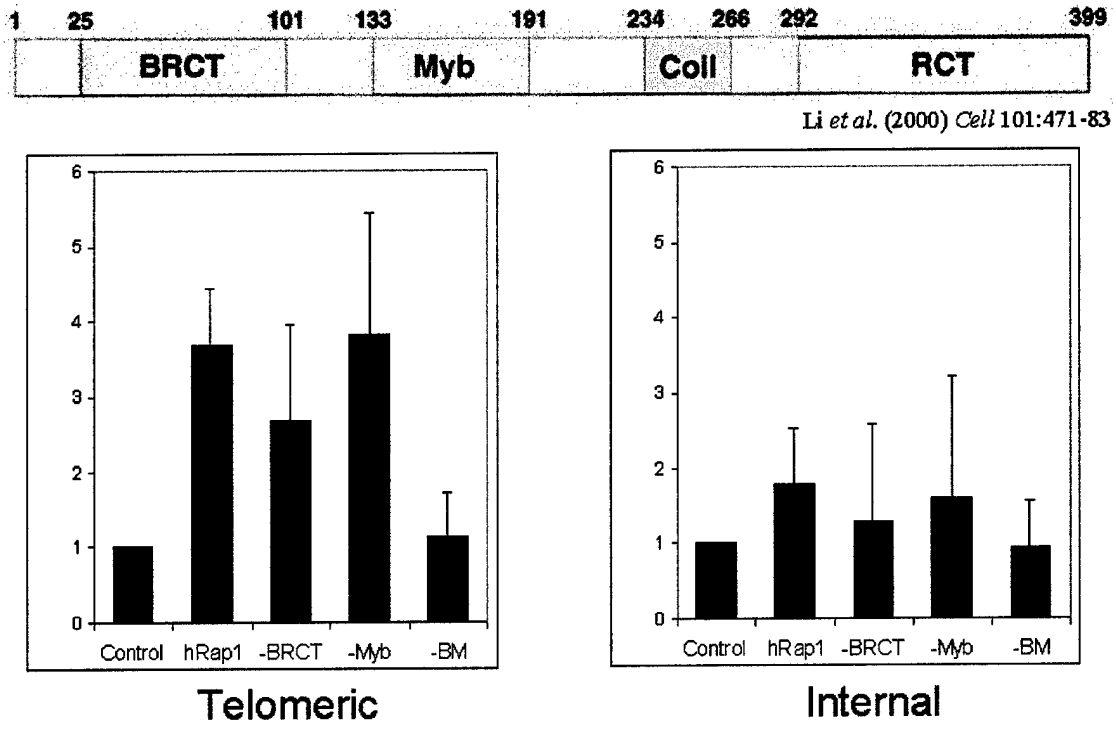
Conclusions

My work during this second year, including experiments outlined for all three original tasks, has resulted in the submission of a second manuscript on human telomere position effect and the development of several new techniques. In addition, I was able to present the work at several meetings and we are in the process of creating a 70-mer array that will greatly accelerate the analysis of sub-telomeric gene expression. The regulation of subtelomeric genes by telomere length could have extremely important implications for breast cancer. Altered expression of subtelomeric genes could indicate the presence of cells with extremely short telomeres that could be expected to be at an increased risk of genomic rearrangements. Increased expression of subtelomeric genes may also have a more active role in the progression of breast cancer. The *in situ* hybridization techniques being developed will allow detailed characterization of telomere lengths and specific gene rearrangements that will complement the expression data to create a detailed picture of the behavior of telomeres in breast cancer

References

1. Hande, M.P., Samper, E., Lansdorp, P., and Blasco, M.A. (1999) Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice. J Cell Biol *144*, 589-601.
2. Blasco, M.A., Lee, H.W., Hande, M.P., Samper, E., Lansdorp, P.M., DePinho, R.A., and Greider, C.W. (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell *91*, 25-34.
3. Sawyer, J.R., Husain, M., Pravdenkova, S., Krisht, A., and Al-Mefty, O. (2000) A role for telomeric and centromeric instability in the progression of chromosome aberrations in meningioma patients. Cancer *88*, 440-453.
4. Zheng, M.H., Siu, P., Papadimitriou, J.M., Wood, D.J., and Murch, A.R. (1999) Telomeric fusion is a major cytogenetic aberration of giant cell tumors of bone. Pathology *31*, 373-378.
5. Wan, T.S., Martens, U.M., Poon, S.S., Tsao, S.W., Chan, L.C., and Lansdorp, P.M. (1999) Absence or low number of telomere repeats at junctions of dicentric chromosomes. Genes Chromosomes Cancer *24*, 83-86.
6. Hazelrigg, T., Levis, R., and Rubin, G.M. (1984) Transformation of white locus DNA in drosophila: dosage compensation, zeste interaction, and position effects. Cell *36*, 469-481.

7. Gottschling, D.E., Aparicio, O.M., Billington, B.L., and Zakian, V.A. (1990) Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. Cell *63*, 751-762.
8. Stavenhagen, J.B., and Zakian, V.A. (1994) Internal tracts of telomeric DNA act as silencers in Saccharomyces cerevisiae. Genes Dev *8*, 1411-1422.
9. Vega-Palas, M.A., Martin-Figueroa, E., and Florencio, F.J. (2000) Telomeric silencing of a natural subtelomeric gene. Mol Gen Genet *263*, 287-291.
10. Stack, M., Jones, D., White, G., Liscia, D.S., Venesio, T., Casey, G., Crichton, D., *et al.* (1995) Detailed mapping and loss of heterozygosity analysis suggests a suppressor locus involved in sporadic breast cancer within a distal region of chromosome band 17p13.3. Hum Mol Genet *4*, 2047-2055.



Appendix A1. Transient overexpression of hRap1 or deletion mutants relieves silencing of a telomeric luciferase reporter. Cells were either mock-treated or transfected with an overexpression construct containing hRap1 or a deletion mutant lacking the BRCT domain, the Myb domain, or both (-BM). Cells were collected 48h after transfection. Although the effect is not completely specific, there is a preferential relief of silencing in the telomeric clone. The double deletion (-BM) initially appeared to be incapable of inducing this loss of silencing but was later found to be only marginally expressed.

Spontaneous reactivation of a silent telomeric transgene

Joseph A Baur, Jerry W Shay and Woodring E Wright

Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390-9039

Correspondence should be addressed to:

Woodring E. Wright, MD, PhD
Department of Cell Biology
UT Southwestern Medical Center
5323 Harry Hines Blvd.
Dallas, TX 75390-9039
Tel: (214) 648-2933
Fax: (214) 648-8694
Woodring.Wright@UTSouthwestern.edu

ABSTRACT

Subtelomeric reporter genes in human cells are silenced in a telomere length-dependent manner. Here we show that a subtelomeric reporter gene is expressed in only a sub-population of cells within a clone and that this heterogeneity is generated by switching between expression states. We observed frequent reversion from the silenced state back to active expression. Although this process was more prominent for subtelomeric transgenes, we also observed cases of spontaneous reversion in control clones bearing the reporter at an internal site. We additionally show that treatment of these cells with 5-bromodeoxyuridine (BrdU) results in a strong activation of the transgene. To our knowledge, this is the first direct observation that variegation in clonal populations of human cells can be generated through ongoing fluctuations in transcription, as opposed to progressive silencing or fixing of expression states at some point after the first few divisions. These data demonstrate the spontaneous activation of genes near human telomeres and imply that telomere shortening during human aging could lead to stochastic activation of subtelomeric genes.

INTRODUCTION

Silencing near telomeres, termed telomere position effect (TPE), was first observed in *Drosophila* (1) and has since been studied extensively in *Saccharomyces cerevisiae* and other lower organisms (2,3). It is characterized by the semi-stable repression of subtelomeric reporter genes in a manner that is proportional to both the length of the telomere tract (4) and the proximity of the reporter to the telomere (5). TPE was recently reported to affect an endogenous *S. cerevisiae* gene of unknown function (6). Although the involvement of telomeric silencing has been suggested in the regulation of surface antigen expression for several parasites (3), no biologically relevant role for TPE has yet been proven.

One of the hallmarks of telomere position effect in yeast is the spontaneous switching of a telomeric gene from an active to a silent state and vice versa. Our initial studies using a luciferase reporter to demonstrate silencing near human telomeres (human telomere position effect, or hTPE) did not allow analysis at the single-cell level. We and others (7) have now confirmed the existence of human TPE (hTPE) using fluorescent reporter genes in order to detect single cells. While the fraction of expressing cells varies widely in internal control clones, cell lines bearing a telomeric reporter consistently show expression in only a few percent of the cells. Such a pattern could conceivably be generated by several mechanisms, including slow progressive silencing, fixing of expression states at some point after the first few divisions (for example the chromatin structure at the integration site might take time to stabilize), or stochastic switching between expression states. While progressive silencing has been observed for transgenes in mammalian cells (8) and likely contributes here, available data on transgene expression at yeast telomeres (2) and at other variegating loci within mammalian cells (9,10) favors the third hypothesis. Switching between expression states was not detected,

however, in the only published report examining TPE in human cells at the single cell level (7), raising the possibility that variegation in these cells is generated through one of the other two mechanisms. Here we show that in HeLa cells, spontaneous reactivation of the transgene can be detected in initially negative subclones, demonstrating that heterogeneity is in fact generated through an ongoing stochastic process of switching between expression states.

RESULTS

In order to detect switching in human cells, we used a linearized plasmid containing telomere repeats (11) to construct a series of clones bearing the fluorescent reporter DsRed2 either next to a newly formed telomere, or randomly integrated into the genome (Fig. 1). While the number of cells expressing the reporter varied from 0 to nearly 100% in our internal controls (random integration), all of the telomeric clones recovered in our initial experiment expressed the dsRed2 protein in only a few percent of the cells, consistent with the findings of Koering *et al* (7). The fact that expression near human telomeres can be increased by the histone deacetylase inhibitor trichostatin A (7,12), but not by the demethylating agent 5-azacytidine (7), suggests that a methylation-independent process causes the majority of the silencing in these cells.

As demonstrated previously using a luciferase reporter (12), elongation of the telomeres in these cells leads to increased silencing specifically in telomeric clones (Fig. 2). The maximal level of expression within each clone remains unchanged while the proportion of expressing cells is reduced, indicating that the reduction in reporter expression observed across the population primarily represents a decrease in the fraction of positive cells, consistent with previous reports in human cells (8,13). After telomere elongation, reporter expression in the internal control clones appears to increase slightly, however this result was not observed in a comparable experiment in which luciferase was the reporter (12). The difference in expression between this telomeric and this internal clone can thus be explained by the combination of different fractions of expressing cells, different levels of expression per cell, and the fact that the telomeric clone is affected by telomere length.

In order to detect spontaneous reactivation of the transgene, we subcloned the progeny of a single cell, following the entire process by fluorescence microscopy. Each subclone was first observed at the 1 to 4-cell stage and was grown for approximately 20 population doublings. In 18 of 19 subclones that were initially negative, dsRed2-expressing cells were detected within 1 to 2 weeks (Fig. 3A-C), with 11 of the subclones becoming similar in appearance to the parent clone by week 3. Switching can go in both directions. Subclones that were initially weakly positive gave both strongly positive and completely negative cells within 1 to 2 weeks (Fig. 3D). Our data suggest that telomere position effect in these cells resembles the variegation observed at yeast telomeres (2) and in the cells of transgenic mice (14), where all-or-none patterns of switching predominate over gradual changes in gene expression (10,15). The fact that Koering *et al* did not observe fluctuations in expression at human telomeres likely indicates that cell type, the site of chromosome truncation, and/or the direction of transcription can influence the rate of switching.

Silencing near telomeres was previously shown to be relieved by treatment with the histone deacetylase inhibitor trichostatin A (TSA) (7,12), but this process was highly toxic to the cells. Here we show that similar results can be obtained with greatly reduced toxicity by treatment with 5-bromodeoxyuridine (BrdU). Relief of silencing for a telomeric DsRed2 reporter was observed after a 72-hour incubation in medium containing 50 μM BrdU (Fig. 4A). Silencing was also relieved in low-expressing internal controls and in cells bearing a luciferase reporter at the telomere (Fig. 4B). Enhancement of internal transgene expression in the presence of BrdU has been reported previously (16), however its mechanism of action remains poorly understood. The fact that repression of telomeric transgenes can be relieved provides additional evidence that this phenomenon is in fact a position effect, rather than the result of mutation within the gene or its promoter (e.g. due to the high frequency of genomic rearrangements in subtelomeric regions (17)).

DISCUSSION

We have shown in human cells that a clonal population derived from a cell bearing a telomeric reporter gene can acquire a variegated phenotype through a stochastic process of switching between expression states. Taken together with previous studies of transgene expression in mammalian cells (8-10,13,18), our data suggests that many repressive loci within the human genome may be similar in this respect. The fact that expression at both telomeric and internal loci is enhanced by treatment with either TSA or BrdU is also suggestive of a common silencing mechanism. At present, telomere position effect is distinguished from other repressive effects in human cells only by its dependence on telomere length and its relative strength (ten-fold lower expression on average relative to internal loci) (12). We observed 100% penetrance of variegation within the first 2 weeks of clonal growth in telomeric clones as opposed to ~50% in internal controls (our unpublished results), although at later time points many of the internal controls that were initially uniform in expression did become more heterogeneous. Some suggestive evidence has been presented that heterochromatin protein 1 (HP1) may be specifically involved in telomeric silencing (7). HP1 overexpression was previously shown to enhance variegation at centromeric loci while suppressing variegation at non-centromeric loci (19). This suggests that variegation may occur by at least two distinct mechanisms in mammalian cells despite the similar outward appearances of various mammalian position effects. It will be interesting in the future to see whether or not a specific set of proteins involved in telomeric silencing emerges.

A change in the expression of endogenous human genes regulated by telomere length remains to be demonstrated. The present results establish that if such a regulation occurs, it will likely be manifested primarily by an "all-or-none" change in expression in a small fraction of cells rather than a quantitative increase in per cell expression. It has been suggested that the high frequency of duplications and rearrangements in subtelomeric regions may facilitate "rapid adaptive evolution" (17). This hypothesis is supported by the finding that a block of subtelomeric sequence containing three olfactory receptor genes is duplicated polymorphically on at least 14 different chromosome ends in humans but is single-copy in non-human primates (20). Telomere position effect may

contribute to this process through silencing of subtelomeric genes and the production of a variegated phenotype, allowing the majority of cells to progress through potentially harmful genetic intermediates while continuously sampling the products of rearranged genes. A similar role has been proposed for prion proteins in the generation of genetic diversity (21). Collapse of the silent domains near human telomeres (i.e. loss of silencing due to telomere shortening) might be predicted to trigger inappropriate expression of a host of rearranged subtelomeric genes as part of the aging process. It is also possible that the telomere shortening accompanying cell division might provide a method for timing changes that occur over many years in long-lived organisms. It is difficult to imagine how conventional timing mechanisms (biochemical oscillations, lunar cycles of changes, changing gradients during early development) could be adapted to count decades.TPE regulated changes in gene expression produced by slow but ongoing cell turnover could provide such a mechanism. Clearly further study is required to elucidate the role (if any) of TPE in human gene regulation, aging, and disease.

MATERIALS AND METHODS

Generation of clones

The Afl III/Bfr I fragment (containing the DsRed1 protein) from pDsRed1-N1 (Clontech, Palo Alto, CA) was blunt ligated into the Sma I/Hpa I backbone from pSXneo1.6T_2AG_3 (11) such that the CMV promoter was placed at the base of the telomere repeats. Next, a blunted fragment containing an IRES (internal ribosome entry site) and the blasticidin resistance gene was ligated into the Hpa I site in the same orientation as the DsRed1 protein. When DsRed2 became available, the new coding region was inserted by exchanging the DsRed fragment defined by Sal I/Not I (this required a partial digest with Not I) and the resulting vector was designated pSXD2. The control vector lacking repeats was generated by blunt ligation after excision of the Cla I/ Sac II fragment. Vectors were linearized with Cla I/Pvu I (with repeats) or Pvu I alone (without repeats) and transfected into HeLa cells using FuGENE 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Stable clones were obtained by ring-cloning after selection in 1 μg/mL blasticidin for 1 week. Individual clones were isolated by placing a glass ring over them (sealed with vacuum grease) and transferred to separate dishes by standard trypsinization methods.

Analysis of clones

Cells were observed primarily on a Zeiss Axiovert 100M inverted microscope attached to a MacIntosh G4 computer using Openlab imaging software. Scanning was performed on a FACScan (Becton Dickinson, San Jose, CA).

Subcloning

Single cells were sorted into each well of a 96 well plate using a MoFlo high-speed cell sorter (Cytomation, Fort Collins, CO) and a FACStar Plus (Becton Dickinson, San Jose, CA). Sorting into 96-well plates was an automated feature of the cell sorters. 100 μL media was placed in each well prior to sorting and cells were selected based on size criteria only (to avoid fragments and doublets). Wells containing cells were identified by

microscopy the following day (2-4 cell stage) and cells were observed every 2-3 days thereafter for at least three weeks.

BrdU Treatment
Cells were treated with 50 μM BrdU for 2-5 days in regular media. A small decrease in growth rate was noted, however toxicity was dramatically reduced as compared to TSA treatment.

ACKNOWLEDGEMENTS

This work was supported by the Department of Defense grant BC000422 (JAB) and NIH grant AG07792 (WEW and JWS).

REFERENCES

1. Hazelrigg, T., Levis, R., and Rubin, G. M. (1984) *Cell,* **36**, 469-481.
2. Gottschling, D. E., Aparicio, O. M., Billington, B. L., and Zakian, V. A. (1990) *Cell,* **63**, 751-762.
3. Tham, W. H., and Zakian, V. A. (2002) *Oncogene,* **21**, 512-521.
4. Kyrion, G., Liu, K., Liu, C., and Lustig, A. J. (1993) *Genes Dev,* **7**, 1146-1159.
5. Renauld, H., Aparicio, O. M., Zierath, P. D., Billington, B. L., Chhablani, S. K., and Gottschling, D. E. (1993) *Genes Dev,* **7**, 1133-1145.
6. Vega-Palas, M. A., Martin-Figueroa, E., and Florencio, F. J. (2000) *Mol Gen Genet,* **263**, 287-291.
7. Koering, C. E., Pollice, A., Zibella, M. P., Bauwens, S., Puisieux, A., Brunori, M., Brun, C., Martins, L., Sabatier, L., Pulitzer, J. F., and Gilson, E. (2002) *EMBO Rep,* **3**, 1055-1061.
8. Walters, M. C., Magis, W., Fiering, S., Eidemiller, J., Scalzo, D., Groudine, M., and Martin, D. I. (1996) *Genes Dev,* **10**, 185-195.
9. Feng, Y. Q., Alami, R., and Bouhassira, E. E. (1999) *Mol Cell Biol,* **19**, 4907-4917.
10. Ronai, D., Berru, M., and Shulman, M. J. (1999) *Mol Cell Biol,* **19**, 7031-7040.
11. Hanish, J. P., Yanowitz, J. L., and de Lange, T. (1994) *Proc Natl Acad Sci U S A,* **91**, 8861-8865.
12. Baur, J. A., Zou, Y., Shay, J. W., and Wright, W. E. (2001) *Science,* **292**, 2075-2077.
13. Walters, M. C., Fiering, S., Eidemiller, J., Magis, W., Groudine, M., and Martin, D. I. (1995) *Proc Natl Acad Sci U S A,* **92**, 7125-7129.
14. Rakyan, V. K., Blewitt, M. E., Druker, R., Preis, J. I., and Whitelaw, E. (2002) *Trends Genet,* **18**, 348-351.
15. Robertson, G., Garrick, D., Wu, W., Kearns, M., Martin, D., and Whitelaw, E. (1995) *Proc Natl Acad Sci U S A,* **92**, 5371-5375.
16. Suzuki, T., Yaginuma, M., Oishi, T., Michishita, E., Ogino, H., Fujii, M., and Ayusawa, D. (2001) *Exp Cell Res,* **266**, 53-63.

17. Mefford, H. C., and Trask, B. J. (2002) *Nat Rev Genet,* **3**, 91-102.
18. Dorer, D. R. (1997) *Transgenic Res,* **6**, 3-10.
19. Festenstein, R., Sharghi-Namini, S., Fox, M., Roderick, K., Tolaini, M., Norton, T., Saveliev, A., Kioussis, D., and Singh, P. (1999) *Nat Genet,* **23**, 457-461.
20. Trask, B. J., Friedman, C., Martin-Gallardo, A., Rowen, L., Akinbami, C., Blankenship, J., Collins, C., Giorgi, D., Iadonato, S., Johnson, F., *et al.* (1998) *Hum Mol Genet,* **7**, 13-26.
21. True, H. L., and Lindquist, S. L. (2000) *Nature,* **407**, 477-483.

Figure Legends

Figure 1. Structure of the constructs used in the generation of clones. Both vectors were transfected into HeLa cells in linear form using FuGENE 6 transfection reagent (Roche, Basel, Switzerland). In the chromosome truncation vector (A), the CMV promoter was located approximately 80 nucleotides from the base of the T_2AG_3 repeats. The fluorescent protein DsRed2 was transcribed away from the telomere and an internal ribosome entry site (IRES) was used to allow expression of the blasticidin resistance gene (Blast) from the same transcript. Transcription was terminated using the SV40 polyadenylation signal (SV40 Poly A). In the vector used to generate internal (control) integrations (B), the T_2AG_3 repeats were replaced with an additional 1 kb of plasmid sequences.

Figure 2. Elongation of telomeres by hTERT overexpression decreases expression of DsRed2 only in telomeric clones. Clones were infected with an empty vector (pBabe) or a retrovirus encoding the telomerase catalytic component (hTERT). (A) FACS analysis of a cell line bearing a telomeric DsRed2 reporter with short telomeres (red outline) or long telomeres (solid blue histogram). (B) Bright field and fluorescent images for the cells analyzed in panel A. (C) FACS analysis of a cell line bearing an internal DsRed2 reporter with short telomeres (red outline) or long telomeres (solid blue histogram). The slight increase in DsRed2 expression upon overexpression of telomerase was not expected and was not observed previously with a luciferase reporter (12). (D) Bright field and fluorescent images for the cells analyzed in panel C.

Figure 3. Spontaneous reversal of silencing in cells bearing a telomeric reporter gene. Each row represents a different subclone derived from a single parental clone in which the gene for DsRed2 fluorescent protein had been placed next to a newly formed telomere. Subclones were initially negative (A-C) or very weak (D) for DsRed2 expression but by 3 weeks after subcloning most had developed sporadic expression in a small fraction of cells, resembling the pattern of expression in the parental cell line. Arrows indicate the positions of cells in the original bright field images. Arrowheads indicate weakly positive cells in the fluorescent image of subclone D.

Figure 4. Loss of telomeric silencing in the presence of 5-bromodeoxyuridine (BrdU). Cells bearing a telomeric reporter were grown in regular media (control) or media supplemented with 50 μg/mL BrdU for 72 hours before analysis. (A) Expression of DsRed2 was dramatically upregulated in the presence of BrdU and toxicity was minimal as compared to that observed when the cells were treated with trichostatin A (TSA). As with TSA (12), BrdU also affected silencing in low-expressing internal control clones (data not shown). (B) Silencing of a luciferase reporter was relieved after BrdU treatment in both telomeric and internal clones. Representative data is presented showing that expression after treatment is higher and more uniform between clones. Occasional resistant clones were noted among the internal controls (middle) and may represent cases in which methylation or rearrangement of the gene have occurred.

Figure 1

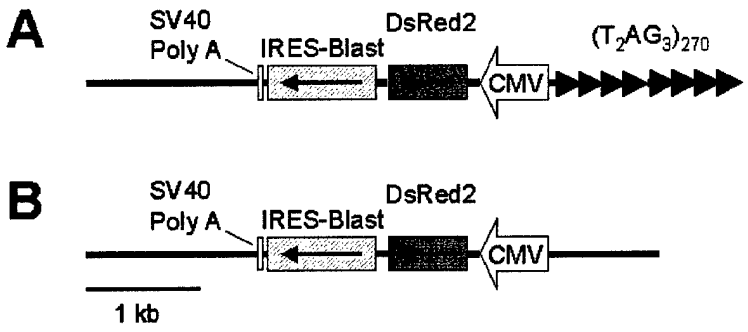


Figure 2

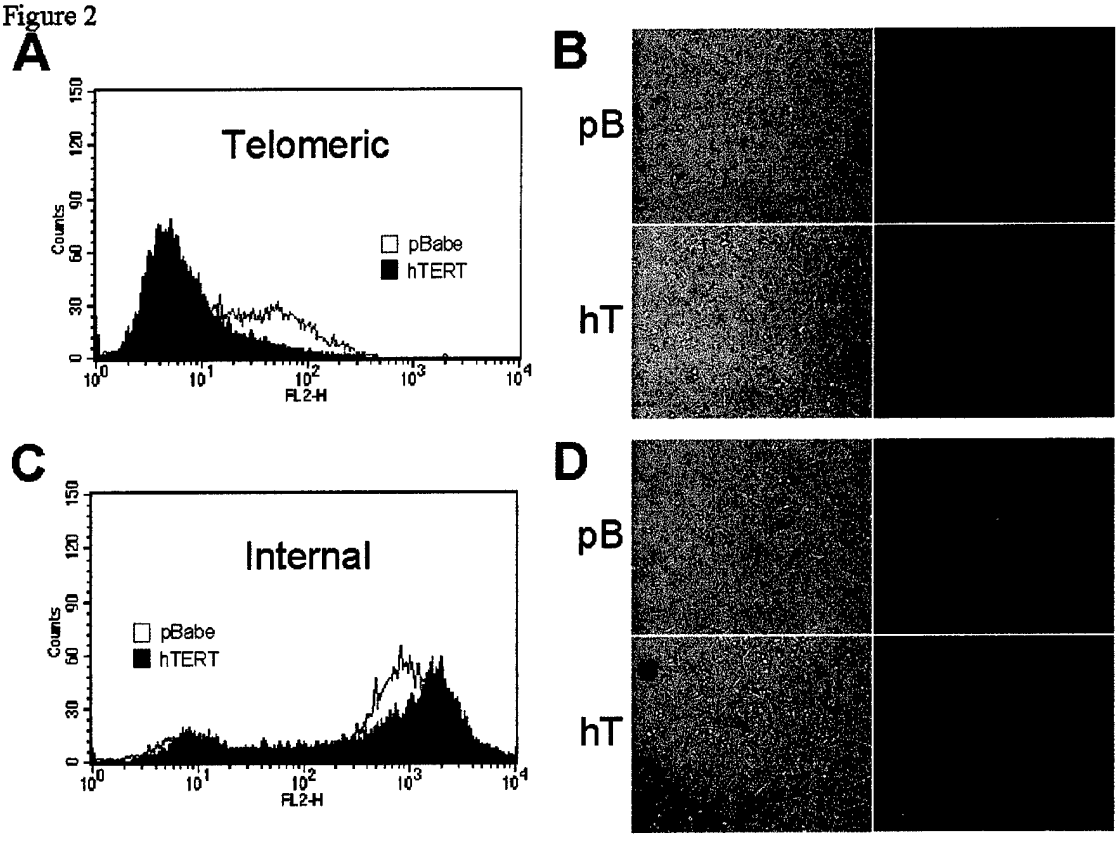


Figure 3

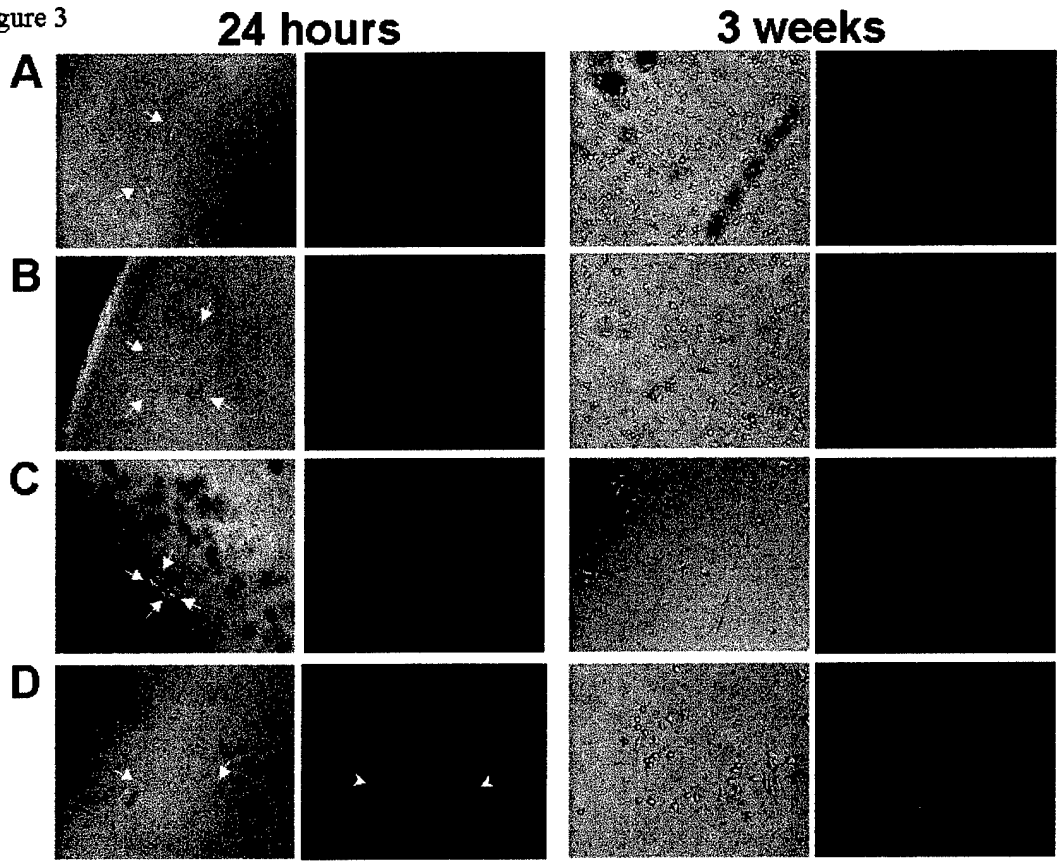
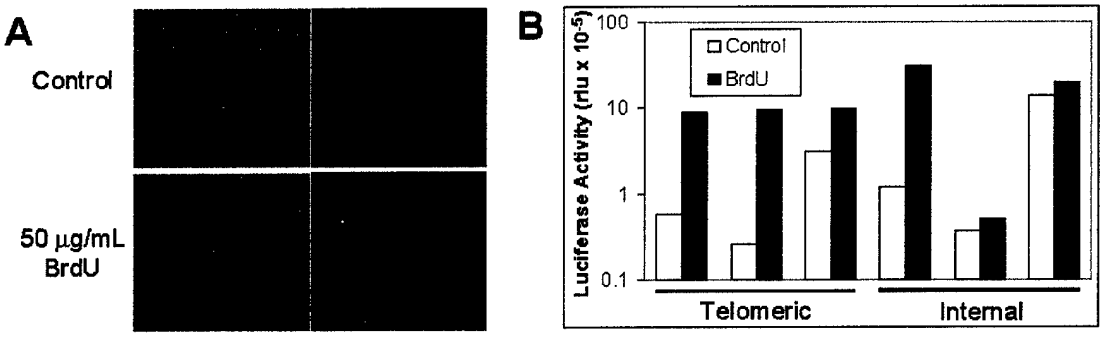


Figure 4



List of Abbreviations

BrdU – 5-bromodeoxyuridine

CMV – cytomegalovirus

DsRed2 – *Discosoma sp.* red fluorescent protein

FACS – fluorescence activated cell sorting

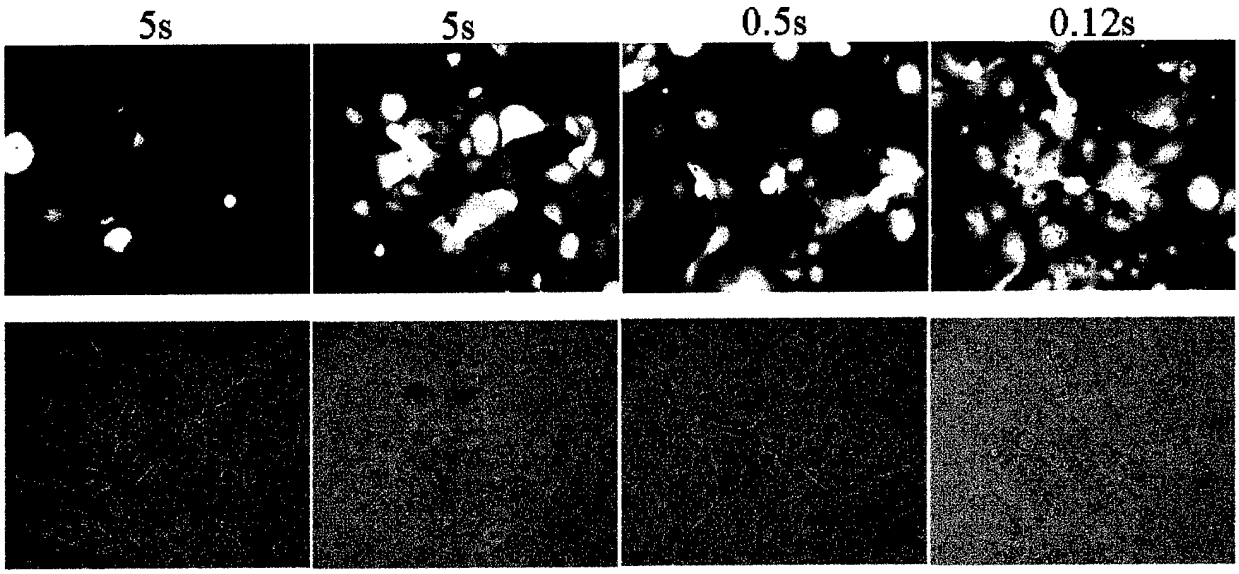
hTERT – human telomerase reverse transcriptase

hTPE – human telomere position effect

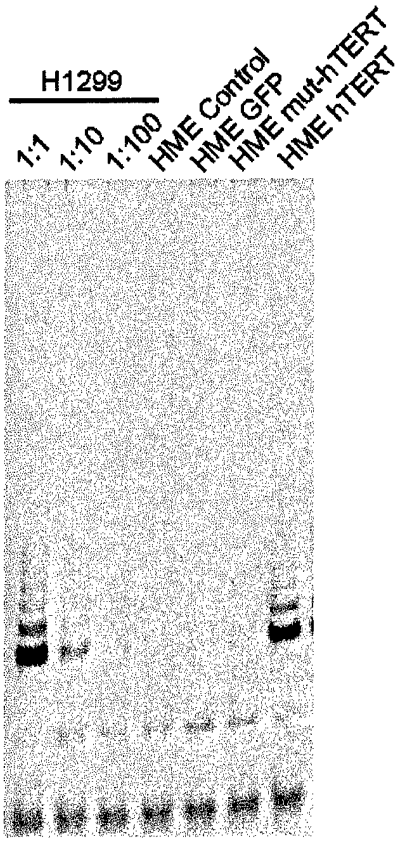
IRES – internal ribosome entry site

TPE – telomere position effect

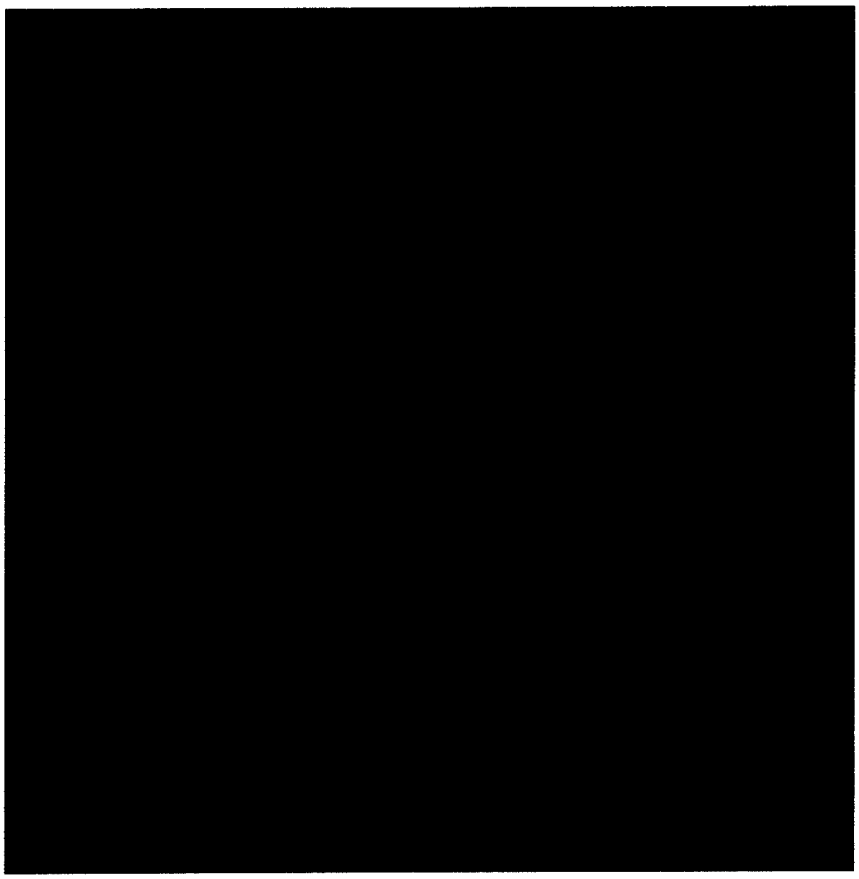
TSA – trichostatin A



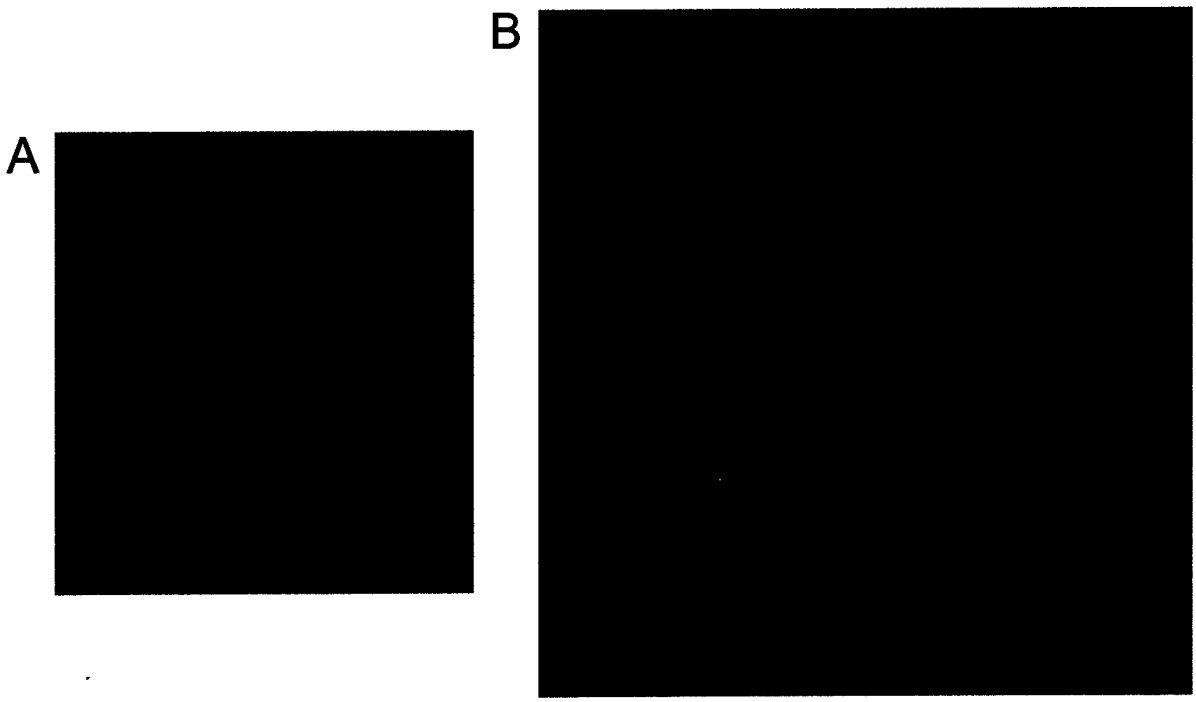
Appendix A3. HMEs can be efficiently infected with adenoviral vectors. Human mammary epithelial cells were infected at the indicated multiplicity of infection (MOI) and examined by fluorescence microscopy after 48 hours. Exposure time is indicated above each image. As shown, 30 MOI was sufficient to infect most cells. Although higher MOIs resulted in greater GFP expression, they also altered cell morphology and over time killed all (10 000 MOI) or some (1000 MOI) of the cells.



Appendix A4. Adenoviral hTERT reconstitutes telomerase activity in HMEs. Human mammary epithelial cells were infected with a vector containing GFP, a mutant (inactive) form of hTERT, or wild type hTERT. Telomerase activity was assessed by telomerase repeat amplification protocol (TRAP) assay. The human tumor line H1299 served as a positive control. Uninfected (control) HMEs, or those infected with GFP or mutant hTERT showed no telomerase activity while hTERT infected cells showed a level of activity comparable to that of H1299.



Appendix A5. Human chromosomes arrested in the metaphase stage of mitosis. Chromosomes were visualized using the general DNA stain DAPI (blue) and a fluorescently labeled $(CCCTAA)_3$ probe (Cy5, shown in pink), was hybridized to telomeres. Four spots are visible because the chromosomes have been replicated but not yet separated into daughter cells. Telomere length can be estimated from the fluorescence intensity of each spot using software-based quantitation.



Appendix A6. The hTERT gene is present in multiple copies after spontaneous immortalization of HMEs. A) *In situ* hybridization for the hTERT gene in the cell line HME50-5, derived from a patient with Li-Fraumeni syndrome (LFS). Only one pair of signals is visible, indicating that the hTERT gene has not been duplicated in this cell line. B) *In situ* hybridization for the hTERT gene in HME50-5E, a spontaneously immortal clone derived from HME50-5. At least four copies of the hTERT gene re present in this clone, indicating that duplication and/or rearrangement of the hTERT gene may play an important role in the progression of HMEs from a phenotypically normal to an immortal phenotype.