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Award Number: DAMD17-02-1-0443

TITLE: Cooperative Interactions During Human Mammary Epithelial Cell Immortalization

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REPORT DATE: July 2005

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2005	Final	1 Jul 02 – 30 Jun 05

4. TITLE AND SUBTITLE
Cooperative Interactions During Human Mammary Epithelial Cell Immortalization

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0443

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California at Berkeley
Berkeley, CA 94720

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. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

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

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Abstract is attached at the following page.

15. SUBJECT TERMS
Telomerase, Immortalization, Senescence, Cell Culture, Oncogenes, Tumor Suppressor Genes

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	15	19b. TELEPHONE NUMBER *(include area code)*

ABSTRACT

Our laboratories have developed and utilized cultured human mammary epithelial cells (HMEC) to gain information on the defects in growth control processes that allow finite lifespan HMEC to overcome all senescence barriers, reactivate telomerase, and gain immortal potential. We hypothesize that, due to the stringency of telomerase repression in humans, attaining these defects may be rate-limiting in human carcinogenesis. Our goal is to define the minimum number of genetic and epigenetic changes that permit telomerase reactivation and immortal transformation of finite lifespan HMEC, *in a manner that models changes observed in breast cancers*. Thus far, we have been able to obtain immortalized HMEC using combinations of oncogenes with pathological relevance to human breast cancer. Although CGH analyses of some of these immortal lines did not show any detectable large-scale changes in gene copy numbers, these lines have all undergone clonal selection, suggesting that additional unknown stochastic changes are necessary for immortalization. These additional changes remain to be discovered. The exact nature of these alterations may vary depending upon the differentiation/epigenetic state of the targeted cells. Better understanding of the underlying molecular changes involved in telomerase reactivation may provide novel prevention strategies and/or targets for therapeutic intervention in breast cancer pathogenesis.

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INTRODUCTION

Immortality allows the progeny of a single cell to accumulate the multiple errors needed to gain invasive and metastatic properties. Long-lived animals such as humans have developed extremely stringent mechanisms of cellular replicative senescence to prevent immortal transformation, presumably as a tumor-suppressor mechanism. Many recent studies suggest that one pathway by which stringent senescence is enforced in normal human cells is through repression of hTERT expression and telomerase activity. Almost all human breast cancers show re-expression of hTERT and telomerase activity. Our laboratories have developed and utilized cultured human mammary epithelial cells (HMEC) to gain information on the defects in growth control processes that allow finite lifespan HMEC to overcome all senescence barriers, reactivate telomerase, and gain immortal potential. Our previous work demonstrated that cultured HMEC could be immortally transformed following exposure to combinations of *pathologically relevant* oncogenic agents. All of these studies generated immortal lines containing unknown errors. We proposed in this grant that the telomere dysfunction-based senescence barrier could be overcome by reactivation of hTERT, but that doing so required the cells to undergo changes in multiple distinct pathways. This requirement may be responsible for the stringency of telomerase repression in humans, and consequently, reactivating telomerase may be a key rate-limiting step in human carcinogenesis. Our previous studies suggested specific defined defects that might be involved in telomerase reactivation. The goal of this project has been to determine the specific genetic and epigenetic changes that permit hTERT reactivation and immortal transformation of finite lifespan HMEC, *in a manner that models changes observed in breast cancers in vivo*.

BODY

Task 1. Perform a semi-quantitative assessment of the efficiency of HMEC immortalization and associated changes in phenotype by GSE22, c-*myc*, and *ZNF217* alone or in combination.

Spontaneous transformation to immortality is virtually non-existent in cultured human cells derived from normal tissues. HMEC readily overcome an RB-mediated, stress associated senescence barrier, stasis, through loss of p16 expression. However, continued telomere erosion with ongoing proliferation of these post-stasis cells (termed post-selection where p16 silencing occurred spontaneously *in vitro*, and extended life when p16 loss occurred after *in vitro* carcinogen exposure) eventually produces widespread genomic instability as a consequence of telomere dysfunction (1-4). Our previous data has indicated that multiple alterations are required for p16(-) HMEC to overcome the telomere dysfunction-based senescence barrier (termed agonescence in p53(+) cells; crisis in p53(-) cells). Overcoming this barrier is associated with gaining the capacity to express hTERT. We therefore proposed that at least two alterations are required to reactivate endogenous hTERT in these p16(-) post-selection HMEC. The likelihood that all the necessary errors would occur in the same cell, even under conditions where widespread genomic errors are generated, is exceedingly small. However, if the telomere dysfunction barrier were approached in cells already harboring one error predisposing to immortality, the genomic instability resulting from telomere dysfunction could give rise to rare additional complementary errors that allow reactivation of endogenous telomerase activity and immortalization. Defining the errors that allow telomerase reactivation could greatly increase our understanding of the derangements required for human breast cancer progression.

We first introduced individual errors into p16(-) HMEC – over-expression of the breast cancer-associated oncogenes c-*myc* or *ZNF217*, and/or inhibition of p53 function by the genetic suppressor element GSE22. Immortal lines resulting from some of these manipulations all contained some karyotypic errors, and most lines emerged during the period of genomic instability accompanying telomere dysfunction. We then proceeded to use combinations of defined errors to see if we could produce immortal transformation without incurring undefined errors. We hypothesized that such immortalized cells would require changes in a minimum of three distinct pathways, resulting in: (a) the hTERT gene being rendered accessible to transactivation; (b) appropriate

transcriptional activator(s) of hTERT being aberrantly expressed; and (c) molecules that inhibit telomerase activity being functionally eliminated. Post-selection p16(-) HMEC from specimen 184, having no known existing genetic defects, were transduced with retroviral vectors containing c-*myc*, *ZNF217*, and GSE22, alone or in combination. We monitored three lineages of each experimental condition independently to control for jackpot effects and overgrowth by rare variants within a population. Resulting populations were assayed for expression of the transgenes, hTERT mRNA levels, telomerase activity, SA-βgal activity, morphology, growth rates, colony forming efficiency, labeling index ± TGFβ, genome copy number changes (by CGH), mean TRF length, the number of foci of growing cells that appear when most cells have ceased growth at agonescence/crisis, and retroviral integration sites.

In two preliminary experiments, transduction of both *ZNF217* and c-*myc* into post-selection 184 HMEC produced what appeared to be uniform immortalization; CGH analyses of the immortal populations did not show any detectable changes in gene copy numbers (data not shown). In separate, NIH-supported experiments, we also demonstrated that although newly immortal p53(+) HMEC lines were capable of expressing telomerase activity, the functional p53 in these cells was able to repress this activity until telomeres became critically short and a conversion process was initiated (2).

In experiments performed with additional controls, we examined the molecular consequences of transducing post-selection 184 HMEC at passages 7-9 with combinations of *ZNF217*, c-*myc*, and GSE22. Three independent lineages were monitored for each combination. The combination of over-expressed *ZNF217* and c-*myc* (ZM) produced homogeneously growing immortalized cells in one lineage (ZM3), heterogeneously growing immortalized cells in a second lineage (ZM2), and no immortalized cultures in the remaining lineage (ZM1). *ZNF217* alone (ZB) produced sporadic immortality in 1/3 lineages. In both the ZM2 and ZB cultures that immortalized, clonal outgrowths appeared at 14p, while the majority of the cells underwent agonescence. No immortal clones arose in any of the three c-*myc* alone (LM) lineages. Assays of telomerase activity showed early reactivation in ZM3, and reactivation during agonescence in ZM2 (**Fig. 1**). CGH analysis showed no changes in gene copy number in ZM3, but amplification of the 8q locus where *c-myc* resides, as well as other alterations, in ZM2 (**Fig. 2A,B**). 8q was also amplified in the newly generated ZB line, as it was in previously examined lines immortalized with *ZNF217* alone.

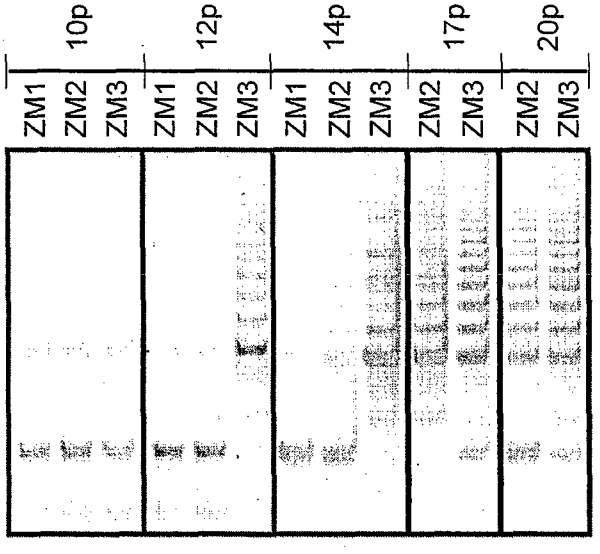

Fig. 1. Telomerase activity increases gradually at different rates in HMEC transduced with both *ZNF217* and c-*myc*. Lysates of three independently transduced populations at indicated passages (p) were assayed for telomerase activity by the PCR-based TRAP assay. The presence of a ladder of PCR products of increasing size indicates positive telomerase activity.

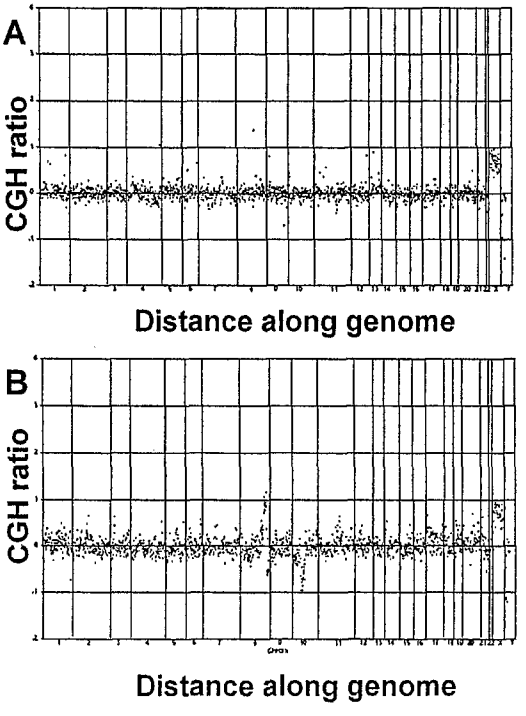

Fig. 2. Comparative genomic hybridization (CGH) results of immortalized HMEC lines (A) 184ZM3 passage 17 and (B) 184ZM2 passage 17. Note that CGH analysis showed no changes in gene copy number in 184ZM3, but amplification of the 8q locus where c-*myc* resides, as well as other alterations, in 184ZM2.

Despite our ability to generate immortal lines without large gene copy number alterations in cultures transduced with *ZNF217* and c-*myc,* southern analysis of retroviral integration sites in our three ZM lines with no detectable CGH alterations indicated a limited number of viral integrations, suggesting that clonal selection had taken place in all three lines. We hypothesize that the variable results obtained in the ZM experiments could be due to: (a) variability in the levels of *ZNF217* and c-*myc* expression achieved; (b) epigenetic influences; (c) stochastic genetic events too small to be detected by CGH (e.g. point mutations, small deletions, etc.). To address possibility (a), we are comparing *ZNF217* and c-*myc* transcript and protein levels in the transduced cells prior to and after immortalization. We are currently addressing possibilities (b) and (c) by (i) investigating a possible role of methylation of the hTERT gene, and (ii) gene expression profiling of finite and immortal HMEC transduced with *ZNF217* and c-*myc* (described in Technical Objective 2).

Since our previously published work (2) has shown that loss of p53 function can accelerate the acquisition of telomerase activity in susceptible HMEC lines, we performed experiments to determine whether inactivation of p53 can increase the level of telomerase activity and frequency of immortalization in HMEC that initially failed to immortalize when transduced with both *ZNF217* and c-*myc*. Specifically, we transduced GSE22 into the ZM1 lineage as well as into ZB and LM lineages (lineages that all did not immortalize). Although the cells transduced with GSE22 did exhibit more population doublings than control cultures prior to agonescence/crisis, we did not observe differences in immortalization or telomerase activity, negating the likelihood that p53 contributed to the poor frequency of immortalization.

Additional information consistent with a role of *ZNF217* and c-*myc* in HMEC immortalization was generated by related experiments designed to assess the effect of p16/RB repression of cell cycle progression in pre-stasis HMEC still capable of p16 expression. Pre-stasis 184 HMEC were transduced with viral vectors encoding p16 shRNA and/or *c-myc* or appropriate control vectors at passage 4. Control cells transduced with empty retroviruses entered stasis and were discarded after >50 days in culture **(Fig. 3)**. In contrast, cells transduced with p16 shRNA or *c-myc* underwent slow heterogeneous growth phases before reacquiring uniform exponential growth kinetics. Cells transduced with both p16 shRNA and *c-myc* proliferated exponentially without a slow heterogeneous phase. CGH analysis of this latter immortal line at passage 25 indicated no gene copy number changes **(Fig.4)**. In contrast, CGH analysis of the immortal line obtained after transduction with p16 shRNA alone showed its clonal origin. Remarkably, the only two regions of high gene copy number increase in this line were at 8q24 - the location of the c-*myc* gene, and 20q13 – the location of the *ZNF217* gene. The immortal line obtained after transduction with c-*myc* alone showed gene copy number changes on chromosomes 5 and 6, but no evidence of changes at 20q13. The relative ease of immortal transformation of pre-stasis vs. post-selection HMEC is consistent with past reports of different HMEC populations displaying differential susceptibilities to immortal transformation by viral oncogenes (5, 6), and suggests that additional repression of the hTERT gene locus occurs by epigenetic means in the cells destined to become post-selection HMEC. These epigenetic differences may pre-exist in the pre-stasis population, or may arise during the selection process itself.

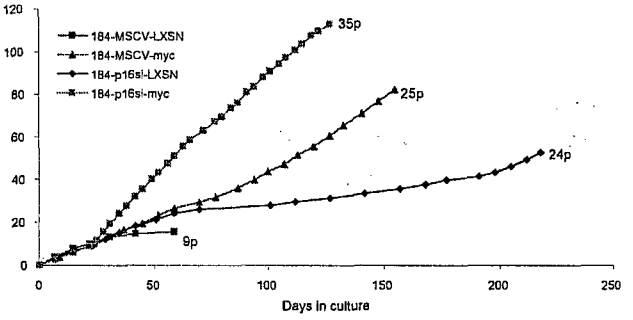

Fig. 3. Indefinite growth of pre-stasis 184 HMEC transduced with p16 shRNA and/or *c-myc*. Control cells transduced with empty retroviruses entered stasis and were discarded after >50 days in culture. In contrast, cells transduced with p16 shRNA or *c-myc* underwent slow heterogeneous growth phases before reacquiring uniform exponential growth kinetics. Cells transduced with both p16 shRNA and *c-myc* proliferated exponentially without a slow heterogeneous phase.

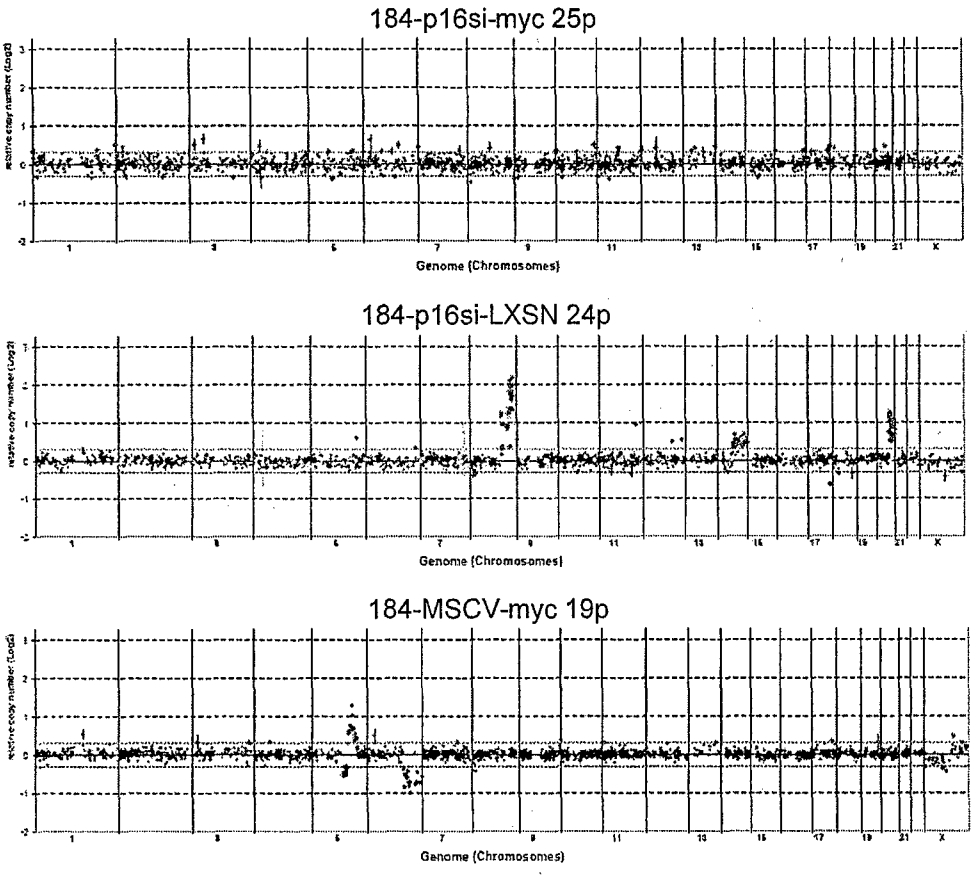

Fig. 4. CGH results of immortalized HMEC lines obtained from pre-stasis 184 HMEC. Gene copy number gains are indicated in green while losses are indicated in red. LXSN and MSCV correspond to empty retroviral vectors. Passage (p) numbers sampled are indicated.

Our studies of hTERT gene methylation have been conducted in collaboration with Jean Benhattar (Institut Universitaire de Pathologie, Lausanne, Switzerland), and in coordination with another DOD supported study (DAMDW81XWH-04-1-0283) of a novel DNA binding protein (BORIS) with possible hTERT gene regulatory activity. Previous studies by Dr. Benhattar have shown a strong positive correlation between methylation of the hTERT gene promoter and telomerase activity in tumors and tumor-derived lines (7). In contrast, however, studies with normal human cells have suggested a negative correlation between hTERT expression and methylation of the hTERT promoter region (8). We have provided Dr. Benhattar with DNA from finite lifespan and immortal HMEC derived from different individuals. His laboratory has examined hTERT promoter methylation by two different methods – methylation-sensitive single-strand conformation analysis (MS-SSCA) and/or a methylation-sensitive DNA dot blot analysis (**Appendix Table 1**). Similar to what has been shown for tumor lines, most of the immortal HMEC lines showed methylation of the hTERT promoter. However, we note that most HMEC that immortalized readily, without undergoing the period of genomic instability that accompanies telomere dysfunction (e.g., lines transduced with both ZNF217 and c-*myc*), did not show methylation. This preliminary data therefore does not support the hypothesis that alterations in hTERT gene promoter methylation patterns are an epigenetic factor contributing to immortalization of lines that show no gross genomic changes by CGH. However, the possibility remains that methylation at alternative sites that have not yet been examined in the hTERT promoter or gene body may play such a role. It is also possible that alterations in hTERT gene promoter methylation may only play a role in immortalization of lines that exhibit genetic instability accompanying telomere dysfunction.

As stated above, we observed an absence of gross genomic changes in three of our HMEC lines immortalized by ectopic expression of ZNF217 and c-*myc*, indicating that it was possible to generate immortal HMEC from finite lifespan HMEC using known, *pathologically relevant* agents. In the past year, we asked whether it was

possible to malignantly transform the ZNF217/c-*myc* immortalized HMEC in the absence of genomic instability. These studies were designed to address the question of whether aneuploidy per se is necessary for malignancy, or whether defined genomic imbalances introduced experimentally are sufficient. In an initial test of this concept, we introduced the mutant erbB2/Her2/neu oncogene into ZNF217/c-*myc* immortalized HMEC. The immortally transformed line 184vZNMY3 at passage 30 was transduced with activated erbB2 (neuT) in the WZL retroviral vector or with empty vector. Transduced cells were selected with blasticidin S at 5 μg/ml. At passage 35 the transduced cells were seeded into 2 x 60 mm POLYHEMA coated dishes in 1.5% methylcellulose at a density of $1x10^5$ cells/dish. The anchorage independent line 184B5ME (obtained by overexpressing wild type erbB2 in the 184B5 line) was plated as a positive control. After 3 weeks, cells capable of forming colonies in methylcellulose were counted. 184vZNMY3 cells transduced with empty WZL vector showed no anchorage independent growth. 184vZNMY3 cells transduced with activated erbB2 had an anchorage independent growth frequency of 0.63% (colonies > 75μm). In comparison, the 184B5ME line had an anchorage independent growth frequency of 5.35%. Thus, over-expression of one additional oncogene associated with mammary cancers was capable of conferring a malignancy-associated property on a genomically stable HMEC population immortalized by over-expression of known breast cancer-associated oncogenes. These cultures are currently being assayed for other malignancy-associated properties.

To determine if the anchorage independent growth conferred by activated erbB2 was associated with gene copy number changes, DNA from 184vZNMY3-neuT was analyzed by CGH. Results of the CGH analysis showed no large gene copy number changes. These preliminary results therefore suggest that defined genomic imbalances introduced experimentally are sufficient to produce a malignant phenotype starting with finite lifespan HMEC from normal tissue. We plan on testing additional ZNF217/c-*myc* immortalized HMEC transduced with wild type or activated erbB2.

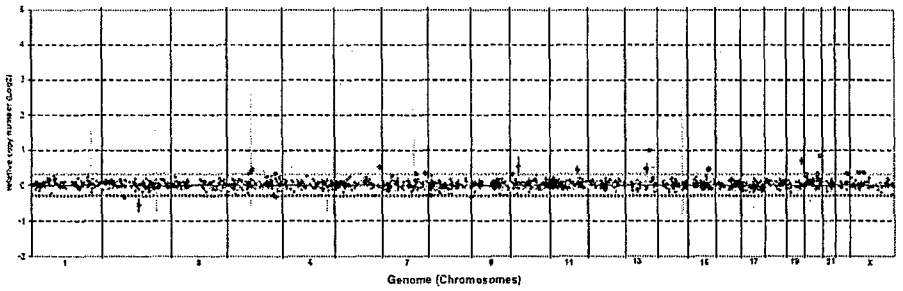

Fig. 5. CGH results of 184vZNMY3-neuT line obtained from 184 HMEC.

Technical Objective 2: Perform differential screening of high density cDNA microarrays to identify genes whose expression is altered in closely related finite lifespan, EL, and immortal HMEC:

To identify gene expression changes that are associated with ectopic expression of ZNF217 and c-*myc*, we have performed expression array analysis comparing finite lifespan 184 HMEC, the 184-ZM3 HMEC prior to the detection of telomerase activity, and fully immortal telomerase(+) 184-ZM3 HMEC. RNA was isolated from duplicate cultures of the indicated cells and analyzed using the Affymetrix Human Genome U133 Plus 2.0 Array in collaboration with Dr. Jeff Gregg at the University of California Davis Medical School. Comparisons of the pre-immortal 184-ZM3 with finite lifespan 184 revealed fewer than 200 transcripts whereas comparisons of the fully immortal 184-ZM3 with the finite lifespan 184 revealed approximately 1300 transcripts showing greater than ±1.6 fold (signal log ratio of ±0.7) differences in expression levels (data not shown). Thus we found that there were further changes in gene expression after immortalization. Changes in gene expression patterns were seen in pathways for integrin signaling, apoptosis, cell cycle regulation, MAP kinase, as well as general metabolic processes. While presumably, most of these additional changes did not directly affect hTERT expression, analysis of the changes may yield insights into pathways responsible for both the changes observed and hTERT expression.

Additionally, we have collaborated with Dr. Steven Haney at Wyeth Pharmaceuticals to profile a larger number of finite lifespan and immortally transformed HMEC. Post-selection HMEC from seven different specimen donors were compared to four different non-malignant immortally transformed lines from one of these specimens, i.e., 184A1and 184B5 (p53+), 184AA2 and 184AA3 (p53-), 184B5Me, and three immortal lines from another individual, MCF10A, MCF10A-2, and MCF12A. The resulting data has been subjected to cluster and pathway analyses. All the finite lifespan specimens cluster independently of all the immortally transformed HMEC. Significant differences include those gene transcripts associated with G1 regulation and DNA damage responses. The gene expression patterns in the non-malignant fully immortalized lines most closely resemble those of DCIS, and are very similar to those of cancer cell lines and tumors. The fully immortalized lines are highly similar in this regard, despite diverse origins. The number of genes exhibiting distinct expression differences from one cell type/line to another at the same stage is small, although such differences could be important. Gene expression patterns that vary include those associated with interferon signaling, HDAC function, and specific transcription factors such as NFY and SP1. Additional differences are seen when erbB2 is introduced, but these are fewer, less directly associated with changes in the cell cycle, and more directly associated with ECM structure and developmental signaling. It may be that the introduction of such an oncogene into a fully immortalized cell line most closely models the events associated with the transition from DCIS to IDC, as opposed to a transition of epithelial hyperplasia to DCIS.

Altogether, these data support our hypothesis that the critical step in human breast cancer carcinogenesis is the one that transforms finite lifespan HMEC into telomerase expressing immortal lines.

Technical Objective 3: Use random homozygous knockout (RHKO) selection method to identify genes that suppress HMEC immortalization.

In collaboration with Dr. Stanley N. Cohen, we have employed his random homozygous knockout (RHKO) selection strategy to try to identify unknown genes whose inactivation promotes immortalization of post-selection p16(-) HMEC. In three independent experiments, a total of 32 plates were infected with both a tTA vector encoding a doxycycline-sensitive transactivator and an RHKO vector encoding a tTA responsive antisense promoter. In the first experiment, 1/9 plates containing benzo[a]pyrene-treated extended-life184Aa cells yielded a clonal outgrowth with additional proliferative potential. Unfortunately, this outgrowth ultimately senesced before sufficient material could be obtained for further analysis. In a subsequent experiment, post-selection 184 cells were infected with GSE22 to inactivate p53 function prior to infection with the RHKO vector. Despite this modification, all the RHKO-infected cells on 11 plates ultimately underwent crisis, yielding no clones with additional proliferative potential. In the third experiment, we attempted to take advantage of the differential sensitivity of finite life span and immortalized HMEC to *raf* oncogene-induced growth arrest (9) to identify immortalized cells in RHKO-infected cultures of finite life span specimen 48RS. We found unexpectedly that the *raf*-induced growth arrest was not stringent – all 12 plates infected with RHKO and tamoxifen inducible *raf-ER* constructs yielded slowly growing *raf*-resistant cells which ultimately senesced. No additional experiments using the RHKO methodology have been attempted.

KEY RESEARCH ACCOMPLISHMENTS

• Immortalized cells were obtained reproducibly from post-selection p16(-) HMEC using a combination of two oncogenes (c-*myc* and *ZNF217*) with pathological relevance to human breast cancer.

• CGH analyses of three immortal populations obtained using c-*myc* and *ZNF217* did not show any detectable changes in gene copy numbers.

• Southern analysis of viral integration sites has indicated that these three lines have undergone clonal selection, suggesting that unknown stochastic changes, in addition to over-expression of these 2 genes, might be necessary for immortalization.

• Pre-stasis HMEC transduced with both p16 shRNA and c-*myc* proliferated exponentially without a slow heterogeneous phase and yielded immortal cells with no gene copy number changes, suggesting that in these cells, the 20q13 (*ZNF217*) amplification was not required for immortalization.

• Methylation of the hTERT promoter correlates with hTERT expression in immortally transformed HMEC lines that have undergone genetic instability accompanying telomere dysfunction. However, in lines that have not undergone genetic instability, methylation of the hTERT promoter is not required for hTERT mRNA expression.

• Immortalized HMEC displayed altered expression of genes involved in integrin signaling, apoptosis, cell cycle regulation, MAP kinase, as well as general metabolic processes.

REPORTABLE OUTCOMES

Abstracts

A Model for Human Mammary Epithelial Cell (HMEC) Senescence and Immortalization in Vitro
Martha Stampfer, James Garbe, Charles Holst, Thea Tlsty, and Paul Yaswen, Lawrence Berkeley National Laboratory, Berkeley, CA, and UC San Francisco, San Francisco CA, presented at the *AACR Special Conference on the Role of Telomeres and Telomerase* in Cancer Dec. 7-11, 2002 in San Francisco, CA

Upregulation of TRF2 during Human Mammary Epithelial Cell Immortalization
Paul Yaswen, Tarlochan Nijjar, Jim Garbe, and Martha Stampfer, Dept. of Cell and Molecular Biology, Lawrence Berkeley National Laboratory, Berkeley, CA, presented at the *AACR Special Conference on the Role of Telomeres and Telomerase* in Cancer Dec. 7-11, 2002 in San Francisco, CA

Overcoming Telomere-based Senescence and Genomic Instability during Human Mammary Epithelial Cell Immortalization
Martha R Stampfer, Tarlochan Nijjar, James Garbe, Sandy DeVries, Fred Waldman, Paul Yaswen, Lawrence Berkeley National Lab., Berkeley, CA; U.C. San Francisco, San Francisco, CA., presented at the *94th AACR Annual Meeting* July 11-14, 2003 in Washington, DC.

Telomerase Repression and Telomere Dysfunction in Human Mammary Epithelial Cell Transformation,
Paul Yaswen, James Garbe, Betty Gardie, Tarlochan Nijjar, and Martha Stampfer, presented at the International Association for Breast Cancer Research, Sacramento, CA, November 1-5, 2003.

Telomerase Reactivation and Genomic Instability during Immortal Transformation of Cultured Human Mammary Epithelial Cells
Martha Stampfer, James Garbe, Koei Chin, Collin Collins, Joe Gray, Fred Waldman, Karen Swisshelm. Thea Tlsty and Paul Yaswen, presented at the conference on Aneuploidy in Cancer, Society for Investigative Research, Oakland, CA, January 23-26, 2004.

Changes in hTERT gene methylation during immortalization of human mammary epithelial cells
Joanna Mroczkowska, Stéphanie Renaud, Martha Stampfer, Jean Benhattar, and Paul Yaswen, presented at the Keystone Symposim on Epigenetic Regulation, Tahoe City, CA, January 21-26, 2004.

Cooperative Changes Resulting in De-repression of Telomerase and Immortalization of Human Mammary Epithelial Cells
Paul Yaswen, James Garbe, Betty Gardie, Tarlochan Nijjar, Genevieve Nonet, Colin Collins, and Martha Stampfer, presented at the 95th AACR Annual Meeting, Orlando, FL, March 28-31, 2004.

Cultured Human Mammary Epithelial Cell Senescence Barriers and hTERT Expression
M. Stampfer, T. Tlsty, A. Bazarov, P.Yaswen, J. Garbe, presented at the 96th AACR Annual Meeting, Anaheim, CA, April, 2005.

Cooperative Interactions During Human Mammary Epithelial Cell Immortalization
Paul Yaswen, James Garbe, Betty Gardie, Tarlochan Nijjar, Genevieve Nonet, Koei Chin, Joe W. Gray, Colin Collins, and Martha R. Stampfer, presented at the DOD Breast Cancer Research Meeting, Philadelphia, PA, June 8-11, 2005.

Papers

Chin, K., Ortiz de Solorzano, C., Knowles, D., Jones, A., Chou, W., Garcia Rodriguez, E., Kuo, W-L. Ljung, B-M., Chew, K., Garbe, J., Myambo, K., Krig, S., Stampfer, M., Yaswen, P., Gray, J.W., and Lockett, S.J. *In situ* analyses of genome instability in breast cancer. Nature Gen. 36: 984-988, 2004.

Stampfer, M, Garbe, J, Chin, K, Collins, C, Gray, J, Waldman, F, Swisshelm. K, Tlsty T, Yaswen, P. Telomerase Reactivation and Genomic Instability during Immortal Transformation of Cultured Human Mammary Epithelial Cells. Cellular Oncology, 26:248-251, 2004.

Rodier, F., Kim, S-H., Nijjar, T., Yaswen, P., and Campisi, J. Cancer and aging: The importance of telomeres in genome maintenance. Int. J. Biochem. Cell Biol.: 37: 977-990, 2005.

Nijjar, T., Bassett, E., Garbe, J., Takenaka,Y., Stampfer, M.R., Gilley, D., Yaswen, P. Accumulation and altered localization of telomere-associated protein TRF2 in immortally transformed and tumor-derived human breast cells. Oncogene 24:3369-3376, 2005.

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Goldstein, J, Rodier, F, Garbe, J, Stampfer, M, Campisi, J, Caspase-independent cytochrome c release is a sensitive measure of low level apoptosis in cell culture models, Aging Cell, in press.

CONCLUSIONS

A high level of telomerase activity, along with telomere-length maintenance, is one of the most common distinguishing features of cancer tissues and tumor-derived immortal cell lines when compared to normal human somatic tissues and finite life span cells. In the absence of high telomerase levels, cells growth arrest or die due to telomere dysfunction before all the errors necessary for invasive cancer can accrue. Our studies are designed to address the crucial question of what errors are responsible for allowing the telomerase reactivation that transforms finite lifespan cultured HMEC to immortality, *in a manner that models changes observed in breast cancers in vivo*. We believe that understanding how telomerase is reactivated in human cells is of critical significance because; (a) overcoming senescence and attaining immortality may be rate-limiting in human carcinogenesis; (b) human and rodent cells have significant differences in telomere biology - the lack of strict telomerase repression and stringent senescence in rodent cells means that they can not model the human mechanisms. A better understanding of the underlying molecular changes involved in telomerase reactivation

may provide novel prevention strategies and/or targets for therapeutic intervention in breast cancer pathogenesis.

The data generated by this grant supports our hypothesis that telomerase is repressed by multiple mechanisms in HMEC. We can repeatedly generate immortal lines lacking detectable genomic copy number alterations by CGH from finite lifespan post-selection HMEC transduced with both *ZNF217* and *c-myc*. However, the clonal nature of these lines suggests that over-expression of *ZNF217* and *c-myc* alone is not sufficient to cause hTERT de-repression and immortalization, even if p53 function is blocked. Other necessary alterations contributing to immortalization remain to be discovered. The exact nature of these alterations may vary depending upon the differentiation/epigenetic state of the targeted cells.

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Appendix 1: Methylation of hTERT promoter and hTERT/TRAP expression

Specimen ID/Cell type	Pass-age	hTERT methylation Dot-blot %	hTERT methylation MS-SSCA %	Genetic instability	hTERT mRNA	TRAP
Finite: Pre-stasis (M85-grown)						
184*	5	0	0	no	0	
184*-p16si	9	0	0	?	0	+/-
Finite: Post-selection						
184v-LXSN-Babe3	9	50	0	no	0	-
184v	12	1-5	0	agonescence	0	-
239 L E1-3	8	10	0	no	0	
239 L E1-3	13	5	0	agonescence	.00065	
161-LXSN	8	0	0	no	0	
161	9	0	0	no	0	-
48RO	6	0	0	no	0	-
Finite: Extended Life (BaP-exposed)						
184Aa	10	0	0	no	0	-
184Aa	16	0	0	agonescence	0	-
Finite: Post-selection transduced with ZNF217 +/or c-myc						
184v-ZNF-Babe2	9	60-70	0		0	-
184v-myc-LXSN1	9	60-70	0		0	-
184v-ZNF-myc1	9	60-70	0		0	-
184@K-ZNF	16	30-40	0		0	-
184@K-myc	15	80	50		.0023	+/-
239-ZNF	12	0	0		.00242	
161-ZNF	9	0	0		.00077	
Immortal Lines						
184@K-HPV16E6	27	90	100	yes	.00043	
184@K-myc						
184@K-myc (*finite*)	15	80	50		.0023	+/-
184@K-myc	29	90	50	yes	.0117	+++
184v-ZNF+myc						
184v-ZNF-myc1 (*finite*)	9	60-70	0		0	-
184v-ZNF-myc1	21	0	0	no	.00026	
184v-ZNF-myc2	33	50	10	no	.0062	
184v-ZNF-myc3	20	20	0	no	2.51	+++
184-ZNF217 series						
184@K-ZNF (*finite*)	16	30-40	0	no	0	-
184ZN4A	22	50-60	0	yes	.00083	-
184ZN4A	28	80	0	yes	.00539	++
184ZN4A	37	90	10	yes	.00463	nd
184ZN4A	48	100	100	yes	.00805	nd
184ZN5	31	80	50	yes	.0036	

184v-ZNF-Babe2 ***(finite)***	9	60-70	0	no	0	-
184v-ZNF (early jackpot)	22	0	0	no	54.25	+++
184Aa-derived lines						
184A1	14	nd	0	no	.00081	-
184A1	56	nd	0	no	.0086	+++
184A1-myc	27	10-20	0		.0144	+++
184AA4	62	50	100	yes	.1077	++
184Aa-derived lines						
184AA2 (p53-)	45	50	100	yes-high	.044	+++
184AA3 (p53-)	43	60-70	100	yes-high		+++
184Aa-derived (myc or ZNF217)						
184Aa-myc1	21	0	0		.000099	++
184Aa-myc2	21	90	100		.01	++
184AaZN1A	35	0	0	yes-low	.007	++
184AaZN1A	55	0	0	yes-low	.000144	++
184AaZN2A	39	0	0	yes-low	.018	++
184AaZN2A	53	0	0	yes-low	.345	++
184Be-derived line						
184B5	43	90-100	100	yes-low	.00082	+++
184B5Y9H	100	10	0		0	-
184B5Y9H	112	10-20	50		.242	++