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Award Number: DAMD17-01-1-0420

TITLE: Development of a Novel in-situ Telomere Length Qualification System to Address Suitability of Telomerase Inhibitor Therapy to Breast Cancer Following Corrective Surgery

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REPORT DATE: April 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
	April 2003	Annual Summary (1 Apr 02 - 31 Mar 03)

4. TITLE AND SUBTITLE
Development of a Novel in-situ Telomere Length Qualification System to Address Suitability of Telomerase Inhibitor Therapy to Breast Cancer Following Corrective Surgery

5. FUNDING NUMBERS
DAMD17-01-1-0420

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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)*

Normal breast epithelial cells undergo progressive telomeric shortening throughout their replicative lifespan culminating in critically short telomeres, which trigger replicative senescence. Breast cancer cells are able to bypass this growth-arrest mechanism through deregulated expression of telomerase, which maintains telomeres above the minimal critical length. Based on this observation the suitability of these tumors to telomerase inhibitors as direct or adjuvant therapy needs to be determined. To do this we have developed a novel 3-D quantitative fluorescence in-situ hybridization system to analyze total nuclear telomeric signal from normal and tumor-derived breast epithelial cells in tissue culture. This technique has been applied to tumor-derived breast epithelial cells successfully and will be applied to two normal Human Mammary Epithelial Cell (HMEC) lines, HME13 and HME17. Collection of regular cell and DNA samples has enabled us to determine the mean telomere lengths and rate of shortening for these HMEC lines. We will use Q-FISH to determine a per nuclei telomere dynamic. These findings will then be applied to 3-D models before finally generating predictions of telomere lengths within resected breast tumor samples. This will allow us to determine the suitability of telomerase inhibitors in vivo.

14. SUBJECT TERMS:
telomerase, telomeres, Q-FISH, HMEC

15. NUMBER OF PAGES
17

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

Cells contain multiple copies of the TTAGGG hexameric DNA repeat sequences, called telomeres, at the end of chromosomes to provide genomic stability and to provide a source of expendable DNA due to the end replication problem where one DNA strand cannot complete its end during replication (1-3). The lengths of telomeres have also been shown to decrease with time and age. The mechanism termed replicative senescence is the means whereby normal cells have a controlled lifespan after which cellular proliferation ceases (4-6). The replicative senescence mechanism is thought to occur as a response to the presence of critically short telomere(s). These critically short telomere(s) in turn are thought to instigate a DNA-damage response, which prevents further proliferation through upregulation of effector molecules (7).

Maintenance of telomeres has been shown to involve telomerase activity, which acts as a reverse transcriptase to add base pairs to the ends of chromosomes (3). It has been proposed that an immortalized cell emerges from a stage called crisis, characterized by a period of genomic instability, when telomerase or another mechanism to maintain telomere stability is activated (8). Once telomerase is activated it elongates and stabilizes telomere length and permits continued cell division. Most cancer cells have been shown to contain short but stable telomeres compared to parental cells. Beside germ line and stem cells of renewal tissues, other cells having telomerase activity are cancer cells (90% of those tested) (9-11). Telomerase activity can be determined using a standard PCR-based assay with a labeled primer and cell extracts(12)

Telomerase has been shown to become active early in breast cancer progression (10, 13-16). This early activation will therefore maintain the majority of the cancer cells telomere population (46 pairs) at a level beyond the critical minimum seen in senescence and crisis. However, this breast cancer telomere population exists as a heterozygous population with mean telomeric size varying over approximately 2.5kb DNA (Dr B-S. Herbert, personal communication). Thus, the proposed study will determine, using both a cell culture model system and a 3-D tissue culture equivalence model, if the precise range of telomere length in breast tumor cells renders a telomerase inhibitor as a viable therapy option.

The approach will utilize quantification of Fluorescence in situ hybridization signals (Q-FISH) obtained with fluorochrome-labelled short telomere repeat sequences to determine value ranges for young, pre-senescent, and senescent HMEC lines. This will then be applied to archived, sectioned breast tumors to predict telomerase inhibitor treatment times, success of these predictions will be determined using the complementary cryogenically archived cell lines to the sections and existing telomerase inhibitors (17, 18).

Body

The second years statement of work was to develop a direct visualization of telomeric and centromeric signal from HMEC through Q-FISH in a 3-D cell culture environment. This required completion of the analysis from the first year's statement of work. The two cell lines we studied, HME13 and HME17, were cultured on both plastic substrate and the stress-reducing substrate provided by culture with 3T3 cells which are irreversibly growth arrested by Mitomycin C treatment. The growth curves for these cells are outlined in Appendices 1 and 2. Culture in low oxygen can extend continuously proliferative lifespan by around 40% in fibroblasts (19) but no observations have been recorded with mammary epithelial cells. With HME13 we observed a marginal extension of lifespan in low oxygen of around 10% which was not significantly extended by culture on feeder layers (A1). Our alternate line, HME17, did not shown any oxygen concentration dependant extension of lifespan. The 3T3 substrate extended proliferation by around 20% over cells cultured on plastic (A2).

In addition to freezing back ampoules of cells at regular intervals we also routinely collect DNA, RNA and protein samples to assist in later studies. The routine collection of genomic DNA enables telomere length analysis by gel electrophoresis for comparison with data sets obtained by Q-FISH. Appendices 3 and 4 show telomere length electrophoresis images for HME13 and HME17, respectively. Quantification of telomeric signal from these gels enables us to derive shortening rates for both populations, shown in Appendices 5 and 6. This demonstrates that for HME13 cells grown on plastic in low oxygen the telomeres shorten at a very slow rate of approximately 23 bp per PD (A3 and A5). This is at the lower end of telomere shortening rates which tend to be around 50 bp per PD for appropriately cultured cells (20). In room oxygen the telomeres shortened by around 90 PD per division which is approximate to our observed value for inappropriately cultured cells (A3 and A5). Low oxygen clearly has an effect on HME13 cells in culture which has not been further characterized at this point. When cultured on a feeder layer substrate in room oxygen the cells appeared to go through a period which displayed the characteristics of selection. The shortening rates for HME17 were approximately similar in room and low oxygen, 85 bp and 99 bp per PD, respectively (A4 and A6).

Self-selection is the process where HMECs inactivate the stress response gene p16 enabling continued proliferation (21). The two observed lines when cultured on plastic, HME13 and HME17, did not display any slow-down in culture until irreversibly arrested at the end of proliferative lifespan. In room oxygen combined with the feeder system HME13 cells went through a cessation of proliferation that displayed features of selection which was not seen in low oxygen. Although our cultures on plastic did not display any features of selection we felt this could suggest that p16 inactivation was taking place at a later point on these cells. The protein analysis of these cells in shown in Appendix 7. No p16 expression was noted at any time in either of our cultures regardless of substrate or oxygen concentration. The levels of p53 remained fairly constant in the lysates tested but p21 expression did undergo significant upregulation in room oxygen with both HME13 and HME17 (A7).

The delay in establishing completed lineages of our selected target lines necessitated the investigation of an alternative line to investigate the applicability of our technology to a 3-D model. We selected the breast cancer derived cell line MDA-MB 231 as suitable for our studies. The 231 cell line has telomeres of approximately 5kb and is telomerase positive. We also artificially elongated telomeres to 12-15 kb using a retrovirally-induced overexpression of telomerase (Appendix 8). The primary and elongated telomere cell lines were both used to form tumors in nude mice; these were removed, embedded in paraffin and sectioned for staining with telomeric and centromeric probes. Metaphase spreads and tissue culture fixed slides were

prepared at the same time. It quickly became apparent that our existing software package (Openlab 2.2.5) which we used to establish initial results was inadequate to study 3-D materials. The condensed nature of nuclei within sections, particularly epithelial layers, leads to a great deal of nuclear overlap which essentially makes it impossible to measure the signal from individual objects. To overcome this shortfall we utilized the 3-D reconstructive software Volocity (Improvision) which enables iterative deconvolution of z-sliced volumes. This process does not discard data from images, qualitative, but retains all quantitative information. Reconstructing z-sliced images, counterstained with dapi, showed us that the minimum thickness of sections required for intact nuclei is 10μm where 10% of nuclei are whole. Increasing section thickness to 20μm sections leads to approximately 40% of nuclei scored as intact. Our analysis establishes that we are able to quantify differences based on population intensity means. Due to continued difficulties with our centromeric probe, which did improve in reliability but was in no way robust, we chose to use nuclear DNA counterstain Dapi as our normalization (22). In spite of this improved normalization we are concerned that within a population there is an observable ≥ 5-fold difference in summed intensity from adjacent nuclei. Although this difference may be predictable based on an analysis of an individual nucleus this is not a reasonable figure from a relatively homogenous population. This suggests that we are not getting uniform probe penetration which could be due to either incomplete digestion of protein complexes packaging the target DNA, unsuccessful denaturation of target sequences and/or insufficient probe concentration. The observed variation is visible with all species tested (A8) which means that we will be able to achieve a high throughput based on analysis from interphase nuclei processed in a metaphase spread manner. Once we established why we are encountering such misleading intensity spreads we will be able to proceed quickly onto our final stated aim outlined in Task 3.

The development of the Q-FISH technology represents an important advancement in the ability to visualize and quantify telomeres both in the tissue culture environment and also *in vivo*. The advantages of this system will become readily applicable once applied to immortal breast cancer cell lines treated with telomerase inhibitors. This in turn should become a valuable and significant diagnostic tool in the outcome prediction and treatment times of breast cancer patients following resection.

Key Research Accomplishments

Original Statement Task 1. The direct visualization of telomeric and centromeric signals from Human Mammary Epithelial Cells (HMECs) via Q-FISH, in a 2-D cell culture environment, Months 1-12:

- Replicative lifespan of HMEC line HME13 is extended by culture of cells in a low oxygen background on a feeder layer, this does not occur on plastic substrate.
- HME13 displays evidence of a p16-independent M0/selection.
- Replicative lifespan of HMEC line HME17 is extended by culture of cells on feeder layers as compared to plastic substrate. Low oxygen does not add any proliferative advantage.
- Telomere shortening rates for HME13 cells cultured on plastic in low oxygen are significantly lower than those seen with room oxygen.
- Telomere shortening rates for HME17 are essentially unaffected by oxygen concentration.
- HME13 and HME17 do not display p16 expression but do show a significant upregulation of p21 in room oxygen that is not seen in low oxygen.

Original Statement Task 2. Direct visualization of telomeric and centromeric signal from HMEC through Q-FISH in a 3-D cell culture environment.
Months 12-24

- Optimization processes are ongoing to eliminate lack of uniformity seen in adjacent nuclei.
- Establishment of Dapi as a robust normalization.
- Establishment of minimal tissue section depth for intact nuclei as 10μm.

Reportable Outcomes

Publications

Forsyth, N. R., Evans, A. P., Shay, J. W., Wright, W. E. Developmental differences in the immortalization of lung fibroblasts by telomerase. Mol.Cell. Biol. In review process

Forsyth, N. R., Wright, W. E., Shay, J. W. Telomerase and differentiation in multicellular organisms: Turn it off, turn it on, and turn it off again. *Differentiation* 69 (4-5), 188-197. 2002

Abstracts

Forsyth, N. R., Evans, A. P., Shay, J. W., Wright, W. E. Oxygen levels can affect telomerase elongation rates and maintenance telomere lengths. AACR Telomere and Telomerase Conference, San Francisco, CA. December 2002.

Forsyth, N. R., LaRue, D. M., Wright, W. E., Shay, J. W. Development of a Novel *in-situ* Telomere Length Quantification System to Address Suitability of Telomerase Inhibitor Therapy to Breast Cancer Following Corrective Surgery. Breast Cancer Research Program Annual Meeting. Era of Hope 2002. Orlando, FLA. September 2002.

Forsyth, N. R., Shay, J. W., Wright, W. E. Appropriate culture conditions extend lifespan of WI38 cells enabling hTERT immortalization. Keystone Symposium entitled 'Genetics and Genomics of Senescence and Cancer. Jan 2002.

Conclusions

In summary we have determined the replicative lifespan of two normal HMEC cell lines, HME13 and HME17. The telomere shortening rates of these lines have been determined and will be used to establish confidence in our Q-FISH methodology. One of our subject lines, HME13, underwent what appeared to be a p16-independent M0/selection in room oxygen on a feeder layer substrate. Interestingly the telomere shortening rate of HME13 cells in low oxygen was greatly reduced as compared to room oxygen. Neither HME13 nor HME17 displays any p16 expression although both cultures in room oxygen display significantly upregulated p21 expression. We have established that we can quantify telomeres in a 3-D setting and this is essential for studying tumor sections. Determination of conditions to reduce the spread of our data points and generate a more uniform distribution is well under way. Once established this will enable us to rapidly progress through our Statement of Work and meet the delineated objectives.

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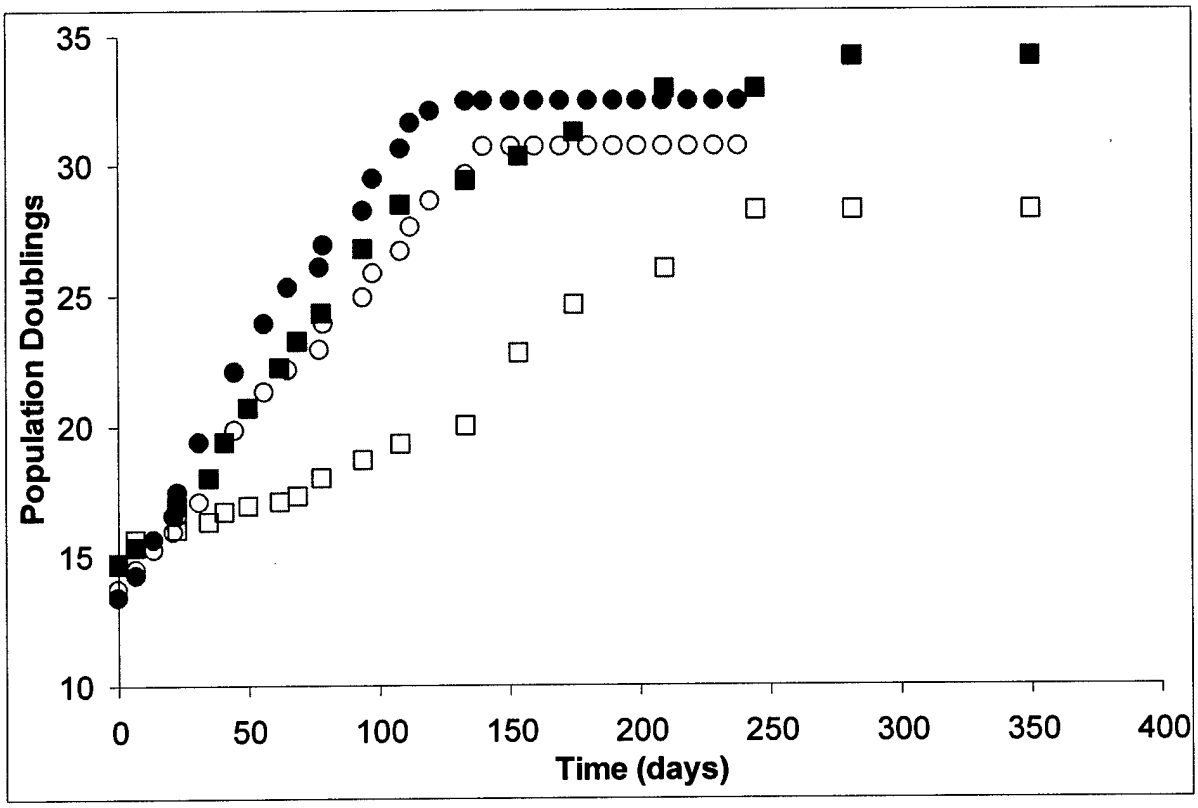
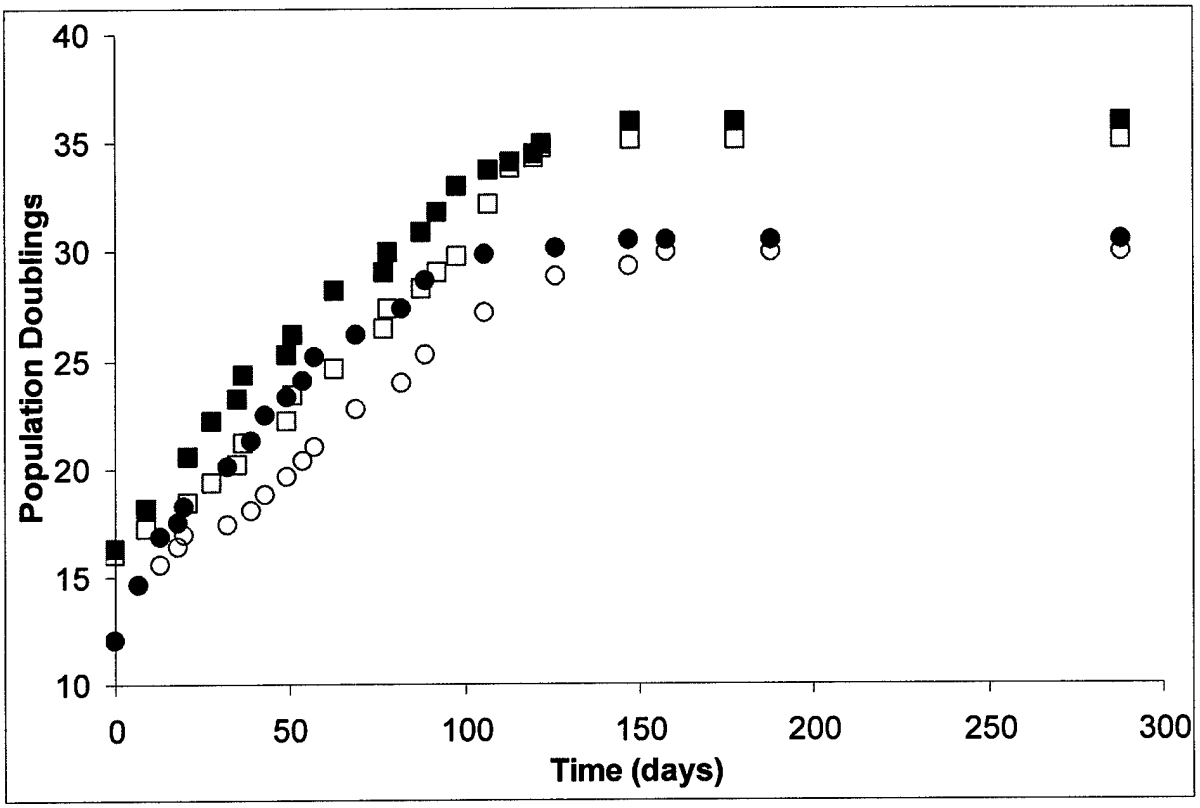
Appendices

A1



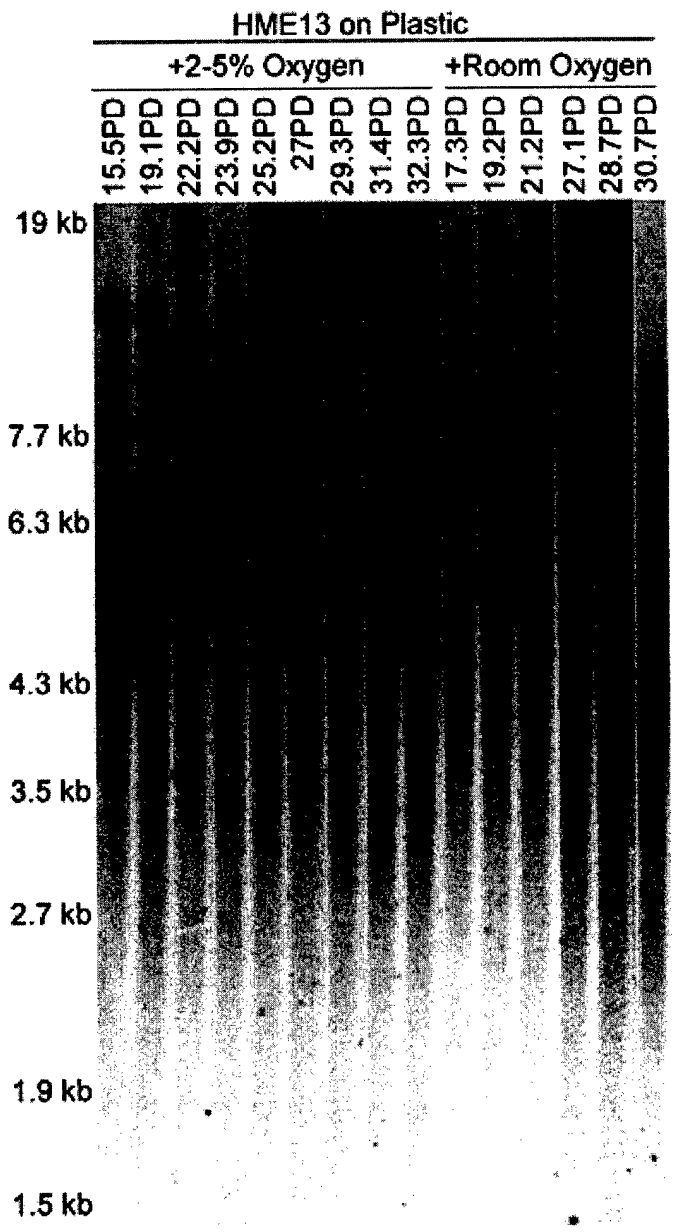
HME13 Growth Curves. Cultures of HME13 cells grown on plastic in low oxygen (2-5%) (○) or room oxygen (21%) (□) and 3T3 feeder layers in 2-5% (■) or 21% O2 (•). Cells grown on plastic or feeder layers in low oxygen proliferate for around 33 population doublings (PD). Room oxygen plastic substrate cultured cells showed a minimal 10% reduction in PDs. However room oxygen cells on feeder layers underwent what resembled the Mo/selection process and performed approximately 20% less PD than their low oxygen counterparts. Cells cultured on plastic have been previously demonstrated to inactivate the stress response gene p16INK4A in the growth period termed M0 or alternatively selection. The behavior of the plastic cultures suggests that self selection may have occurred prior to our thawing the cells. Culture of cells on 3T3 feeder cells is thought to prevent this occurring so it remains to be established what mechanism is responsible for the observed slow down with HME13 in room oxygen.

A2



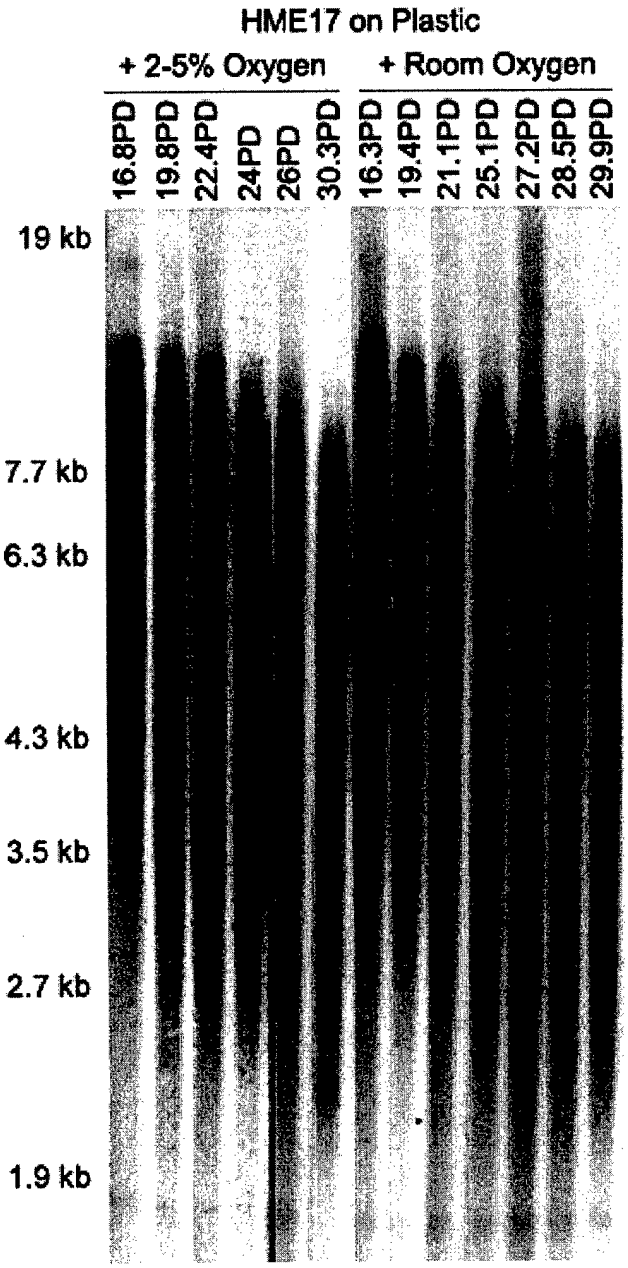
HME17 Growth Curves. Cultures of HME17 cells grown on plastic in low oxygen (2-5%) (○) or room oxygen (21%) (□) and 3T3 feeder layers in 2-5% (■) or 21% O2 (•). Initial proliferative rates of cells cultured on alternate substrates with different oxygen concentrations did not differ. Matched substrate pairs also did not differ in their proliferative capacity irrespective of the oxygen concentration used to culture the cells. However, cells cultured on feeder layers performed approximately 20% more PDs than cells cultured on plastic substrate. The growth rates of both populations were initially identical with no observance of any slow down period. This argues against selection and suggests that this culture may also have lost p16 expression prior to our thawing of the cells.

A3



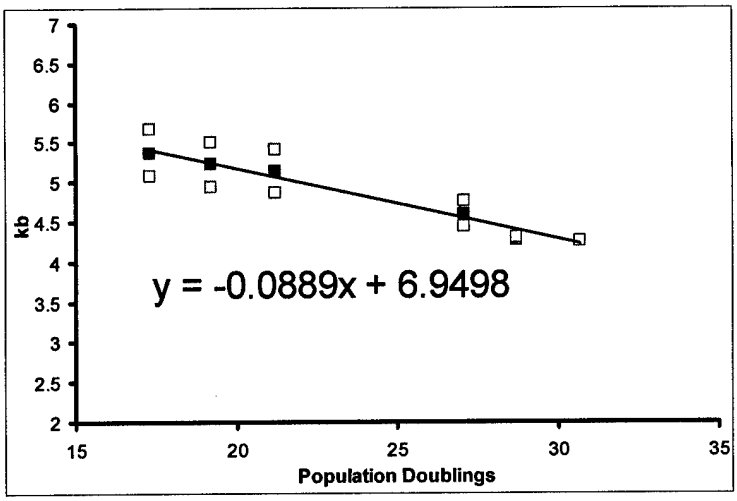
HME13 Telomere Analysis. Genomic DNA was extracted at regular intervals, digested with a cocktail of restriction enzymes, resolved using gel electrophoresis and telomeric DNA visualised according to the protocol previously described (23). The y-axis details known molecular weight standards, the upper x-axis describes the sequential population doublings and the respective oxygen concentration. Telomere length qunatififcation is described in Appendix 5.

A4

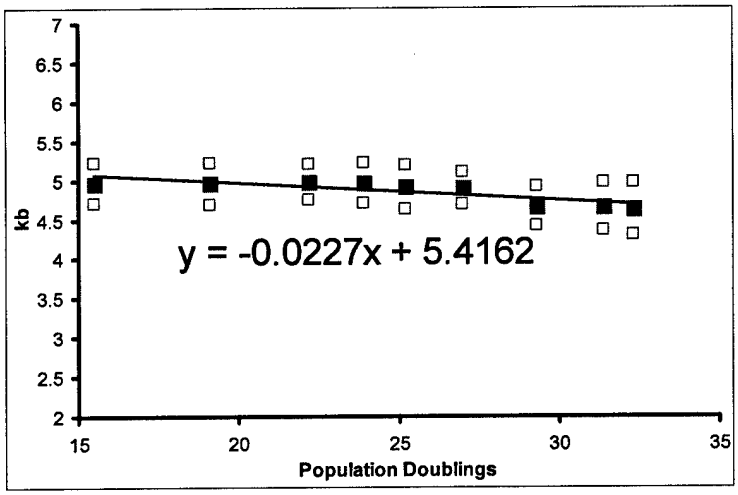


HME17 Telomere Analysis. Genomic DNA was extracted, resolved using gel electrophoresis and telomeric DNA visualized according to the protocol previously described (23). The y-axis details known molecular weight standards, the upper x-axis describes the sequential population doublings and the respective oxygen concentration. Telomere length qunatififcation is described in Appendix 5.

A5

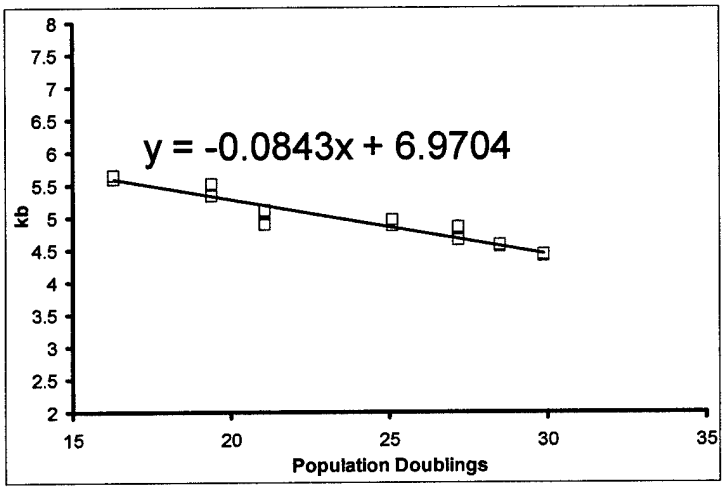


HME13 Telomere Shortening Quantification: Room Oxygen. This was performed using known molecular weight markers (y-axes in A3 and A4) and TeloRun software available at 'http://www.swmed.edu/home_pages/cellbio/shay-wright/research'. Telomeres shortened at approximately 90 bp per PD in room oxygen as indicated by the equation outlined above where y = telomere length and x = population doubling point. Individual points (□) and the weighted average (■) of minimally two individual experiments are shown.

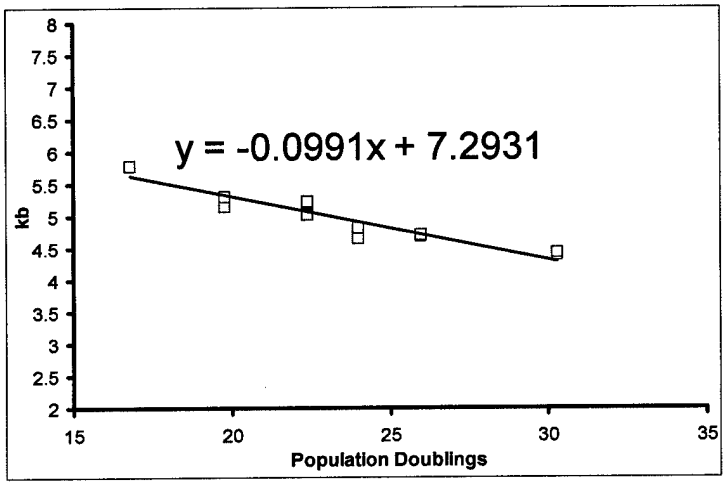


HME13 Telomere Shortening Quantification: Low Oxygen. With the methodology outlined above the low oxygen telomere shortening rate was revealed to be approximately 23 bp per PD.

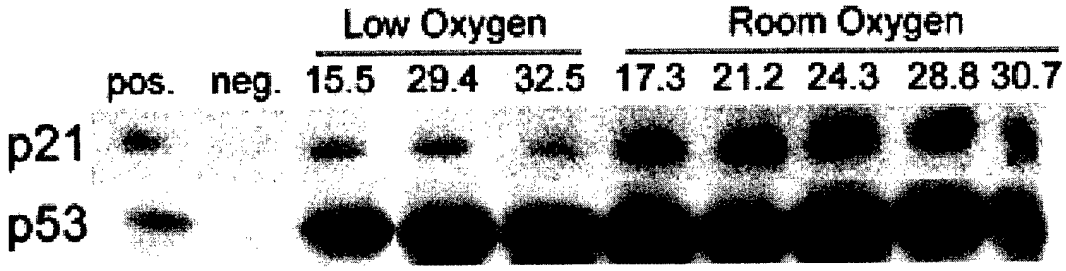
A6



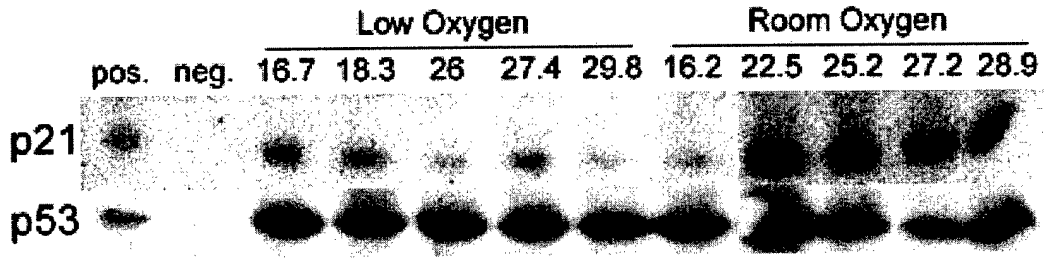
HME17 Telomere Shortening Quantification: Room Oxygen. Methodology as outlined in A5. HME17 telomeres in room oxygen shorten by around 84 bp per PD.



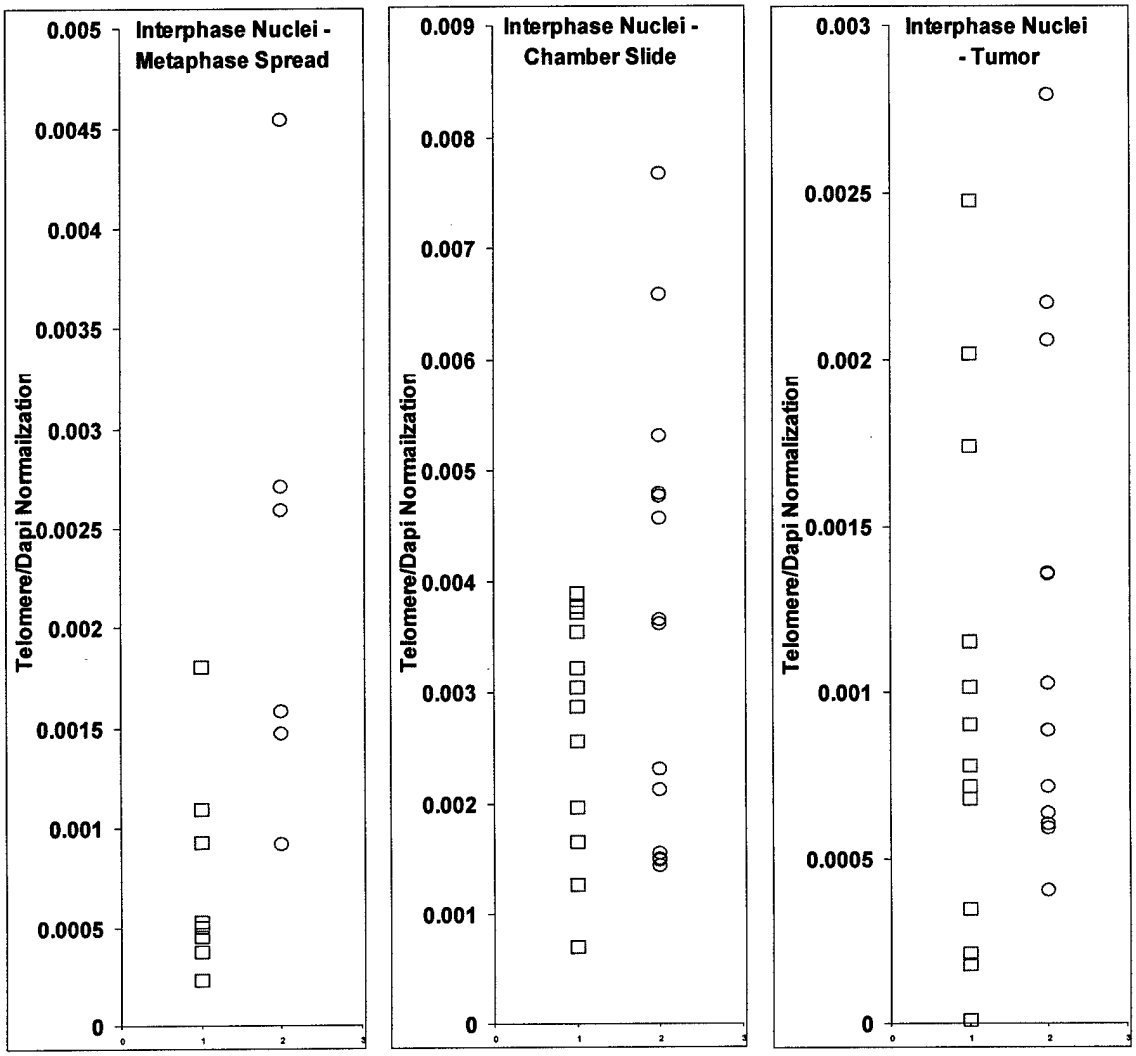
HME17 Telomere Shortening Quantification: Low Oxygen. HME17 telomeres shorten in low oxygen by around 99 bp per division.



HME13 Protein Analysis. We analyzed cell lysates for p16, p21, and p53 expression and this was done for both low and room oxygen following standard protocols (21). This was done for both plastic and feeder layer cultured cells, plastic results are shown but both displayed similar phenotypes. No p16 expression was observed for either plastic or feeder layer cultured cells indicating that, as suspected, self-selection had occurred prior to our thawing of the cells. No significant upregulation of p53 was noted although expression was fairly high throughout. Interestingly the p53 downstream target p21 was upregulated significantly in room oxygen but only underwent a slight increase in low oxygen. The y-axis describes the protein tested, the x-axis describes PD tested and oxygen concentration.



HME17 Protein Analysis. Methodology and axes are as described above. No p16 expression was observed in these cells on either plastic or feeder layer substrate. The levels of p53 remained relatively constant throughout time in culture but p21 levels were again upregulated significantly in room oxygen cells.



	Metaphase Spread	Chamber Slide	Tumor
231	7.36E-04	2.68E-03	8.85E-04
231hTERT	2.24E-03	3.84E-03	1.23E-03
Fold Difference	3	1.4	1.4

MDA-MB 231 Q-Fish Analysis. Interphase nuclei were z-sliced, captured and deconvolved using Volocity software. The software generates a 3-D reconstructed view of individual nuclei from which mask generation is carried out directly onto the 3-D telomere spots. Background noise is negligible due to the iterative deconvolution which reassigns or removes light noise according to a Point Spread Function protocol. The 231hTERT cells have telomeres 2-3 times larger than those in 231 parental cells. Dapi has recently been utilized as a robust normalization and we chose to use this as a control in this instance (22). The standard deviation from a mixed population of Dapi intensity/area gives an approximate 10% value which is excellent for a biological system. In all instances we get a positive fold-difference change, scatter plots show summed telomeres per nucleus from a mixed population. Open box = 231, Open circle = 231hTERT. The table displays the mean sum of intensities for each data set in each tested condition.