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PRINCIPAL INVESTIGATOR: Paul Yaswen, Ph.D.

CONTRACTING ORGANIZATION: University of California at Berkeley
Berkeley, California 94720

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California at Berkeley Berkeley, California 94720 E-Mail: p_yaswen@lbl.gov			8. PERFORMING ORGANIZATION REPORT NUMBER	
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13. ABSTRACT (Maximum 200 Words) The candidate oncogene ZNF217 was originally identified based on its core location in an amplicon on chromosome 20q13.2 in breast cancer cell lines and primary tumors. To understand how ZNF217 overexpression contributes to breast cancer progression, in vitro studies were performed on cultured human mammary epithelial cells (HMEC). Expression of a retrovirally transduced ZNF217 gene in normal finite lifespan and carcinogen-treated extended-life HMEC cultures was found to lead reproducibly to immortalization, a potentially rate-limiting step in the progression of human cancers. HMEC that overcame senescence initially exhibited heterogeneous growth and continued telomere erosion, followed by increasing telomerase activity, stabilization of telomere length, and resistance to TGFβ growth inhibition. Comparative genomic hybridization analysis of ZNF217-immortalized cell lines showed common low level regional DNA-sequence copy number variations on chromosomes 1 and 8 that may be sites of genes that cooperate with ZNF217 in facilitating immortalization. The results obtained support the hypothesis that ZNF217 gene amplification is frequently found in breast cancers because it is involved in enabling breast cells to overcome senescence, thus allowing the cells to continue growing and accumulating other changes necessary for malignant progression. ZNF217 may prove to be a clinically useful marker and a novel therapeutic target.				
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TABLE OF CONTENTS

FRONT COVER	1
STANDARD FORM	2
TABLE OF CONTENTS.....	3
INTRODUCTION.....	4
BODY	4-5
KEY RESEARCH ACCOMPLISHMENTS.....	5
REPORTABLE OUTCOMES	5-6
PERSONNEL	6
CONCLUSIONS.....	6
REFERENCES	7
APPENDICES	8-50

INTRODUCTION

The candidate oncogene ZNF217 (previously designated *ZABC1*), predicted to encode alternately spliced Kruppel-like transcription factors, was originally identified based on its core location in an amplicon on chromosome 20q13.2 in breast cancer cell lines and primary tumors, and its recurrent pattern of expression in tumors (1). 20q amplification, common in many human cancers, is also associated with overcoming senescence and p53-independent genome instability in cultured human uroepithelial cells (2, 3). The current experiments were initiated with the aim of determining which specific cellular functions altered by ZNF217 overexpression may be responsible for causing selection of cells with increased gene copy number and tumor progression. Several cell biological assays useful in distinguishing normal HMEC from immortal and tumorigenically transformed cells have been used to compare ZNF217-transduced cells with control cells. Alteration of specific phenotypic properties in HMEC overexpressing ZNF217 will provide direct evidence of the gene's oncogenic potential, and provide information about the biochemical pathways affected. Such information should be valuable in evaluating the prognostic implications of ZNF217 overexpression, and in designing new therapeutic strategies to combat the effects of such overexpression.

BODY

Technical Objective 1: Express ZNF217 cDNA in normal and immortalized HMEC.

The coding sequence of ZNF217 was subcloned into a standard, widely used retroviral vector, LXS_N (4), for efficient uptake and expression in HMEC. High titer amphotropic stocks of ZNF217 and control retrovirus were prepared using a transient packaging system (5) and used to infect recipient HMEC cultures. Northern analysis showed clear evidence of ZNF217 mRNA overexpression, and immunoblot analysis using a crude antibody preparation showed evidence of ZNF217 protein expression, in the infected cells. A separate experiment employing a ZNF217-EGFP fusion construct in a plasmid vector showed preferential nuclear localization of fluorescent signal in transfected COS7 cells, confirming the presence of a functional nuclear localization signal in the ZNF217 sequence. Interestingly, the ZNF217-EGFP fusion protein exhibited a particularly punctate, speckled pattern of fluorescence in some nuclei.

Technical Objective 2: Determine whether ZNF217 expression influences the growth rate of adherent normal HMEC.

Experiments were conducted in finite lifespan HMEC that had or had not received exposure to a chemical carcinogen prior to ZNF217 transduction. Growth rates in complete growth medium, in the absence of EGF, or in the presence of TGF β were compared in cells transduced with ZNF217 or control virus (LXS_N). In each case, no statistically significant differences in growth rates were noted (data not shown).

Technical Objective 3: Determine whether ZNF217 expression extends the replicative lifespan of or immortalizes normal HMECs when expressed alone or in combination with viral oncogenes.

In five independent experiments (see Nonet et al., Cancer Research 61: 1250-1254, 2001 in Appendix 1 for details), ZNF217-transduced cultures maintained growth beyond the point where control cells senesced. HMEC that overcame senescence initially exhibited heterogeneous growth and continued telomere erosion, followed by increasing telomerase activity, stabilization of telomere length, and resistance to TGF β growth inhibition. This pattern is similar to what we have observed in rare HMEC lines immortalized following exposure to a chemical carcinogen, where telomerase reactivation and attainment of good uniform growth occurred in an incremental, apparently epigenetic manner, a process we have termed "conversion," as a consequence of overcoming senescence.

The data demonstrate that constitutive aberrant expression of ZNF217 can immortalize finite lifespan HMEC. However, the precise frequency of immortalization has not yet been determined. Southern analysis of retroviral integration sites in ZNF217-transduced HMEC growing past senescence suggested that these cultures were rapidly overgrown by distinct clonal populations. In an effort to determine whether distinct chromosomal alterations might be conferring growth advantages on clones immortalized with ZNF217, DNA from three different immortalized cultures was used for quantitative measurement of DNA copy number using comparative genomic hybridization (CGH) (6). CGH analysis showed low level regional DNA-sequence copy number variations on chromosomes 1q and 8q common to all three cell lines. The region amplified on 8q included the c-myc oncogene, which itself has been shown to cause HMEC immortalization when overexpressed (7). In addition, each line showed unique regions of high and low level DNA-sequence copy number variations. These sites of regional copy number variation, some of which have also been frequently observed in breast cancer cell lines and primary tumors (6), may contain genes that cooperate with ZNF217 in facilitating growth and immortalization.

Technical Objective 4: Determine whether ZNF217 expression influences the ability of immortalized HMEC to grow under anchorage independent conditions.

Three independently derived ZNF217-immortalized HMEC lines were assayed for anchorage-independent growth by suspension of single cells in methylcellulose and incubation on PolyHEMA-treated plates for four weeks. Colony forming efficiency was found to be < 0.03% for all three lines (data not shown). Moreover, the cells in the few colonies obtained were not enriched for anchorage independence when re-analyzed. This data indicates that although ZNF217 can promote immortalization of HMEC, it does not confer anchorage independence.

Technical Objective 5: Determine whether ZNF217 expression affects growth/differentiation/invasion response to extracellular matrix.

These experiments have not yet been performed.

Technical Objective 6: Determine whether ZNF217 expression influences the ability of HMEC to undergo apoptosis when forced to express adenovirus E1a.

These experiments have not yet been performed.

Technical Objective 7: Determine whether ZNF217 expression alters HMEC tumorigenicity in nude mice.

Three independently derived ZNF217-immortalized HMEC lines were assayed for tumorigenicity in nude mice and SCID mice. For the nude mice, 1.0×10^7 cells per mouse were injected along with Matrigel into 9, 10, and 8 nude mice respectively. Although in each case, the cells initially formed palpable lumps at two weeks after injection, all the lumps started to regress by week 3. By 40 days, there were no signs of tumor growth. For the SCID mice, 2.0×10^7 cells per mouse were injected along with Matrigel into 5 animals each. The SCID mice also showed palpable lesions soon after injection, but these lesions also regressed. We conclude from these experiments that overexpression of ZNF217, by itself, is not sufficient to confer tumorigenicity in normal or carcinogen-treated HMEC.

KEY RESEARCH ACCOMPLISHMENTS

- Overexpression of a retrovirally transduced ZNF217 gene in normal finite lifespan and carcinogen treated extended-life HMEC cultures leads reproducibly to immortalization.
- Immortalization of ZNF217-transduced HMEC occurred without changes in p53 inducibility or function, and without changes in Rb expression.
- Reactivation of telomerase and attainment of uniform good growth +/- TGF β occur incrementally after ZNF217-transduced HMEC have overcome senescence.
- CGH analysis of three cell lines shows common low level regional DNA-sequence copy number variations on chromosomes 1 and 8 that may be sites of genes that cooperate with ZNF217 in facilitating growth and immortalization.
- Overexpression of ZNF217, by itself, is not sufficient to confer anchorage independence or tumorigenicity in HMEC.

REPORTABLE OUTCOMES

Nonet, G.H., Stampfer, M.R., Chin, K., Gray, J.W., Collins, C.C., and Yaswen, P. The ZNF217 Gene amplified in breast cancers promotes immortalization of human mammary epithelial cells. *Cancer Res.* **61**: 1250-1254, 2001.

Stampfer, M.R. and Yaswen, P. Immortal transformation and telomerase reactivation of human mammary epithelial cells in culture, in: *Advances in Cell Aging and Gerontology: Telomerase, Aging and Disease* (M. Mattson and T. Pandita, eds.) Elsevier, Amsterdam, In press.

Abstract - G. H. Nonet, M.R. Stampfer, C.C. Collins, J.W. Gray, and P. Yaswen. Immortal transformation of human mammary epithelial cells following overexpression of ZNF217: a gene amplified and overexpressed in breast cancer. *Proc. Amer. Assoc. Cancer. Res.* **41**, 318, 2000.

Abstract - P. Yaswen., G. H. Nonet, C.C. Collins, J.W. Gray, and M.R. Stampfer. Immortalization of human mammary epithelial cells by ZNF217: a novel gene amplified and overexpressed in breast cancers. *Proc. DOD Breast Cancer Research Program Meeting I*, 83, 2000.

Abstract - P. Yaswen, G. H. Nonet, C.C. Collins, J.W. Gray, and M.R. Stampfer. Human mammary epithelial cell immortalization by ZNF217: a novel gene amplified and overexpressed in breast cancers. *Telomerase and Telomere Dynamics in Cancer and Aging* June 24-28, 2000 San Francisco, CA.

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PERSONNEL

Paul Yaswen, Ph.D., Principal Investigator

Genevieve Nonet, Ph.D., Post-doctoral Research Associate

Tarlochan Nijjar, B.Sc., Graduate Research Assistant

CONCLUSIONS

The results obtained support the hypothesis that ZNF217 gene amplification is frequently found in breast cancers because it is involved in enabling breast cells to overcome the restraints of senescence, thus allowing the cells to continue growing and accumulating other changes necessary for malignant progression. The slow gradual changes in telomerase activity and growth in ZNF217-transduced cells after they have overcome senescence resemble the changes seen during the conversion process in carcinogen-immortalized HMEC, where measurable telomerase reactivation follows rather than precedes the overcoming of senescence. While viral oncogenes HPV E6 and E7 can also immortalize HMEC (8), HPV is not associated with most human cancers, other than those of the cervix. ZNF217 transduction, on the other hand, represents a biologically relevant model for one of the changes involved in immortalization and in cancer progression. Moreover, ZNF217 may prove to be a clinically useful marker as well as a novel therapeutic target.

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APPENDICES

1. Nonet, G.H., Stampfer, M.R., Chin, K., Gray, J.W., Collins, C.C., and Yaswen, P. The ZNF217 Gene amplified in breast cancers promotes immortalization of human mammary epithelial cells. *Cancer Res.* 61: 1250-1254, 2001.
2. Stampfer, M.R. and Yaswen, P. Immortal transformation and telomerase reactivation of human mammary epithelial cells in culture, in: *Advances in Cell Aging and Gerontology: Telomerase, Aging and Disease* (M. Mattson and T. Pandita, eds.) Elsevier, Amsterdam, In press.

The *ZNF217* Gene Amplified in Breast Cancers Promotes Immortalization of Human Mammary Epithelial Cells¹

Genevieve H. Nonet, Martha R. Stampfer, Koei Chin, Joe W. Gray, Colin C. Collins, and Paul Yaswen²

Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720 [G. H. N., M. R. S., P. Y.], and University of California San Francisco Cancer Center, San Francisco, California 94143-0808 [K. C., J. W. G., C. C. C.]

Abstract

The functional consequences of overexpression of a candidate oncogene on chromosome 20q13.2, *ZNF217*, were examined by transducing the gene into finite life span human mammary epithelial cells (HMECs). In four independent experiments, *ZNF217*-transduced cultures gave rise to immortalized cells. HMECs that overcame senescence initially exhibited heterogeneous growth and continued telomere erosion, followed by increasing telomerase activity, stabilization of telomere length, and resistance to transforming growth factor β growth inhibition. The incremental changes in telomerase activity and growth that occurred in *ZNF217*-transduced cultures after they overcame senescence were similar to the conversion pattern we have described previously in rare HMEC lines immortalized after exposure to a chemical carcinogen. Aberrant expression of *ZNF217* may be selected for during breast cancer progression because it allows breast cells to overcome senescence and attain immortality.

Introduction

The candidate oncogene *ZNF217*, predicted to encode alternatively spliced Krüppel-like transcription factors, was originally identified based on its core location in an amplicon on chromosome 20q13.2 in breast cancer cell lines and primary tumors and its recurrent pattern of expression in tumors (1). 20q amplification, common in many human cancers, is also associated with overcoming senescence and p53-independent genome instability in cultured human uroepithelial cells (2, 3). We investigated the functional consequences of *ZNF217* overexpression by transducing the gene into finite life span HMECs³ (4). In four independent experiments, *ZNF217*-transduced cultures gave rise to immortalized cells. HMECs that overcame senescence initially exhibited heterogeneous growth and continued telomere erosion, followed by increasing telomerase activity, stabilization of telomere length, and resistance to TGF- β growth inhibition. The incremental changes in telomerase activity and growth that occurred in *ZNF217*-transduced cultures after they overcame senescence were similar to the conversion pattern we have described previously in rare HMEC lines immortalized after exposure to a chemical carcinogen (5).

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² To whom requests for reprints should be addressed, at Lawrence Berkeley National Laboratory, Mailstop 70A-1118, 1 Cyclotron Road, Berkeley, CA 94720. Phone: (510) 486-4192; Fax: (510) 486-4475; E-mail: P_Yaswen@lbl.gov.

³ The abbreviations used are: HMEC, human mammary epithelial cell; TGF, transforming growth factor; CGH, comparative genomic hybridization; DAPI, 4',6'-diamidino-2-phenylindole; SA β -gal, senescence-associated β -galactosidase; TRF, terminal restriction fragment.

Materials and Methods

HMEC Culture. Finite life span 184 HMECs were obtained from reduction mammaplasty tissue and were cultured in serum-free MCDB 170 medium (Clonetics) as described previously (6, 7).⁴ Extended life span culture 184Aa cells emerged from 184 HMECs after benzo(a)pyrene exposure of primary cultures growing in MM medium as described previously (8). Cells that showed no evidence of growth after 6 weeks were considered to have become senescent. To assay growth heterogeneity of single cell-derived colonies in the absence or presence of TGF- β , cultures were maintained for 14–20 days after seeding 200–1000 cells/100-mm dish. [³H]Thymidine (0.5–1.0 μ Ci/ml) was then added for 24 h 4–7 h after refeeding, and labeled cells were visualized by autoradiography as described (9). Colony forming efficiency was determined by counting the number of colonies containing >50 cells and growth capacity by counting the percentage of labeled nuclei in these colonies. Uniform good growth was defined as a labeling index of >50%. To determine growth capacity in TGF- β , 5 ng/ml TGF- β (R&D Systems) in the presence of 0.1% BSA (Sigma) were added to some cultures for 10–14 days once the largest colonies contained 100–250 cells, and the cultures were then labeled.

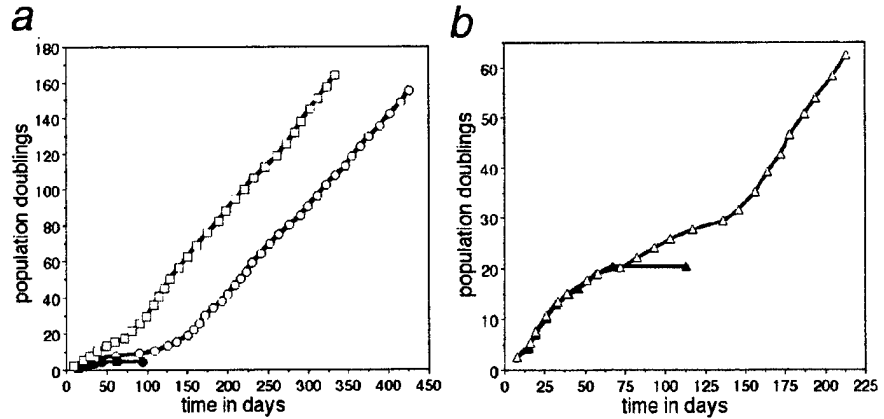
Retroviral Construction and Infection. A 3.1-kb cDNA encoding the complete *ZNF217* open reading frame flanked by a hemagglutinin tag (TAC CCA TAC GAC GTC CCA GAC TAC GCT) and *Eco*RI sites was constructed by PCR using a high-fidelity Taq polymerase (Expand High Fidelity Taq; Boehringer Mannheim). The PCR product was cloned into the *Eco*RI site of the retroviral vector pLXSN (10) using STBL2 competent cells (Life Technologies, Inc.). High-titer amphotropic stocks of *ZNF217* and control retrovirus were prepared using a transient packaging system (11) and used to infect parallel cultures of recipient HMECs. After 24 h in normal medium, cells were selected with G418 (400 μ g/ml) medium for 10 days and subsequently maintained in 100 μ g/ml G418. High *ZNF217* mRNA and protein expression in the retrovirally transduced cells, comparable with that seen in breast tumor cell lines, were confirmed by Northern and immunoblot analyses (data not shown).

Telomerase and Telomere Length Assays. Cell extracts for telomerase assays were prepared by a modification of the detergent lysis method (12). Telomerase activity was measured using the TRAP-EZE telomerase detection kit (Oncor) using 2 μ g of protein/assay. The Sybr Green-stained telomerase products were detected using a Storm 860 fluorescence imager (Molecular Dynamics). DNA for mean TRF analysis was isolated using a genomic DNA isolation kit (Qiagen), and the TRF analysis was performed as described previously (13) with the following modifications. Two μ g of genomic DNA were restriction digested and resolved on a 0.5% agarose gel. The DNA was then transferred to a nylon membrane and hybridized with the ³²P-labeled telomere-specific oligonucleotide (CCCTAA)₄. The ³²P signal was detected using a PhosphorImager (Molecular Dynamics). Mean TRF length was calculated as described (14).

p53 Analysis. Protein lysates were collected in 2 $\times$ SDS lysis buffer (4% SDS, 20% glycerol, and 0.126 M Tris-Cl, pH 6.8) with protease inhibitors (20 μ g/ml aprotinin, 5 μ g/ml leupeptin, and 5 μ g/ml pepstatin A). The lysates were boiled for 10 min and sheared by several passages through 23-gauge needles. Thirty μ g of each protein sample were resolved on a 10% Tris bis-polyacrylamide Nupage minigel (Novex). Protein was transferred to polyvinylidene difluoride membrane, and total p53 protein was detected using the anti-p53 antibody Ab-6 (Oncogene Research). To test for p53-dependent GADD45 expression, subconfluent HMECs were exposed to UV irradiation

⁴ Internet address: <http://www.lbl.gov/~mgrs>.

Fig. 1. ZNF217-transduced HMECs continue to grow indefinitely after control cultures have senesced. The cumulative population doublings of 184Aa (a) or 184 (b) cells infected with either vector alone (LXSN; ●, ■, ▲) or ZNF217 (○, □, △) were plotted against time in days. The LXSN controls senesced and were discarded after >60 days in culture without net increases in cell numbers. In one 184Aa experiment shown, a single immortal clone grew out of an otherwise senescent LXSN-infected population and is not plotted; this clone was distinct from ZNF217-transduced immortal clones in both morphology and growth characteristics. Note that the population doublings indicated are underestimates, because they do not take plating efficiencies into account.



(37 joules/m²). Samples were then collected at 0- and 4-h time points by lysis directly into buffered guanidine thiocyanate solution. Total cellular RNA was then purified, and Northern blots were prepared as described previously (9). Blots were then hybridized with a ³²P-labeled, GADD45-specific probe (15). GADD45 signal was measured and quantitated using a PhosphorImager. The values for relative hybridization were normalized by subsequent hybridization of the blot to a ³²P-labeled probe specific for a constitutively expressed human acidic ribosomal protein transcript (16).

CGH. Genome copy number changes were analyzed as described previously (17). Briefly, DNA samples isolated from normal human lymphocytes and from a test cell line were labeled by nick translation with fluorescein-12-dUTP and Texas Red-dUTP, respectively. Two hundred ng of each DNA probe were mixed with 20 µg of unlabeled Cot-1 DNA and hybridized to normal lymphocyte metaphase spreads for 3 days. The preparations were washed and counterstained with DAPI for chromosome identification. DAPI, fluorescein, and Texas Red images were acquired for several metaphases for each hybridization as described previously (18). Chromosomes were segmented based on the DAPI image, and green:red ratio profiles along the segmented images were calculated for each chromosome. The results from 8 to 10 chromosomes of each type were combined for each hybridization to determine a mean ($\pm 1\sigma$) for each chromosome type. Mean profiles for the 23

chromosome types (the Y chromosome was not analyzed) were arranged from short arm to long arm and from chromosomes 1 to 22, then X, to produce a genome-wide CGH profile.

Results and Discussion

Two HMEC strains were used for these experiments: 184Aa, an extended but finite life span culture obtained from reduction mammaplasty-derived HMECs exposed to a chemical carcinogen (8); and postselection 184, a population of reduction mammaplasty-derived HMECs capable of long-term growth in serum-free medium before reaching senescence (6). The cyclin-dependent kinase inhibitor p16^{INK4a}, thought to serve as one block to immortal transformation, is not expressed in either cell strain because of mutation and/or epigenetic silencing (19). Senescence occurs reproducibly after ~16 passages (~64 population doublings) in 184Aa and 20 passages (~80 population doublings) in 184 HMECs. In Fig. 1a, the cumulative population doublings of transduced cell cultures are plotted against time in culture for two experiments using 184Aa. In both experiments, the ZNF217-transduced cells showed no initial growth advantage over

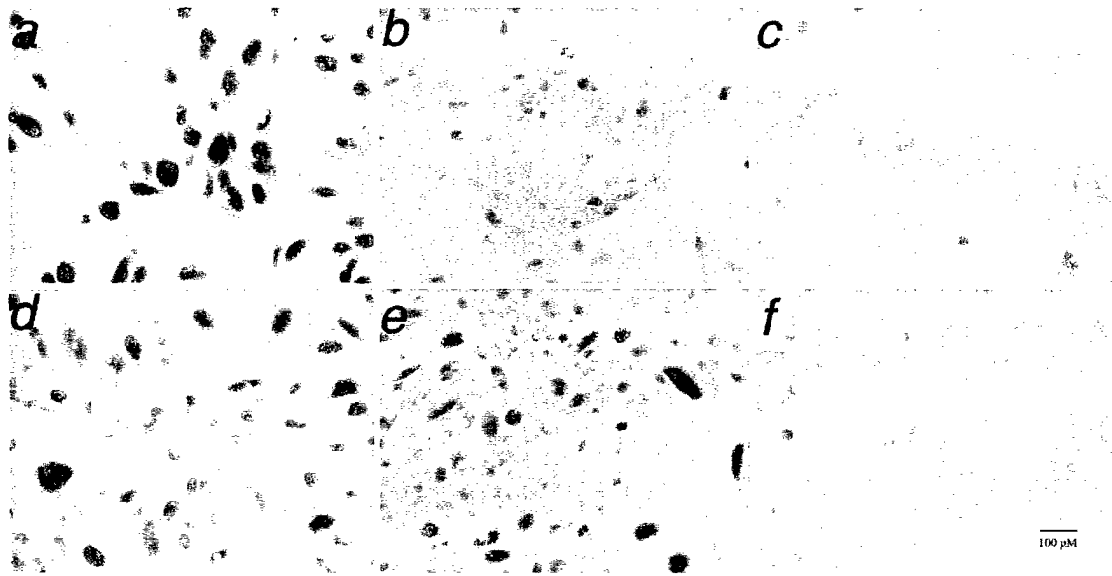


Fig. 2. ZNF217-transduced cultures show gradual loss of SA β -gal activity. 184Aa (a-c) and 184 (d-f) HMECs infected with LXSN control (a and d) or ZNF217-containing (b, c, e, and f) retrovirus were stained for SA β -gal activity (pH 6.0) at the following passages: a, p14; b, p17; c, p35; d, p20; e, p20; f, p25. Control cultures showed large, flat cells with abundant SA β -gal staining when they reached senescence at passages 14-16 for 184Aa cells or passage 20 for 184 cells. At this point, ZNF217 cultures began showing the presence of small, mitotic, SA β -gal-negative cells in a background of positive senescent cells. By later passages, most of the cells were SA β -gal-negative and growing well.

Table 1 Growth of 184ZN4 and AaZN1A colonies at different passage levels in the absence or presence of TGF- β

Single cells (200–10,000) were seeded per 100-mm dish, and the labeling index $\pm$ TGF- β in the ensuing colonies, which contained >50 cells, was determined as described in "Materials and Methods."

Labeling index (%)									
Passage	TGF- β (-)				TGF- β (+)				CFE ^a (%)
	<10	10-25	26-50	>50	<10	10-25	26-50	>50	
184									
13									
LXSN	0	3	14	83	100	0	0	0	9.8
ZNF217	0	9	31	60	95	5	0	0	12.8
17									
LXSN	86	14	0	0	100	0	0	0	7.3
ZNF217	9	16	28	47	100	0	0	0	4.1
20									
ZNF217	4	5	35	56	93	7	0	0	4.8
23									
ZNF217	0	14	0	86	100	0	0	0	0.75
28									
ZNF217	0	0	1	99	100	0	0	0	8.4
43									
ZNF217	0	0	0	100	63	21	5	11	11.8
184Aa									
23									
ZNF217	0	0	0	100	62	11	11	16	5.5
30									
ZNF217	0	0	0	100	71	11	11	7	12.5
44									
ZNF217	0	0	0	100	53	8	13	26	9.5
50									
ZNF217	0	0	0	100	44	3	2	51	16.8

^a CFE, colony forming efficiency.

the control cultures, but while the latter cultures senesced after 50–100 days, the ZNF217-transduced cells continued to grow beyond this point. The control cultures showed large, flat cells with abundant SA β -gal activity (20) when they reached senescence (Fig. 2a). At similar passages, the ZNF217-transduced cultures, termed AaZN1A and AaZN2A, began showing numerous foci of small, mitotic, SA β -gal-negative cells among SA β -gal-positive senescent cells (Fig. 2b). AaZN1A and AaZN2A growth was at first slow and heterogeneous but became faster and more uniform within four to six passages. By later passages, most cells were SA β -gal negative (Fig. 2c) and grew well.

Because 184Aa cultures have given rise to rare immortal clones spontaneously or by insertional mutagenesis (8; Fig. 1 legend),⁵ we repeated the ZNF217 transgene experiments using 184 HMECs, which have never yielded spontaneous immortal clones in numerous experiments using large numbers of cells. Similar to the ZNF217-transduced 184Aa cells, the ZNF217-transduced 184 cells (Figs. 1b and 2, d–f) showed no initial growth advantage over control cells but continued to grow after the control cells senesced, heterogeneously at first, and faster and more uniformly in later passages, producing the 184ZN4 line (Table 1). In the second experiment with 184 HMECs, a single morphologically distinct colony appeared one passage prior to senescence, and again initial passages beyond this point showed very heterogeneous, but continued, growth.

In postselection HMECs that lack p16 expression, as in other cell types, senescence has been correlated with shortened telomeres, and overcoming senescence has been correlated with derepression of telomerase (21, 22). Telomerase activity was not detectable in newly ZNF217-transduced 184Aa and 184 cultures, and mean TRF size, an indicator of telomere length, continued to decrease in the transduced cultures in the initial passages past control cell senescence (Fig. 3). Telomerase activity then increased incrementally, and mean TRF length stabilized at comparatively short lengths, similar to the lengths found in many carcinoma-derived cell lines (23).

All finite life span HMECs cease proliferation in response to TGF- β . In contrast, most immortal and malignant epithelial cell lines can maintain growth in its presence, and this trait is thought to contribute to the malignant phenotype (24). We examined 184ZN4 and AaZN1A at different passages for growth capacity in TGF- β . No growth was seen prior to and just after overcoming senescence (Table 1). However, with increasing passage, some cells capable of maintaining growth in the presence of TGF- β began emerging. This gradual, heterogeneous acquisition of TGF- β resistance is similar to what is observed during conversion of our carcinogen-immortalized HMECs, where the ability to maintain growth in the presence of TGF- β is acquired incrementally in both mass cultures and clonal isolates (5). The incremental nature and reproducibility of this change suggest that it is attributable to epigenetic changes in gene expression after immortalization.

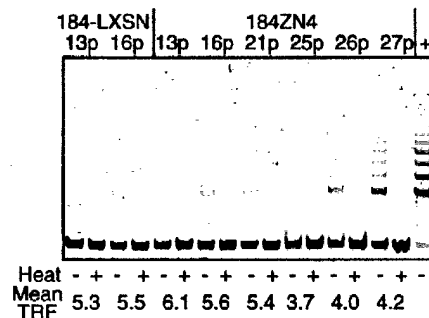


Fig. 3. Finite life span 184 HMECs transduced with ZNF217 show gradual acquisition of telomerase activity and stabilization of telomere lengths. Telomerase activity at indicated passages was measured in 2- μ g extracts of 184 HMECs transduced with LXSN alone (negative control) or LXSN containing the ZNF217 gene (184ZN4). This representative telomerase assay gel reveals the characteristic 6-bp ladder indicative of enzymatic activity that is prominent in an immortalized human kidney cell line (+, positive control) and later passage (26p and 27p) 184ZN4 cells. Heat-treated extracts were used as negative controls. Mean TRF size (an indicator of telomere length) was calculated from Southern blots (data not shown) of genomic DNA harvested from cells at the indicated passages.

⁵ M. R. Stampfer *et al.*, manuscript in preparation.

Loss of function of the tumor suppressors, p53 and pRb, has been observed in numerous immortal cell lines and is thought to play a role in the immortalization process. To determine whether loss of p53 function contributed to the immortalization of the *ZNF217*-transduced HMECs, induction of p53 expression by the DNA-damaging agent actinomycin D was measured. Induction of p53 similar to that in the finite life span cells was observed in all three *ZNF217*-transduced immortalized HMECs tested (Fig. 4a). For a second confirmation of p53 activity, we analyzed p53-dependent induction of GADD45 transcripts by UV irradiation (15). GADD45 mRNA levels were increased 4 h after UV exposure in both the finite life span 184 and immortalized 184ZN4A cultures (Fig. 4b). pRb was also present and underwent normal cycles of phosphorylation and dephosphorylation in these cells (data not shown). Thus, as shown previously for the carcinogen-immortalized HMECs (25, 26), alterations in p53 and/or pRb are not obligatory for immortalization of the *ZNF217*-transduced HMECs.

The above data demonstrate that constitutive aberrant expression of *ZNF217* can immortalize finite life span HMECs, and that overcoming senescence is separable from subsequent changes in telomerase activity and TGF- β resistance. The precise frequency of *ZNF217*-induced immortalization remains to be determined. Southern analysis of retroviral integration sites in *ZNF217*-transduced HMECs growing past senescence suggested that these cultures were rapidly overgrown by distinct clonal populations (data not shown). In an effort to determine whether distinct chromosomal alterations might be conferring growth advantages on clones immortalized with *ZNF217*, DNA from three different immortalized cultures was used for quantitative measurement of DNA copy number using CGH (27). CGH analysis showed low-level regional DNA sequence copy number variations on chromosomes 1q and 8q common to all three cell lines (Fig. 5). The region amplified on 8q included the *c-myc* oncogene, which itself has been shown to cause HMEC immortalization when overexpressed (28). In addition, each line showed unique regions of high- and low-level DNA sequence copy number variations. These sites of regional copy number variation, some of which have also been observed frequently in breast cancer cell lines and primary tumors (27), may contain genes that cooperate with *ZNF217* in facilitating growth and immortalization.

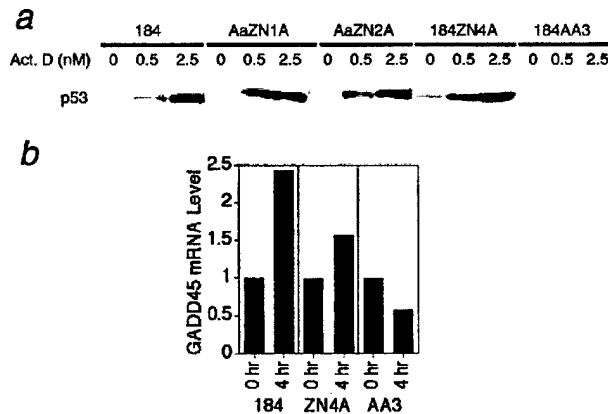


Fig. 4. p53 expression and function are intact in HMECs immortalized after *ZNF217* transduction. **a**, immunoblot of p53 expression in response to DNA damage by 24 h treatment with indicated concentrations of actinomycin D. 184 has wild-type p53. 184AA3 is a negative control HMEC line in which one *TP53* allele has been inactivated by insertional mutagenesis, and the other allele has been inactivated by unknown means.⁵ The cells were assayed at passages 14 (184), 52 (AaZN1A), 49 (AaZN2A), 39 (184ZN4A), and 45 (184AA3). **b**, the relative abundance of GADD45 mRNA in indicated cell types 4 h after exposure to UV irradiation (37 joules/m²) was measured by Northern analysis, normalized to the levels of a ribosomal protein transcript, and is presented in graphical form as induction relative to that in the same cells at 0 h.

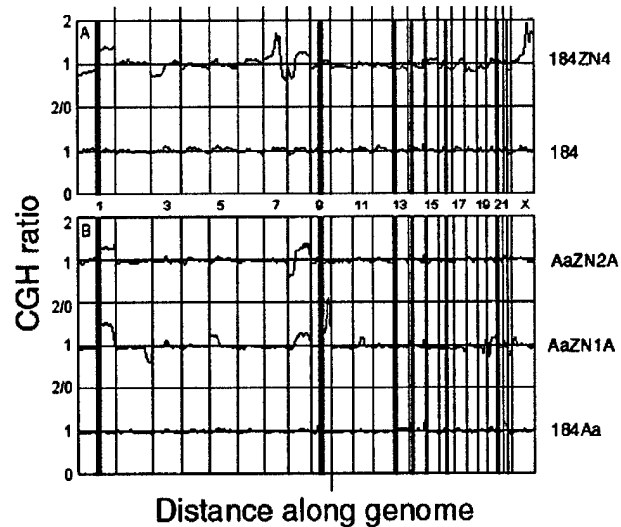


Fig. 5. CGH analyses of genome copy number in HMECs before and after *ZNF217*-induced immortalization. CGH ratios are arranged from short arm to long arm and from chromosomes 1 to 22, then X. Thin vertical black lines, chromosome boundaries. Heavy gray vertical bars, regions containing repeated sequences that were not reliably analyzed in these CGH analyses. Locations of the odd-numbered chromosomes are indicated between A and B. **A**, CGH profiles for finite life span normal 184 HMEC at passage 13 and *ZNF217*-immortalized cell line 184ZN4 at passage 37. **B**, CGH profiles for extended life span 184Aa HMEC at passage 8 and *ZNF217*-immortalized cell lines AaZN1A and AaZN2A at passages 55 and 54, respectively.

These results support the hypothesis that *ZNF217* gene amplification is found frequently in breast cancers because it enables breast cells to overcome senescence, allowing the cells to continue growing and accumulating other changes necessary for malignant progression. The slow gradual changes in telomerase activity and growth in *ZNF217*-transduced cells after they have overcome senescence resemble the changes seen during the conversion process in carcinogen-immortalized HMECs, where measurable telomerase reactivation follows rather than precedes the overcoming of senescence. *ZNF217* now can be added to the small list of cellular (*e.g.*, *c-myc*; Ref. 28) and viral (HPV16 *E6* and *E7*; Ref. 29) oncogenes that cause HMEC immortalization. Unlike *E6*, which may alter several cellular functions simultaneously, both *ZNF217* and *c-myc* immortalize HMECs inefficiently and are likely to require additional changes for immortalizing activity. Further studies are needed to elucidate the mechanism(s) by which *ZNF217* acts. However, one possibility is that *ZNF217* overexpression interferes with one or more checkpoint functions that normally operate to eliminate senescing cells [*e.g.*, the p53-dependent, DNA damage-sensing pathway that prevents continued proliferation with short telomeres (30)].

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CHAPTER 6

IMMORTAL TRANSFORMATION AND TELOMERASE REACTIVATION OF HUMAN MAMMARY EPITHELIAL CELLS IN CULTURE

MARTHA R. STAMPFER and PAUL YASWEN

Table of contents

1.	Introduction	104
2.	Growth of finite lifespan HMEC <i>in vitro</i>	105
2.1.	Growth properties of finite lifespan HMEC	105
2.2.	Senescence of finite lifespan HMEC	106
2.3.	Extended life HMEC following exposure to the chemical carcinogen benzo(a)pyrene	107
3.	Conversion of conditionally immortal p53(+) HMEC lines	108
3.1.	Telomerase reactivation and mean TRF length stabilization of carcinogen-immortalized p53(+) HMEC	108
3.2.	Expression of p57 ^{KIP2} in conditionally immortal p53(+) HMEC and loss of p57 expression in fully immortal HMEC	109
3.3.	TGF β -induced growth inhibition in conditionally immortal HMEC and gradual gain of resistance during conversion to full immortality	110
3.4.	Other molecular changes associated with conversion	111
3.5.	Immortalization of HMEC with the putative breast cancer oncogene, ZNF217.	112
3.6.	Telomerase control in fully immortal HMEC	114
4.	Immortalization of p53(-) HMEC	114
4.1.	Derivation of p53(-/-) immortal lines	114
4.2.	Attainment of full immortality in p53(-) HMEC	115
4.3.	Effect of inhibition of p53 function	116
5.	Effect of hTERT on growth of finite lifespan and conditionally immortal HMEC ..	117
6.	Immortalization of HMEC with viral oncogenes	119
7.	An overall model of HMEC immortalization and telomerase reactivation	121
7.1.	Alterations involved in immortalization of HMEC by different methods	121
7.2.	Potential role of conversion <i>in vivo</i> carcinogenesis	125
8.	Conclusion	126
9.	References	127
	Figures	211

1. Introduction

High levels of telomerase activity are one of the most consistent distinguishing features of cancer tissues and tumor-derived immortal cell lines when compared to normal human somatic tissues and finite lifespan cells. The attainment of unlimited proliferative potential conferred by telomerase activity is thought to be critical for malignant progression because tumor cells need to undergo sufficient cell divisions to accumulate the multiple errors necessary for invasive and metastatic potential. In the absence of high telomerase levels, replicative senescence halts growth before such error accumulation is possible. The cellular senescence normally observed in somatic cells from long-lived species such as humans may therefore have developed as a tumor suppressor mechanism.

The studies to be described in this chapter concern the mechanisms by which normal, finite lifespan human mammary epithelial cells (HMEC) overcome replicative senescence and activate telomerase activity. We suggest that these steps may be key rate-limiting events in human breast carcinogenesis. Figure 1 summarizes our current model for the processes involved in HMEC growth and immortalization. In our HMEC culture system, attaining an indefinite proliferative potential requires cells first to overcome two senescence barriers. The first barrier can be overcome by alterations in the pathways governing RB. In culture, the most common means by which this is effected is the downregulation of the cyclin dependent kinase inhibitor (CKI) p16^{INK4a}. The second barrier appears to be regulated by telomere length, and is extremely stringent. It involves generation of abnormal metaphases and mitotic catastrophes. The means by which the second barrier is overcome have not yet been fully defined. Loss of p53 function appears to contribute to this process, but is not required or sufficient. Likely, more than one error is necessary. However, even after overcoming both senescence barriers, the resultant cells with indefinite proliferative *potential* must still progress through further changes. In HMEC that retain p53 function, cells proceed through a gradual epigenetic process, which we have termed conversion, in order to reactivate telomerase and attain uniform good growth. Conversion appears to be triggered by the critically short telomeres that occur in p53(+) HMEC that maintain proliferation after overcoming stringent senescence. In HMEC immortalized with loss of p53 function, the conversion process is greatly accelerated, and some aspects are not expressed. As a result, uniformly good-growing telomerase-expressing cells appear much more rapidly.

Our observations of cultured HMEC will be discussed in the context of what is known about human breast carcinogenesis *in vivo*. We propose that attainment of short telomeres may play an essential role in human carcinoma progression. Additionally, we describe how different methods of producing immortal HMEC can yield cell lines with significantly different histories and phenotypes. These methods may vary in the extent to which they model human malignant progression *in vivo*.

2. Growth of finite lifespan HMEC *in vitro* (see Figure 2 for schematic chart)

2.1. Growth properties of finite lifespan HMEC

Large quantities of finite lifespan HMEC are readily obtained as discard material of the commonly performed surgery for reduction mammoplasty. Epithelial and isogenic fibroblast cells can be isolated and stored frozen for repeated experiments with cells from the same individual [1, 2]. Our laboratory has introduced two types of culture media for long-term growth of cultured HMEC. The earliest studies used MM medium, which contains serum, growth factors, and conditioned media from other human cell lines [1, 3]. MM supports active HMEC growth for 3-5 passages, or ~15-25 population doublings [4]. As the population becomes non-proliferative, larger, flatter senescence-associated β -galactosidase (SA- β -gal) positive cells predominate. Cultures that maintain growth beyond passage 5 display uneven proliferation. Pockets of small, actively growing cells may appear, but these cells soon become larger and less proliferative.

MM-grown cultures show a mixed morphology and cell composition. Cells with both basal (keratin 5/14 positive, α -actin positive, keratin 19 negative) and luminal (keratin 8/18/19 positive, polymorphic epithelial mucin positive) markers are initially present [5]; however, the keratin 19 positive cells show little proliferative potential in culture. As the population becomes non-proliferative, expression of the CKI p16 increases sharply [6]. The mean TRF (terminal restriction fragment) length of the largely growth-arrested population is ~6-8 kb [7, 8].

In collaboration with Richard Ham's laboratory, a serum-free medium, MCDB 170, was developed for HMEC. MCDB 170 supports a mixed cell population in primary cultures, but cells that maintain proliferation express a predominately basal phenotype [5]. Active division of cobblestone-appearing cells occurs for 2-3 passages. The cells then gradually become larger and flatter, with expression of SA- β -gal and reduced proliferative capacity. High levels of p16 are also present in this growth-arrested population, and the mean TRF is ~6-7 kb [6, 7].

In MCDB 170, a small subpopulation of cells is able to spontaneously overcome this p16-associated growth arrest, which we originally termed selection [9]. After 1-3 weeks with no visible mitotic activity, pockets of actively proliferating cobblestone-appearing cells appear. These cells no longer express p16 and show specific methylation of the p16 promoter [6]; they also contain wild-type p53 with a longer half-life [10]. We have called the process associated with p16 down-regulation, self-selection, and the p16(-) cells, post-selection HMEC [9]. Although selection was later referred to as M0 in the context of viral oncogene transformation [11], we believe it most closely resembles the previously described M1 senescence block (see below) [8, 12]. The absence of spontaneous p16 downregulation in MM vs. MCDB 170-grown HMEC indicates the influence of culture conditions on this process. We have also noted that even in MCDB 170, HMEC from some individual specimen donors required a cAMP stimulator to generate p16(-) post-selection cells. Epigenetic silencing of p16 has been associated with many human cancers, and *de novo* methylation of the p16 promoter has been found in approximately a third of primary breast tumors and breast cell lines [13].

Depending upon the individual specimen, post-selection cells may proliferate for an additional 30-70 population doublings before reaching a second block to further growth. Growth slows around 3 passages prior to the passage at which no further net gains in cell number are observed, with increasing numbers of SA- β -gal positive cells displaying a larger, vacuolated, but still cobblestone epithelial morphology. This block is extremely stringent, as we have never observed any HMEC spontaneously able to overcome this block and maintain growth, although many cultures from over 20 individuals have reached this block. The mean TRF of this growth-arrested population is ~4-5 kb [14].

The finite lifespan HMEC cultures have been examined for telomerase activity using the TRAP assay. We have never observed any activity in the post-selection HMEC [14]. However, in MM-grown HMEC from some individuals, a low level of telomerase activity has been detected [7]. Similar results have been reported from other laboratories [15, 16].

2.2. Senescence of finite lifespan HMEC

Recent studies in Thea Tlsty's laboratory have shed new light on the nature of the two growth arrest barriers encountered by the HMEC (see Figure 1 for a schematic outline of overall HMEC growth, senescence, and immortalization) [8]. Experiments were performed directly comparing isogenic human mammary epithelial and fibroblast cells. Both cell types showed an initial growth arrest associated with high levels of p16 and SA- β -gal expression. In growth-arrested cultures, there was little ongoing DNA synthesis, with fewer than 2% of cells incorporating bromodeoxyuridine during a four-hour pulse, little cell death, with only 1% Annexin-V staining, and retention of normal diploid karyotypes. The arrested cells displayed a FACS DNA profile consistent with G0/G1 arrest, with a 2N to 4N ratio of ≥ 4 . When directly compared, both cell types arrested with a mean TRF of ~6 kb. The fibroblasts differed from the epithelial cells in displaying a larger number of population doublings (~40) before reaching this growth arrest, and never showed spontaneous escape from this arrest. In fibroblasts, this arrest has been previously termed senescence or M1. Given the overall similarities between the two cell types at this growth arrest, we suggest that the selection block in HMEC is most equivalent to what has been called senescence in human fibroblasts [8]. Whether or not a short telomere length is critical for triggering this block has not been definitively determined for HMEC. The nature of the culture conditions, as well as possibly other unknown variables, clearly have an influence on both how many population doublings occur before this block is engaged, and whether some HMEC can overcome this block.

The growth arrest encountered by the p16(-) post-selection HMEC population is notable for its extreme stringency in culture. Cytogenetic analysis of post-selection HMEC showed gross chromosomal abnormalities in metaphase spreads beginning 10-20 population doublings before the final passage. The abnormalities included abundant telomeric association and chromosome fusion and breakage events, polyploidy and aneuploidy. This onset of genomic aberrations coincided with the slow-down in proliferation rate. At the point of no net proliferation, 100% of metaphases exhibited structural abnormalities. Further analysis indicated that the post-selection HMEC at

the final passage showed ~16% bromodeoxyuridine incorporation, ~20% staining with Annexin-V, a 2N to 4N ratio of ~1, and a substantial polyploid subpopulation. There was no net increase in cell number since continued DNA synthesis was associated with mitotic catastrophes and resulted in cell death or non-proliferative multi-nucleated cells. This second senescence barrier has been termed agonescence [17].

On gross inspection, HMEC at agonescence exhibit long-term viability. Metabolically active cultures have been maintained for over a year. This overall viability contributed to the assumption that this second growth arrest was similar to the previously reported viable senescence block in fibroblasts, and unlike the viral oncogene-induced crisis or M2 stage [18]. However, the more recent studies offer a new perspective. *In vitro* model systems of crisis have utilized cells lacking functional RB and/or p53, e.g., cells exposed to SV40 LgT or Human Papilloma Virus (HPV)16 E6/E7, or derived from Li-Fraumeni patients. *In vivo*, normal cells would retain p53 and RB function upon reaching senescence barriers, as do our HMEC cultures at agonescence. Unlike cells at crisis, the HMEC at agonescence do not spontaneously transform to immortality in culture. Similar to crisis, there are widespread chromosomal aberrations, as well as some ongoing DNA incorporation. However, the percentage of cells showing DNA incorporation is significantly lower than in crisis [19], and most cells remain arrested in G1 or G2. The retention of wild type p53 may constitute the difference between the largely viable agonescence observed in post-selection HMEC, and the crisis seen in cells lacking functional p53. The nature of the agonescence block can account for the observed stringent senescence in cultured human epithelial cells; cells which fail to maintain a G1 or G2 arrest at agonescence will eventually die or become non-proliferative via mitotic catastrophe. Unlike an arrest based upon blocking cell cycle progression (e.g., elevated levels of CKIs), the agonescence barrier involving structural failures at mitosis can not be readily overcome and is irreversible. This block also appears to be tightly correlated with telomere length, and thus may serve as a telomere-based clock for limiting cell division.

2.3. Extended life cultures following exposure to the chemical carcinogen benzo(a)pyrene

With the goal of developing a series of progressively transformed HMEC from one individual, primary cultures of MM-grown cells from specimen 184 were exposed to the chemical carcinogen benzo(a)pyrene [4, 20]. We chose to use a chemical carcinogen to induce transformation because we wished to develop a model system that might as closely as possible model human breast carcinogenesis *in vivo*. Chemical carcinogens were known to induce mammary carcinomas in rodents, benzo(a)pyrene is an abundant pollutant in the environment, and we first ascertained that HMEC could metabolize the pro-carcinogen, benzo(a)pyrene, to its active carcinogenic form [21, 22].

MM-grown cells normally cease active growth by passage 5, with high expression of p16, and without spontaneous down-regulation of p16. In the benzo(a)pyrene-treated cultures, there were numerous instances in which growth was extended. These extended life (EL) cells showed heterogeneity with respect to morphology and growth potential. Subsequent testing indicated that all EL cultures examined did not express p16 and

contained a stable form of p53. In only one case tested, EL 184Aa, was the absence of p16 expression due to a detectable mutation. These data provide additional evidence supporting the role of p16 in mediating the first senescence barrier and implicating it as a possible target of carcinogen-induced changes.

In almost all cases, the EL cultures subsequently ceased proliferation. Mean TRF at this growth arrest was ~4-5 kb, similar to the value of post-senescent HMEC at agonescence. Although we have not yet examined the karyotypes of growth-arrested EL cultures, it is most likely that they too are aberrant. However, unlike the post-selection cells, these cultures have been exposed to a carcinogen, and may harbor unknown derangements in addition to their down-regulation of p16. Consequently, they also differ from post-selection cells in their ability to give rise, very rarely, to immortally transformed lines, and in their increased ability to immortalize when exposed to further immortalization promoting agents (see Section 7).

3. Conversion of conditionally immortal p53(+) HMEC lines (see Figure 3A for schematic chart)

3.1. Telomerase reactivation and mean TRF length stabilization of carcinogen-immortalized p53(+) HMEC

We have observed three instances where EL cultures gave rise to HMEC lines of indefinite lifespan without any further treatment [2, 23]. Two lines, 184A1 and 184AA4, were derived from the EL 184Aa culture and were first detected at passages 9 and 14 respectively. One line, 184B5, appeared in the EL 184Be culture at passage 6. Karyotypic analysis indicated distinct clonal origins, and a very low level of genomic instability, in both 184A1 and 184B5 [24]. 184AA4 exhibited numerous abnormal karyotypes when first assayed; however later passages contained fewer abnormalities [23]. Like most breast tumor cells, none of these lines has a known defect in the expression or phosphorylation of RB, or in the sequence of p53 [10, 25]. Similar to their finite lifespan EL precursors, these lines lack expression of p16 and contain a stable form of the p53 protein. None of the lines displays sustained anchorage-independent growth, growth factor independence, or tumorigenicity. These lines allow examination of the process of human cell immortalization without the possible confounding factors of malignant transformation, genomic instability, or viral oncogene exposure.

Although mass cultures of 184A1 and 184B5 consistently displayed an indefinite lifespan, we were initially puzzled to note that many individual cells in these immortal lines lost proliferative potential during early passage culture. Careful examination of these p53(+) lines then uncovered the process of conversion, a previously undescribed, presumably epigenetic step in the transformation of HMEC [14]. Early passage cells of the immortal, p53(+) 184A1, 184AA4, and 184B5 lines show no telomerase activity and have continued telomere erosion with passage (Figure 4A,B). When the mean TRF declined to ≤ 2.5 kb, low levels of telomerase were first detectable. Telomerase levels gradually increased thereafter, and the mean TRF stabilized at 3-7 kb. During the prolonged period when the mean TRF was ≤ 3 kb, and before telomere length was stabilized, growth in

culture was heterogeneous and slow (see Figure 4C). Since many individual cells did not maintain proliferation, we termed early passage cultures of these lines "conditionally immortal." As we have subsequently seen, several variables can influence the percentage of, and speed with which, conditionally immortal cells are able to manifest their pre-existing potential for uniformly good-growing indefinite proliferation. Conversion of telomerase(-) conditionally immortal HMEC to telomerase(+) uniformly good-growing fully immortal HMEC is reproducibly observed in both mass cultures and repeatedly subcloned populations of these early passage p53(+) HMEC lines. This observation, along with the very gradual nature of conversion, suggests an epigenetic rather than a mutational cause.

Our studies of the past few years have indicated that the conversion of telomerase(-) conditionally immortal HMEC to telomerase(+) fully immortal HMEC is accompanied by a variety of alterations in cellular biology, as discussed below.

3.2. Expression of p57^{KIP2} in conditionally immortal p53(+) HMEC and loss of p57 expression in fully immortal HMEC

The presence of a slow growth phase in the conditionally immortal HMEC led us to examine these cells for expression of growth inhibitory molecules. Examination of the CKI p57 mRNA and protein found a tight association between p57 expression and slow growth [26]. p57 belongs to the CIP/KIP family of CKIs which also include p21 and p27. The p57 gene has been localized to chromosome 11p15.5, a region displaying frequent allelic loss in cancers of the breast, lung, and bladder [27]. LOH and microsatellite instability at 11p15 have been associated with rapid proliferation, DNA aneuploidy, and poor prognosis in primary breast tumors [28]. In epithelia *in vivo*, p57 is reported to be expressed in regions of differentiated cells, but not in regions of actively dividing cells, suggesting that p57 may be up-regulated when cells exit the cell cycle and start their differentiation programs. p57 is not detectable in most immortal cell lines. Our examination of p57 expression in our cultured HMEC has found the following [26]:

- a) No p57 mRNA was detected in finite lifespan HMEC, including MM-grown and post-selection HMEC, and the EL cultures 184Aa and 185Be.
- b) p57 was expressed in conditionally immortal p53(+) HMEC arrested in G0 by blockage of EGF receptor signal transduction. When the mean TRF was > 3 kb and the cells displayed good growth, p57 was down-regulated upon mitogenic stimulation and entry into G1. (Figure 5A).
- c) When the mean TRF of conditionally immortal p53(+) HMEC declined to ≤ 3 kb, p57 expression remained high even after mitogenic stimulation and exit into G1 (Figure 5B). This failure to down-regulate p57 coincided exactly with the beginning of the slow, heterogeneous growth phase.
- d) The level of p57 expression in G0 and cycling populations was gradually reduced as the conditionally immortal cells underwent conversion and gradually regained uniform good growth. Fully immortal HMEC lines expressed little or no detectable p57 in G0 or when cycling (Figure 5C).

We do not yet know what is responsible for the initial activation of p57 expression in G0-arrested conditionally immortal HMEC. Perhaps the events that enable cells to overcome agonescence give rise to p57 expression. Finite lifespan HMEC with mean TRF lengths equivalent to those in early passage 184A1 do not express p57, indicating that telomere length alone does not dictate p57 expression. We do know that the expression of p57 is dependent upon the expression of p53 (see Section 4 below).

The failure to down-regulate p57 after release from G0 appears tightly correlated with the telomere length of conditionally immortal HMEC, suggesting that development of extremely short telomeres (mean TRF ≤ 3 kb) causes changes resulting in increased steady state p57 levels. When the mean TRF of early passage pre-conversion 184A1 is kept > 3 kb by transduction with agents that provide or reactivate telomerase activity (e.g., hTERT, or inhibitors of p53 function), p57 is not expressed during G1, the level of p57 in G0-arrested cells declines, and the cell populations never undergo a prolonged period of slow heterogeneous growth [23, 26].

The loss of p57 expression as cells convert to fully immortal HMEC can involve both genetic and epigenetic mechanisms. The p57 gene is imprinted with preferential expression of the maternal allele [29]; loss of the maternal allele by itself can severely reduce p57 expression. The conditional immortal growth constraint exhibited by MCDB170-grown 184A1 during passages 16-20 is particularly severe, and the major (presumably maternal) p57 allele is frequently lost during this period. Deletion of the initially expressed p57 allele coincides with upregulation of the previously imprinted paternal p57 allele [26]. We do not know how expression of this remaining p57 allele is lost as 184A1 gains full immortality. Where the slow growth phase is not as severe, as in 184B5, and in 184A1 grown in the nutritionally richer MM medium, no p57 allele loss is detected. Preliminary results suggest that, in these cases, downregulation of p57 expression is accompanied by increasing methylation of the p57 genes.

Collectively, these results indicate that p57 is expressed by p53(+) HMEC that have recently overcome agonescence. The data suggest that p57 may play an important role in the observed slow heterogeneous growth of conditionally immortal HMEC when the mean TRF declines to ≤ 3 kb, thus inhibiting the conversion of conditionally immortal cells to the fully immortal state. The absence of p57 expression in almost all human tumor-derived cell lines is consistent with the hypothesis that downregulation of p57 is required for full immortalization. As discussed later, if a conversion process occurs during *in vivo* breast carcinogenesis, the expression of p57 in p53(+) cells could have a significant influence on cancer growth and progression.

3.3. TGF β -induced growth inhibition in conditionally immortal HMEC and gradual gain of resistance during conversion to full immortality

Malignant progression in human carcinomas is commonly associated with acquiring the ability to maintain growth in TGF β [30]. In some cases, this acquisition can be attributed to loss of functional TGF β receptors or other mutations in the associated signal transduction pathways, but in most instances, including most breast cancers, no such mutations are detected. In contrast to carcinoma-derived cells, cells derived from normal human epithelial tissues are severely growth inhibited by TGF β . We have not

observed any finite lifespan HMEC with the ability to maintain growth in the presence of TGF β . However, all the fully immortal lines can maintain growth in TGF β , although the rate of growth is generally somewhat slower in its presence. To emphasize this distinction in TGF β growth responsiveness of finite lifespan vs. fully immortal HMEC, we have defined TGF β resistance as the ability to maintain growth in TGF β , even with reduced growth rates. Our fully immortal HMEC that maintain active growth in TGF β have not lost the ability to recognize and respond to TGF β . They maintain expression of TGF β receptors and respond to TGF β with increased protein synthesis and secretion, including induction of extracellular matrix associated proteins such as fibronectin and plasminogen activator inhibitor 1 [31]. In this manner, they resemble mesenchymal cells that can respond to TGF β while maintaining active growth. Possibly, the metabolic demands of the greatly increased protein synthesis, as well as other aspects of the cells' on-going TGF β responsiveness, may be indirectly affecting the fully immortal HMEC growth rate in presence of TGF β .

Examination of conditionally immortal HMEC indicated an absence of any ability to maintain growth in the presence of TGF β at the earliest passages. As the conversion process proceeded, and low levels of telomerase became detectable, there was a gradual increase in the number of cells with progressively better growth capacity in TGF β . For example, in the 184A1 line (see Figures. 4C & 8B), only rare cells capable of maintaining very poor growth in TGF β were present by around passage 28. By passage 44, ~75% of cells were capable of good growth in TGF β . In general, uniform good growth in the presence of TGF β was present around 10-20 passages after telomerase activity was first detectable. Subsequent experiments (see Section 5 for more details) have shown that expression of an exogenously introduced hTERT gene induces the gain of TGF β resistance in these HMEC [32]. This induction of TGF β resistance by hTERT expression may account for the tight correlation between telomerase activity and TGF β resistance in isolated clones of conditionally immortal HMEC in the process of conversion [14].

Since our assays for TGF β resistance allow us to examine the fate of individual cells, we could use this phenotype to acquire information about the heterogeneity of conditionally immortal HMEC. One of the more remarkable aspects of conversion is the degree to which heterogeneity is rapidly generated from repeatedly cloned populations [14]. Thus, single cell-derived colonies at passage 25 of a subclone derived at passage 20 from a clone derived at passage 15 of the clonally-derived line 184B5 varied from showing none to uniform good growth in TGF β . By passage 38, this subclone displayed uniform good growth in TGF β . Any mechanistic explanation for conversion must be able to account for this rapidly generated heterogeneity.

3.4. Other molecular changes associated with HMEC conversion

The conversion of HMEC to full immortality appears to be a complex set of interactive changes. Our laboratory has been engaged in defining what these changes are, and in determining the nature of the interactive processes. The following are some additional cellular phenotypes that change in the process of attaining full immortality:

- a) Finite lifespan HMEC show reduced levels of the proto-oncogene c-myc mRNA and protein when they are arrested in G0 [33]. The fully immortal lines display equivalent levels of c-myc in G0-arrested and cycling cell populations. Early passage conditionally immortal HMEC behave like the finite lifespan HMEC, but then gradually show increased levels of c-myc in G0 as conversion progresses. The overall level of c-myc expression in the cycling population is also increased in fully immortal HMEC.
- b) Finite lifespan cells have been reported to be growth inhibited when exposed to overexpressed H-Ras or Raf-1, whereas the growth of immortal cells is often enhanced under the same conditions [34, 35]. We have utilized a retroviral expression vector encoding the catalytic domain of human Raf-1 fused to the hormone-binding domain of the human estrogen receptor, to demonstrate that our p16(-) finite lifespan HMEC are also severely growth inhibited when oncogenic Raf-1 is induced with 4-hydroxy-tamoxifen [Olsen, Yaswen, & Stampfer, in preparation]. In contrast, the fully immortal lines transduced with oncogenic Raf-1 are able to maintain growth, even in the absence of signal transduction through the EGF receptor, and gain some anchorage independent growth. Assay of good-growing pre-conversion 184A1 indicated that these cells are still severely growth-inhibited by oncogenic Raf-1. As has been reported for other cell systems [36], hTERT-immortalized HMEC, which do not undergo conversion, are also growth inhibited by oncogenic Raf-1 [Olsen, Yaswen, & Stampfer, in preparation]. Thus, the change in response to oncogenic Raf-1 occurs not with the overcoming of agonescence, or by acquiring telomerase activity, but as a consequence of the conversion process to full immortality.
- c) Actively growing finite lifespan HMEC do not express SA- β -gal, while cells arrested at the selection/M1/senescence and at the agonescence blocks, are all positive for SA- β -gal staining. Curiously, the good-growing pre-conversion conditionally immortal HMEC (mean TRF > 3 kb) remain positive for SA- β -gal. Conditionally immortal HMEC retain SA- β -gal staining as conversion begins, and then gradually lose expression of SA- β -gal as conversion proceeds [23]. Fully immortal HMEC are largely negative for SA- β -gal, though some positive staining cells are present.

Altogether, these studies indicate that major changes occur in cellular signal transduction pathways as HMEC transform from finite lifespan to full immortality. It is likely that further examination will uncover additional cellular changes. These differences between finite lifespan, conditionally immortal, and fully immortal HMEC could have implications for the design of therapeutic interventions in cancer. They also serve as a reminder that immortally transformed cells may not model normal finite lifespan cell behavior in key pathways.

3.5. Immortalization of HMEC with the putative breast cancer oncogene, ZNF217 (see Figure 3B for schematic chart)

More recent studies have used another potentially pathologically relevant means, the candidate oncogene ZNF217, to immortally transform finite lifespan HMEC [37].

ZNF217 was originally identified based on its location on chromosome 20q13.2, an amplicon common in breast cancers and associated with poor prognosis [38]. Extra copies of this chromosomal region occur in approximately 18% of breast tumors and 40% of breast cancer cell lines [39]. ZNF217 encodes a conserved member of the C2H2 Kruppel family of transcription factors, with a DNA-binding domain of eight C2H2 zinc fingers and a separate proline-rich domain. Members of the Kruppel family have been implicated in both neoplastic and developmental disorders [40].

We investigated the functional consequences of ZNF217 overexpression by transducing the gene into finite lifespan 184 HMEC and EL 184Aa. In five independent experiments, ZNF217-transduced cultures gave rise to immortalized cells. The ZNF217-transduced HMEC showed no initial growth advantage over the control cultures, but continued to grow beyond the point where the control population growth arrested at the agonescence barrier. Numerous foci of small, mitotic, SA- β -gal negative cells appeared among the SA- β -gal positive agonescent cells. Growth was at first slow and heterogeneous, but became faster and more uniform with continued passage. After ~5-15 passages, varying among experiments, most cells were SA- β -gal negative and grew well.

Telomerase activity was not initially detectable in the ZNF217-transduced 184 and 184Aa cultures that maintained growth past agonescence, and the mean TRF length continued to decrease (Figure 6). Telomerase activity was detectable within 10 passages and then gradually increased, and mean TRF length stabilized at ~4 kb. When assayed for growth in TGF β , ZNF217-transduced 184 and 184Aa were initially completely growth-inhibited prior to and just after overcoming agonescence. With increasing passage, there was a very gradual increase in the number of cells with progressively better growth capacity in TGF β . Assay for p57 showed some expression in G0-arrested and cycling populations in the earliest passages after overcoming agonescence, when growth was slow and heterogeneous, but none in the later good-growing cultures.

Southern analysis of retroviral integration sites in ZNF217-transduced HMEC growing past agonescence suggested that these cultures were rapidly overgrown by distinct clonal populations. To determine whether distinct chromosomal alterations might be conferring growth advantages on clones immortalized with ZNF217, DNA from three different immortalized cultures was used for quantitative measurement of DNA copy number using comparative genomic hybridization [39]. Analysis showed low level regional DNA-sequence copy number variations on chromosomes 1q and 8q common to all three cell lines. The region amplified on 8q included the c-myc oncogene. In addition, each line showed unique regions of high and low level DNA-sequence copy number variations. These sites of regional copy number variation, some of which have also been frequently observed in breast cancer cell lines and primary tumors [39], could contain genes that cooperate with ZNF217 in facilitating immortalization.

To determine whether loss of p53 function contributed to the immortalization of the ZNF217-transduced HMEC, p53 function was assayed by measuring p53 expression after exposure to the DNA damaging agent actinomycin D, and p53-dependent induction of GADD45 transcripts following UV irradiation. Induction of p53 similar to that in the finite lifespan cells was observed in all three ZNF217-transduced immortalized HMEC tested, and GADD45 mRNA levels were increased 4 hrs. after UV exposure in

both finite lifespan 184 and ZNF217 immortalized 184Aa. pRB was also present and underwent normal cycles of phosphorylation and dephosphorylation in these cells.

Thus, the ZNF217-immortalized HMEC showed many similarities to the immortal HMEC lines derived by carcinogen exposure. Alterations in p53 and/or RB were not obligatory for immortalization. Most significantly, these p53(+) immortal lines underwent a conversion process before attaining a fully immortal phenotype. There was a gradual reactivation of telomerase activity and an incremental gain of TGF β resistance, and a transient expression of p57 associated with a period of slow heterogeneous growth.

3.6. Telomerase control in fully immortal HMEC

Telomerase-expressing cells, from unicellular organisms such as yeast to human tumor-derived immortal cell lines, have been shown to maintain control of telomere length within a set range through regulation of telomerase access/activity [41, 42, 43]. The short regulated telomere lengths observed in most human tumor-derived lines is consistent with a model of cells overcoming agonescence and undergoing conversion, followed by a mechanism to regulate the resulting short telomere length. Our immortal HMEC lines display a mean TRF around 4 kb, however analysis of individual clones has indicated a range of ~3-7 kb [14]. Additionally, we have observed that clones at the short end of this range may not express TRAP activity, associated with a slowdown in growth to a brief period (a few weeks) of total loss of proliferation, followed by a rapid re-expression of TRAP activity and a resumption of growth. We have not yet examined the mechanisms governing this behavior. It is likely that the telomeric ends of fully immortal HMEC have undergone an irreversible change into a conformation and set of telomere-associated proteins that allow assessment of telomere length in order to maintain stable, short telomeres.

4. **Immortalization of HMEC associated with loss of functional p53 (see Figure 3A for schematic chart)**

4.1. Derivation of p53(-/-) immortal HMEC lines

Many immortal and malignant human cells show loss of normal p53 function, however, 70-85% of human breast tumors contain wild-type p53 [44, 45], and our immortally transformed 184A1 and 184B5 HMEC lines also contain wild-type p53. We attempted to use expression selection of genetic suppressor elements (GSEs) to define alterations responsible for generating these lines. Retroviruses containing short random cDNA fragments were introduced into 184Aa and 184Be, the EL cultures that gave rise to 184A1 and 184B5. Our goal was to generate new lines with indefinite growth potential by inactivating potential tumor suppressor genes, and then identifying the inactivated genes. Although we were unsuccessful at the goal of defining the alterations responsible for generating the p53(+) lines, these experiments did provide us with two p53(-/-) immortally transformed HMEC lines, 184AA2 and 184AA3, derived from EL 184Aa

[23]. Comparison of these two p53(-/-) lines with the three closely related p53(+) lines suggests a novel role for p53 loss in immortal and malignant transformation.

184AA was infected with retroviruses at passage 12. 184AA2 appeared at passage 13 as tight patches of refractile cells with many mitoses, as well as larger flatter cells. It maintained good growth and a somewhat heterogeneous morphology thereafter. 184AA3 appeared at passage 14 as areas of small densely packed, grossly vacuolated and extremely slow-growing cells. By passages 16-18, mass cultures began a gradual increase in growth rate, and some colonies became less densely packed with fewer grossly vacuolated cells. When seeded at very low densities to permit visualization of the growth of individual colonies, these gradual changes in growth and morphology could be observed in single cell outgrowths. After a few additional passages, mass culture growth increased more rapidly, with uniform good growth attained by passages 20-24. Coincident with the better growth, 184AA3 cell morphology changed to more rounded and refractile, without the tightly packed colonial cell growth seen initially.

Since the one retroviral insertion in 184AA3 lacked detectable HMEC cDNA, we tested the possibility that immortalization resulted from insertional mutagenesis. Inverted PCR analysis showed that the virus had inserted into the p53 gene. Immunoblot analysis for p53 protein showed no p53 expression in either 184AA2 or 184AA3, suggesting that both p53 alleles were inactivated in both lines. Southern hybridization indicated viral integration into one p53 allele, and the absence of a normal second p53 allele in both lines; these HMEC lines are thus p53(-/-).

4.2. Attainment of full immortality in p53(-) HMEC

The good initial growth of 184AA2, and the more rapid attainment of good growth in 184AA3, compared to 184A1 and 184AA4, indicated that these p53(-/-) lines did not undergo a very gradual conversion process. Indeed, some telomerase activity was present in both 184AA2 and 184AA3 at the earliest passages that could be tested (Figure 4A) and the mean TRF length never declined below 3.5 kb (Figure 4B). In both p53(-/-) lines, with increasing passage there was a gradual increase in the number of cells with progressively better growth capacity in TGF β (see Figure 8B), a result consistent with the induction of TGF β resistance by the presence of hTERT.

Unlike the three p53(+) lines, no p57 mRNA expression was detected in either 184AA2 or 18AA3 at early or late passages, in G0-arrested or in cycling populations. Consistent with the absence of p57, neither line displayed a prolonged slow growth phase. Although some large vacuolated cells were initially present in 184AA2, it maintained good growth from the outset. Initial 184AA3 growth was very poor, but good uniform growth was quickly attained by passages 20-24. We do not know what is responsible for this initial short period of slow growth in newly emerged p53(-/-) lines with mean TRF > 3 kb and no p57 expression.

Both 184AA2 and 184AA3 displayed anchorage-independent growth when examined at passage 50. They were also capable of forming tumors in nude mice, although the tumors started to regress by ten days post-injection. Karyotypic analysis indicated that both p53(-/-) lines had both a high level of initial chromosomal derangements, as well as increased chromosomal complexity and instability with continued passage. Both lines

were able to maintain growth after transduction with oncogenic Raf-1.

These data indicate that the behavior of early passages of both p53(-/-) lines significantly differed from that displayed by early passages of the three p53(+) lines with respect to the conversion process. The p53(-/-) lines showed rapid telomerase reactivation, mean TRF lengths which did not decrease below 3.5 kb, and early acquisition of uniform good growth potential. Thus, although the fully immortal p53(-/-) lines shared many similarities with the fully immortal p53(+) lines, they acquired these properties rapidly after overcoming agonescence. The p53(-/-) lines completely differed from the p53(+) lines in their total absence of both p57 expression and an extended period of poor heterogeneous growth, and in their expression of the malignancy-associated properties of anchorage-independent growth, tumorigenicity, and ongoing genomic instability.

4.3. The effect of functional inactivation of p53

The capacity of both p53(-/-) lines to rapidly attain full immortality, relative to the p53(+) lines, and to not express any p57, suggested that these properties were the result of the lack of p53 function. To directly test this possibility, we inactivated p53 function in early passage pre-conversion 184A1 by transduction with the p53-inactivating GSE, GSE22 [46].

184A1 transduced at passage 12 with GSE22 rapidly gained full immortality [23]. A moderate level of telomerase activity was present seven days after infection, and strong activity was present by passage 19. The mean TRF length in 184A1-GSE22 showed a modest decline from ~5 kb at passage 13 to a stabilized length of ~4 kb by passage 25, similar to the stabilized length in 184AA2 and 184AA3. The TRF signal never became faint nor declined below 4 kb. 184A1-GSE22 assayed up to passage 25 showed a gradual increase in the capacity to maintain growth in the presence of TGF β . Analysis for p57 showed no p57 protein expression in cycling populations at passages 14-22, in contrast to the abundant p57 by passage 14 in the 184A1 populations infected with control virus. Very low levels of p57 compared to controls were seen in the G0-arrested cells. 184A1-GSE22 also did not have a prolonged slow growth phase. Good uniform growth was present by passage 25, although, as with the p53(-/-) lines, growth capacity progressively increased to that point.

These results indicate that (1) p53 may be acting to suppress telomerase activity in newly immortalized p53(+) HMEC lines; (2) p57 expression in conditionally immortal HMEC is dependent upon expression of functional p53. The absence of p57 expression in p53(-) HMEC lines may in turn be responsible for the absence of a prolonged conversion-associated slow growth phase. Altogether, these data indicate that an absence of functional p53 can directly accelerate the conversion process, and produce more aggressively growing cells more rapidly. *In vivo*, breast cancers that show p53 loss have a poorer prognosis [44, 45, 47]. If a conversion process occurs in breast cancer development *in vivo*, our results suggest a significant additional mechanism whereby p53 loss may contribute to more aggressive cancer progression.

5. Effect of hTERT on growth of finite lifespan and conditionally immortal HMEC (see Figures. 3C and 2B for schematic charts)

The identification of the human catalytic subunit of the telomerase complex, hTERT, has made it possible to experimentally activate cellular telomerase activity, since hTERT is the limiting factor for such activity in human cells. Exogenous introduction of hTERT has been reported to render some cells immortal, e.g. human retinal epithelium, BJ fibroblasts, and post-selection HMEC [48, 49]; however other cell types remained mortal, e.g. pre-selection HMEC and p16(+) human keratinocytes [49, 50]. Initial published reports on hTERT-immortalized human cells showed no alterations in growth control in response to serum deprivation, high cell density, specific pharmacological inhibitors, or oncogenic Ras, nor were gross chromosome instability, anchorage-independent growth or tumorigenicity reported [36, 51, 52]. These data suggested that hTERT-induced immortalization does not affect normal cell behavior. However, long-term culture of hTERT-transduced post-selection HMEC was reported to be associated with increased expression of c-myc [53]. Additionally, ectopic hTERT expression in conjunction with ectopic expression of the oncogenes SV40-T and H-ras, was able to malignantly transform normal human cells [54, 55].

We have examined the consequences of transducing hTERT into both pre-selection and post-selection finite lifespan HMEC, and into conditionally immortal 184A1 both pre-conversion and during conversion [32]. In particular, since conversion of conditionally immortal HMEC to full immortality was consistently associated with acquisition of the ability to maintain growth in the presence of TGF β , we wanted to examine TGF β responses. Our results indicate that the expression of exogenously introduced hTERT alone can be responsible for inducing resistance to TGF β inhibition in HMEC lacking expression of p16.

hTERT was transduced into three different post-selection HMEC at differing passage levels. All hTERT-transduced cultures showed telomerase activity when examined one passage after infection, and the mean TRF rose rapidly to ~10-12 kb (Figure 7A). Cultures exposed to vector alone had no telomerase activity, showed continued telomere erosion, and senesced as expected. All the hTERT-exposed post-selection HMEC have maintained rapid continuous growth. Similar to other reports, the hTERT-transduced cells exhibited no anchorage-independent growth, and remained severely growth inhibited when exposed to oncogenic Raf-1 [Olsen, Yaswen & Stampfer, in preparation]. Assay for growth in TGF β indicated that these post-selection HMEC rapidly gained TGF β growth resistance (Figure 8A). We have also noted an increased expression of c-myc with continued passage (unpublished observations).

Pre-selection p16(+) HMEC transduced with hTERT at passage 3 did not become immortal nor acquire TGF β resistance. Transduced and control cells senesced similarly around passage 5. However, one hTERT-transduced culture dish did give rise to a continuously growing population. With increasing passage, this population displayed decreasing levels of p16 expression. By passage 20, almost all cells were p16(-), and the earliest indications of the ability to maintain growth in TGF β were detectable. Subsequently, there was a gradual increase in the number of cells with progressively better growth capacity in TGF β . These results showing hTERT failure to immortalize

p16(+) human epithelial cells are similar to what has been reported for human keratinocytes [50].

hTERT transduction into good-growing, pre-conversion conditionally immortal 184A1 at passage 12 produced rapid telomere elongation (Figure 7B). Consistent with the prevention of telomere erosion to < 3 kb, hTERT transduction eliminated elevated p57 protein expression as well as the associated slow heterogeneous growth phase [26]. hTERT also conferred TGF β resistance, well before it would have been acquired as part of the conversion process (Figure 8B). Similar to the kinetics observed in our five immortally transformed HMEC lines following endogenous reactivation of telomerase activity, TGF β resistance was acquired gradually over 10-20 passages.

hTERT transduction of conditionally immortal 184A1 at passage 22, which had already begun the conversion process (mean TRF < 2.5 kb, poor heterogeneous growth, elevated p57 levels) also resulted in rapid telomere elongation (Figure 7C). However, in this case there was no significant effect relative to control cultures on the existing levels of p57, nor on growth capacity in the absence of TGF β . hTERT again conferred TGF β resistance gradually over 10-20 passages (Figure 8B). Thus, the acquisition of good growth in TGF β following hTERT expression is distinct from attainment of good growth in the absence of TGF β .

To determine what functions of the ectopic hTERT were required for induction of TGF β resistance, two different hTERT mutants were introduced into post-selection 184 HMEC at passage 12. One mutant contains inactivating amino acid substitutions in the reverse transcriptase domain [56], and the other is a wild type hTERT with a carboxyl-terminal HA epitope tag that shows *in vitro*, but not *in vivo*, telomerase activity [57]. Neither mutant induced TGF β resistance in recipient cells, indicating that in addition to being catalytically active, telomerase must be capable of telomere maintenance *in vivo* in order to confer TGF β resistance.

The mechanism responsible for hTERT induction of TGF β resistance remains to be elucidated. Our data indicate that there is no correlation between telomere length and TGF β resistance. The incremental acquisition of TGF β resistance in conditionally immortal HMEC suggests that the effect of hTERT is likely to be indirect, possibly involving cumulative changes in chromatin structure and/or soluble factors. hTERT expression might indirectly change the abundance, modification, and/or spatial arrangements of signalling molecules involved in TGF β growth inhibition through altering telomere association with nuclear matrix, or affecting the activities of telomere-associated proteins. Although the hTERT-transduced HMEC were no longer sensitive to TGF β -induced growth inhibition, like our other finite lifespan and immortal HMEC, they were still capable of responding to TGF β with differentiated functions.

These studies provide another cause-and-effect link in our understanding of HMEC immortalization and the conversion process; the gradual acquisition of TGF β resistance observed in the HMEC which have recently overcome agonescence is likely a consequence of their reactivation of telomerase activity. An obligate gain of TGF β resistance as a result of telomerase reactivation could explain why this phenotype is common to carcinoma cells. However, the very gradual nature of both the conversion-induced telomerase reactivation, and the telomerase-induced TGF β resistance, along with the potentially strong growth advantage provided by the loss of TGF β inhibition

during carcinogenesis, could also promote selection of the observed mutation-associated mechanisms of TGF β resistance.

The ability of hTERT to induce TGF β resistance suggests that immortality could be more than a passive facilitator of malignant progression. However, although TGF β resistance may be a tumor promoting property for immortal epithelial cells, it is not a malignant property *per se*, since normal mesenchymal cells may be TGF β responsive but not growth inhibited by TGF β . Furthermore, hTERT-induced immortalization did not produce other phenotypes characteristic of malignancy (e.g., anchorage-independent growth), or of the full immortality resulting from overcoming agonescence and undergoing conversion. Unlike our p53(+) fully immortal HMEC, hTERT-induced indefinite lifespan HMEC remained sensitive to oncogenic Raf-1 induced growth inhibition, did not express p57 nor the associated period of slow heterogeneous growth, and most importantly, never underwent an extended period with critically short telomeres. hTERT transduction may therefore generate the least deviant indefinite lifespan human cells. However, some changes in signal transduction do occur, such as the responsiveness to TGF β and possible alterations in c-myc regulation. Consequently, cells immortalized by hTERT are not totally equivalent to normal finite lifespan HMEC.

Conversely, hTERT transduction may not provide the best model for understanding human carcinogenesis. Ectopic hTERT produces long telomeres [32, 54], while most human cancers and tumor-derived cell lines contain short telomeres [58, 59]. In addition, the hTERT immortalization process bypassed agonescence and conversion. We propose that the reactivation of telomerase during human carcinogenesis may involve an obligate stage of very short telomeres, as part of overcoming senescence and undergoing conversion. Overcoming agonescence requires cells to sustain as yet undefined error(s) that most likely occur only after very short telomeres and chromosomal aberrations have been generated. Conversion in p53(+) HMEC provides a prolonged period where cells possess extremely short telomeres, exhibit slow heterogeneous growth and high p57 levels, and undergo other changes in signal transduction pathways. *In vivo*, such conditions might favor generation and growth of rare aberrant cells that have acquired malignancy-promoting advantages such as anchorage-independent growth, growth factor independence, or angiogenesis. Furthermore, conditionally immortal cells, and cells with extremely short telomeres, might possess unique properties that are vulnerable to therapeutic interventions. Methods of HMEC immortalization that do not model overcoming senescence and undergoing conversion and do not produce cells with critically short telomeres, do not allow testing of therapeutic interventions that target these potentially rate-limiting steps in immortalization and tumorigenicity.

6. Effect of viral oncogenes on growth of finite lifespan and conditionally immortal HMEC

The virtual lack of spontaneous immortal transformation of normal human cells in culture, coupled with the rarity of carcinogen-induced *in vitro* transformation, has led to the use of viral oncogene exposure in order to achieve more reproducible and efficient immortalization. Exposure of HMEC to the SV40 LgT oncogene yields inefficient but

reproducible immortalization [18, 60]; while exposure to the high risk HPV E6 and E7 oncogenes provides reproducible and efficient immortalization [61, 62, 63]. Similarly, other human epithelial cell types have been readily transformed to immortality following exposure to HPV16 E6 and E7 [64, 65]. The HPV16 E6 oncogene alone is also capable of efficient immortalization of the p16(-) post-selection HMEC [7, 66]. These reports of viral oncogene-induced immortal transformation of human epithelial and fibroblast cells did not describe a gradual conversion process. It was difficult to reconcile the M1/senescence $\square$ M2/crisis model presented for this mode of immortalization [67], with what we were observing in our HMEC cultures. As discussed previously in this chapter, we are now aware that some of this difficulty was due to: (a) assuming that the viable agonescence block resembled M1/senescence; (b) the absence of any event obviously resembling crisis (associated with situations where p53 is non-functional) in the immortalization of our p53(+) HMEC.

To address the effect of viral oncogenes on the conversion process, we examined the consequences of exposing good-growing pre-conversion conditionally immortal 184A1 to SV40 LgT, HPV16 E6, or HPV16 E7 [7]. E6 immediately and efficiently converted 184A1 to telomerase(+) cells that maintained good uniform growth in the absence or presence of TGF β (Figure 9A). This activity of E6 was not due simply to its ability to inactivate p53 because: (a) LgT, which also inactivates the p53 gene, did not produce immediate strong telomerase activity or uniform good growth, and (b) a mutant E6 which does not target p53 for degradation [68], also caused immediate conversion to full immortality.

The ability of HPV16 E6 to cause immediate conversion to full immortality may be due to its capacity to subvert many cellular functions at once. In addition to its ability to bind p53, HPV16 E6 has been demonstrated to bind at least six additional cellular proteins in *in vitro* and *in vivo* assays [69], only a few of which have been identified [70, 71]. E6 can reactivate telomerase activity, independent of p53 inactivation [15], and has also been reported to increase the level of c-myc protein expression [53]. One or more of these independent transforming functions may be related to its ability to confer immediate full immortality to conditionally immortal 184A1.

SV40 LgT and HPV16 E7 did not produce immediate conversion of 184A1 to full immortality. They did greatly accelerate aspects of the conversion process (reactivation of telomerase activity and attainment of uniform good growth) and also induced a rapid gain of resistance to TGF β growth inhibition (Figure 9B&C). The inactivation of p53 could be responsible for some of these effects of SV40 LgT, as the data on telomerase reactivation and growth are similar to what we see in the p53(-/-) lines. The means by which HPV16 E7 accelerates conversion are unknown. It is possible that the rapid gain of TGF β resistance could be related to the ability of these oncogenes to bind and inactivate the CKI p27 [72, 73], which is associated with TGF β growth inhibition in our HMEC system and other cell types [25, 74]. The role of p57 in these viral oncogene-transduced 184A1 is not yet clear. Cycling cultures of HPV16-E7 transduced 184A1 cells expressed more p57 than parallel cultures infected with control virus (unpublished data). This finding suggests that this viral oncoprotein might also be able to bind and inactivate p57, or might alter the function of cell-cycle mediators downstream of p57, causing aberrant feedback regulation of p57.

Understanding how these viral oncogenes function may help elucidate the mechanisms of immortal transformation of human cells. However, systems of immortalization that employ viral oncogenes with pleiotropic effects, some of which are still unknown, may not accurately model the gradual changes that occur during human carcinogenesis *in vivo*. Additionally, the common use of oncoproteins which inactivate p53 function has prevented understanding of the mechanisms by which p53(+) human cells overcome senescence, undergo conversion, and gain full immortality.

7. An overall model of HMEC immortalization and telomerase reactivation

7.1. Alterations involved in immortalization of HMEC by different methods

Our laboratory has been developing HMEC culture systems in order to understand the normal processes controlling growth and differentiation in these cells, and how these normal processes are altered in the course of immortal and malignant transformation. The studies described in this chapter represent the information we have gained on this subject in the past 25 years. Many outstanding gaps remain in our knowledge of the mechanisms underlying HMEC immortalization *in vitro*. Nonetheless, the great strides of the past 10 years in elucidating the mechanisms and molecules involved in cell cycle progression, signal transduction networks, tumor suppression and oncogenesis, and the key role of telomerase in immortalization, have enabled us to begin to see the outlines of a coherent picture.

An important emerging concept in cell and molecular biology is the extent to which the many pathways of cellular communication form an interacting network, and the pivotal roles some molecules play as nodes of this network. RB and p53 serve as pivotal network nodes of pathways involving growth regulation, and monitoring and directing of cellular status. The biological consequences for the cell may be significantly different depending upon whether a pivotal molecule like RB or p53 is inactivated, or whether a smaller subset of the pathways they govern is altered. This point may be important with regard to human breast carcinogenesis *in vivo*, and the *in vitro* systems which try to model this process, since inactivation of the RB and p53 molecules themselves is not required nor usually occurs in HMEC immortalization *in vitro* or *in vivo*.

The first senescence barrier encountered by the HMEC *in vitro* appears to be governed by the RB pathway. Agents that inactivate RB e.g., HPV E7, are able to overcome this barrier [11, 66]. However, the large majority of human breast cancers retain wild type RB [75]. Around a third of breast cancers do contain a known alteration in the RB pathway □ absence of p16 expression associated with hypermethylation of the p16 promoter [13]. Absence of p16 expression, either through mutation or associated with p16 promoter methylation, appears to be the favored route of overcoming this barrier in cultured HMEC in which RB has not been targeted by viral oncogenes. It is most likely that this *in vitro* phenomenon is modeling a pathway used *in vivo*. The mechanism by which p16(+) breast cancers overcome this barrier is unknown, but may involve amplification of cyclin D1 expression [76, 77], or alterations in other cyclins [78]. Loss of p16 expression through epigenetic means would not have the

same impact on a cell as total loss of RB function, as p16 mediates only a small portion of the information affecting RB. p16(-) post-selection HMEC remain responsive to other factors influencing whether or not growth is appropriate, e.g., RB remains unphosphorylated in the absence of required growth factor-induced signal transduction. Consequently, cells, including tumor cells, which lose p16 expression but retain normal RB would be expected to express a more normal phenotype. Immortalization of HMEC by methods that totally eliminate RB function may not model the state of a large percentage of human breast cancers. RB(+) cancers may resort to other errors as ways of evading conditions that would normally prevent growth.

An important question for which there is currently little definitive data is whether the senescence barrier associated with high p16 expression plays a role *in vivo*. Mutations and/or methylation of p16 genes in a wide variety of human cancers strongly suggest that there is selection against this growth regulator during malignant progression. However, comparatively little is known about the circumstances or stimuli that normally induce p16 expression *in vitro* or *in vivo*. Telomere attrition has been postulated as a key determinant of the p16-associated senescence/M1 block in both fibroblasts and epithelial cells. However, recent findings complicate this hypothesis: a) the mean TRF length is ~6-8 kb in the senescing HMEC at selection, while in post-selection HMEC mean TRF can decrease to ≤ 5 kb [7], and b) introduction of hTERT alone is insufficient to extend the lifespan of pre-selection HMEC, although it readily extends the lifespan of post-selection HMEC [32, 49]. These findings suggest that telomere shortening can not be solely responsible for the p16-associated growth arrest in the pre-selection cells. Nonetheless, there is some consistency in the mean TRF length at this block. It is possible that TRF length could influence the expression of p16, or other growth inhibitors which might be playing a role in the G1 arrest expressed by cells at this barrier. It remains to be determined whether the selection/M1/senescence barrier depends upon an intrinsic clock-like mechanism or whether p16 expression and growth arrest are primarily cumulative responses to the presence or absence of extrinsic factors. There may also be differences between the M1/senescence arrest experienced by fibroblast cells vs. selection in HMEC, for example in the role of the CKI, p21. Upregulation of p21 has been reported to play a role in fibroblast senescence [79, 80], but has not been reported in HMEC [8].

We do not currently know if p16(-) cells play a normal role in epithelial tissues. *In vivo*, epithelial cells may require much more extensive proliferative capacity than fibroblast cells. It is possible that down-regulation of p16 aids in this cell-type specific difference by providing a pool of cells able to proliferate beyond the barrier imposed by the selection/M1 block. However, the down-regulation of p16 in HMEC exposed to a carcinogen suggests that this process may occur under pathologic conditions. If p16 is normally down-regulated *in vivo*, then it would be beneficial to the organism for the p16(-) cells to have extremely vigilant error recognition and repair processes, and stringent barriers to immortalization, since p16(-) cells are one step closer to immortalization. Possibly, the enhanced stability of wild type p53 protein in the p16(-) cells may increase the efficiency of remaining surveillance mechanisms.

In contrast to the first senescence barrier, the second barrier to indefinite growth, agonescence, does appear to be largely or exclusively due to telomere attrition. Although

we have not been able to define the alterations required for overcoming agonescence, our existing data and ongoing experiments (Garbe, Yaswen & Stampfer, in preparation, see below) do provide some clues from which we venture the following hypotheses. We suggest that two to three alterations are required to overcome agonescence. In cultured HMEC under no selective pressures, the likelihood that all the necessary errors would occur in the same cell, even under the conditions at agonescence where widespread genomic errors are generated, is exceedingly small. Thus, the senescence arrest observed in cultured p16(-) HMEC is extremely stringent. However, if one error is already present, e.g., overexpressed ZNF217, or inactivated p53, the probability greatly increases that the required additional error(s) may be generated by the genomic instability produced by agonescence. EL cultures that gave rise to immortal lines, such as 184Aa, may harbor one predisposing error.

This hypothesis is based upon the data already presented as well as the following preliminary results:

(a) EL 184Aa is readily immortalized by transduction with c-myc or inactivation of p53 by the p53-inhibiting GSE22, whereas post-selection 184 HMEC are poorly, if at all immortalized by these methods. 184 HMEC infected with GSE22 do exhibit a brief extension of proliferative capacity, followed by large-scale cell death.

(b) 184 HMEC and EL 184Aa show a similar low capacity to be immortalized by ZNF217. Evidence of immortalization is first detected at agonescence, and the lines contain numerous chromosomal aberrations.

(c) GSE22 transduction leads to telomerase reactivation in p53(+) pre-conversion conditionally immortal 184A1 but not in finite lifespan 184 HMEC or EL 184Aa.

The p53 pathway appears to play a key role in overcoming agonescence. However, as with the case of RB, total loss of p53 is not required. It is possible that a subset of functions controlled by p53 is involved, and alterations in molecules responsible for these functions may be as effective as total p53 loss in overcoming agonescence. Such alterations would not have the same impact on a cell as total loss of p53 function. Similar to the situation with RB, cancer cells that immortalize while retaining normal p53 might be expected to express a more normal phenotype, and immortalization of HMEC by methods that totally eliminate p53 function may not model the state of a large percentage of human breast cancers. Our data indicate that whether or not p53 is lost as part of the immortalization process can have significant consequences for the cell. p53(-) immortal HMEC attained an aggressive growth potential much more rapidly than p53(+), and also evidenced ongoing genomic instability.

Our data generated from the p53(+) immortal HMEC initially suggested that the transformation to an indefinite lifespan was not coincident with the reactivation of telomerase activity. Our data with the p53(-/-) lines suggest a refinement of this model. Overcoming agonescence may involve induction of the *potential* to express telomerase activity. Where p53 remains present, it may inhibit telomerase activity, possibly through direct association with other proteins in the telomerase complex [81]. In this situation, telomerase reactivation is dependent upon telomere length. As the ongoing proliferation produces critically short telomeres, some as yet undefined change occurs when the TRF

reaches <2.5 kb, possibly in gene expression or in conformation of the telomeric ends, that gradually releases this p53-imposed inhibition. In the absence of p53, there is no requirement for telomeres to decline to <2.5 kb for telomerase reactivation. There is still a short conversion period for telomerase to be fully reactivated and uniform good growth to be attained. The more rapid telomerase reactivation stabilizes telomere length at ~ 4 kb. The nature of the events producing this more rapid, but still not immediate conversion, is currently unknown.

The ability of 184A1 to maintain growth with critically short telomeres, without generating widespread chromosomal aberrations, suggests that one of the alterations required to overcome agonescence is protection of short telomeric ends to permit continued proliferation with critically short telomeres. Changes in telomere-associated proteins and/or their conformation may be involved. It is also possible that a mechanical-tensile constraint is engendered by very short telomeres, contributing to mitotic failures. Alterations that relieve such a constraint might then be involved in overcoming agonescence. If the alterations that prevent mitotic failures occur prior to agonescence, as in 184A1, resulting immortal lines may be near diploid and stable. If they occur as cells enter agonescence, as is likely the situation for 184B5, or well into agonescence, as in 184AA4, the resulting lines will start out with chromosomal aberrations generated by the agonescence process rather than by immortalization. Once rearrangements and translocations are present, as exist in early 184B5 and 184AA4, further fusion-bridge-breakage cycles could give rise to the low level of genomic instability observed in these p53(+) lines. However, the widespread instability seen in the p53(-/-) lines is not present.

We have not yet been able to delineate all the cause-and-effect relationships among the changes that occur during conversion. It is also likely that further research will uncover additional alterations. At this point, it appears that:

- (a) p53 positively controls expression of p57.
- (b) Telomere length of < 3 kb, in the presence of p53, positively controls p57 expression after release from G0, which in turn leads to slow growth. We do not know what controls the down-regulation of this p57 expression as conversion proceeds.
- (c) p53 negatively controls telomerase activity when the mean TRF is >2.5 kb.
- (d) Telomerase activity induces acquisition of TGF β resistance, and possibly changes in c-myc regulation,

The factors controlling the change in the effect of oncogenic Raf-1 expression during conversion, from being growth inhibitory to promoting malignancy, are not known.

The development of an extensive series of normal and transformed cells from one human epithelial cell type has allowed us to compare the effects of a variety of different agents. Our data indicate that different pathways of producing an indefinite lifespan give rise to cells with widely varying phenotypes. Immortal and tumorigenic HMEC may or may not: express RB, p16, or p53, be growth-inhibited or malignancy-promoted by oncogenic Raf, express p57 and undergo a prolonged period of slow heterogeneous growth, possess very short telomeres, or exhibit karyotypic aberrations or genomic instability. These many variables may have profound effects on the nature of the tumor

produced by such a cell, the progression of such a tumor *in vivo*, and how such a tumor may respond to therapeutic interventions. Our recent studies have particularly emphasized the importance of whether or not cells possess wild type p53. This one variable may distinguish between agonescence and crisis, and between very gradual or relatively rapid telomerase reactivation. Since most model systems of immortalization *in vitro* have obligately induced inactivation of p53 function, some of these important differences have been obscured. All the methods of immortalization that we have studied produce obligate alterations of some of the growth control processes found in normal finite lifespan cells. These data emphasize the importance of recognizing that normal human cells possess a finite lifespan and have wild type p53 and RB. Cells that are immortal and/or p53(-) and RB(-) are not normal, and their growth control processes may not accurately reflect the normal situation.

7.2. Potential role of conversion during *in vivo* carcinogenesis

There is currently no data clearly demonstrating that a conversion process occurs during *in vivo* human breast carcinogenesis. However, there are several ways in which the data we have obtained from our *in vitro* systems may model the development of human breast tumors *in vivo*.

- (a) Telomerase activity is detectable early in breast cancer progression. It is seen in ~50% of ductal carcinomas *in situ*, and in most primary tumors [82, 83, 84, 85]. These data are consistent with a conversion process beginning early in breast carcinogenesis, in ductal carcinoma *in situ*, prior to acquisition of invasive and metastatic potential. Conditionally immortal cells undergoing conversion may express detectable telomerase activity, yet continue to exhibit slow non-uniform growth for an extended time. An extended period of conversion would provide a continuous pool of slowly dividing cells able to accumulate errors that both provide a selective growth advantage and promote malignant behavior.
- (b) Many primary carcinomas (particularly those like breast that are mainly p53 wild type) exhibit an extended period of slow, heterogeneous growth prior to the appearance of more aggressive and metastatic tumors [86, 87]. A gradual conversion process, with its generation of extensive heterogeneity, could at least partially account for this heterogeneous growth and clonal selection. Conversion to full immortality might not even be necessary for metastasis; an extended period of conditional immortality could be sufficient. Our data indicate that conditionally immortal cells can undergo a very large number of population doublings before becoming fully immortal. We have also observed the rare stochastic emergence of more aggressively growing fully converted cells [14].
- (c) p53(-) breast tumors have a poorer prognosis [44, 45, 47]. Where examined, loss of p53 occurs early in breast cancer, in ductal carcinoma *in situ*. These findings are consistent with a model in which p53 loss precedes and promotes full immortalization. The rapid conversion exhibited by the p53(-/-) immortal HMEC lines, with early telomerase reactivation, no p57 expression, and no prolonged slow growth phase, produces aggressively growing cells more rapidly. Our newly

uncovered role of p53 in retarding full immortalization could at least partially account for why p53(-) tumors have a poorer prognosis.

- (d) Abnormal karyotypes are found in most human carcinomas. The generation of widespread chromosomal aberrations during agonescence could at least partially account for this finding, particularly for p53(+) tumors. Consistent with this hypothesis, karyotypic abnormalities are seen early in breast carcinogenesis, in ductal carcinoma *in situ* [88]. Pathways of immortalization that require generation of errors during agonescence might be expected to give rise to tumors that exhibit a greater degree of chromosomal aberrations and have a poorer prognosis.
- (e) Human epithelial cancers show a steep age-dependent increase in incidence, and tumor tissues and tumor-derived cell lines express short, regulated telomere lengths. The need to undergo sufficient population doublings to reach and overcome senescence, and undergo conversion $\square$ processes that produce cells with short telomeres $\square$ could at least partially explain these findings. All of our immortal lines, other than those obtained from exogenous introduction of hTERT, express a mean TRF length of $\sim$ 3-7 kb, similar to what is observed in most tumor derived lines.
- (f) As discussed in Section 3.4, the common finding that tumor cells can maintain some growth in TGF β even in the absence of detectable mutations in TGF β pathways may be due to an obligate gain of TGF β resistance following telomerase reactivation.

Although our HMEC model systems may provide a closer approximation of *in vivo* processes of carcinogenesis, it is important to recognize that they are also only partial approximations. They do not address the important issue of cell-cell, and cell-matrix interactions, and our immortally transformed cells lack certain phenotypes commonly found in breast cancer cells, such as expression of keratin 19 and the estrogen receptor.

8. Conclusions

Our data on immortal transformation of HMEC support a model whereby attaining full immortality by overcoming replicative senescence and undergoing conversion may be rate-limiting steps in cells $\square$ acquiring invasive and metastatic potential. Multiple alterations are necessary to overcome both the selection and agonescence barriers, and further limitations are imposed by conversion, particularly in p53(+) immortal cells. In contrast, we and others have shown that once HMEC have undergone immortal transformation and reactivated telomerase activity, further transformation to anchorage-independent growth and/or tumorigenicity is readily attained with exposure to one or two potential oncogenes e.g., SV40 LgT and H-Ras [89, 90], erbB2 [91, 92], or insulin receptor [93].

The model we have presented for telomerase reactivation and immortal transformation is only applicable where there is stringent imposition of replicative senescence, including stringent control of telomerase expression. Exogenous introduction of high

levels of hTERT, and immortalization via HPV16-E6/E7 do not accurately model what occurs as a result of overcoming senescence and undergoing conversion. Immortalization via other oncogenes, such as SV40-T antigens, also introduces multiple unknown cell-cycle deregulating functions, in addition to p53 inactivation, that may not occur during breast carcinogenesis *in vivo*. Short-lived animals, such as rodents, lack stringent barriers to indefinite growth potential. Telomerase activity is not strictly suppressed and immortal transformation is not rate-limiting. Consequently, other changes, which may be less rate-limiting or significant in human cells, may be more prominent in rodent cell systems.

The potential to translate discoveries seen *in vitro* into useful therapeutic approaches may require utilization of model systems that as accurately as possible model the biological process of intrinsic interest. Information gathered from the study of human cancer tissues and derived cells can provide the necessary guidance for evaluating the extent to which culture systems accurately model human carcinogenesis. In particular, the reactivation of telomerase activity appears to be the most crucial event necessary for attaining an indefinite lifespan, which in turn appears crucial for malignant progression. In our *in vitro* model systems, telomerase reactivation required overcoming agonescence and undergoing conversion $\square$ processes that entail the attainment of very short telomeres. This mode of telomerase reactivation may be critical for modeling *in vivo* carcinogenesis.

Further studies will be required to fill in some of the currently outstanding gaps in our knowledge of the mechanisms by which telomerase is reactivated, e.g., (a) how do very short telomeres induce the instability seen at agonescence; (b) what are the alterations involved in overcoming agonescence, including the suppression of chromosomal rearrangements and the acquisition of telomerase activity; (c) how does p53 suppress telomerase activity in conditionally immortal HMEC; (d) how do the critically short telomeres present in conditionally immortal HMEC alter gene expression and/or telomerase activity. The normal and transformed HMEC systems that we have developed may facilitate such studies.

9. References

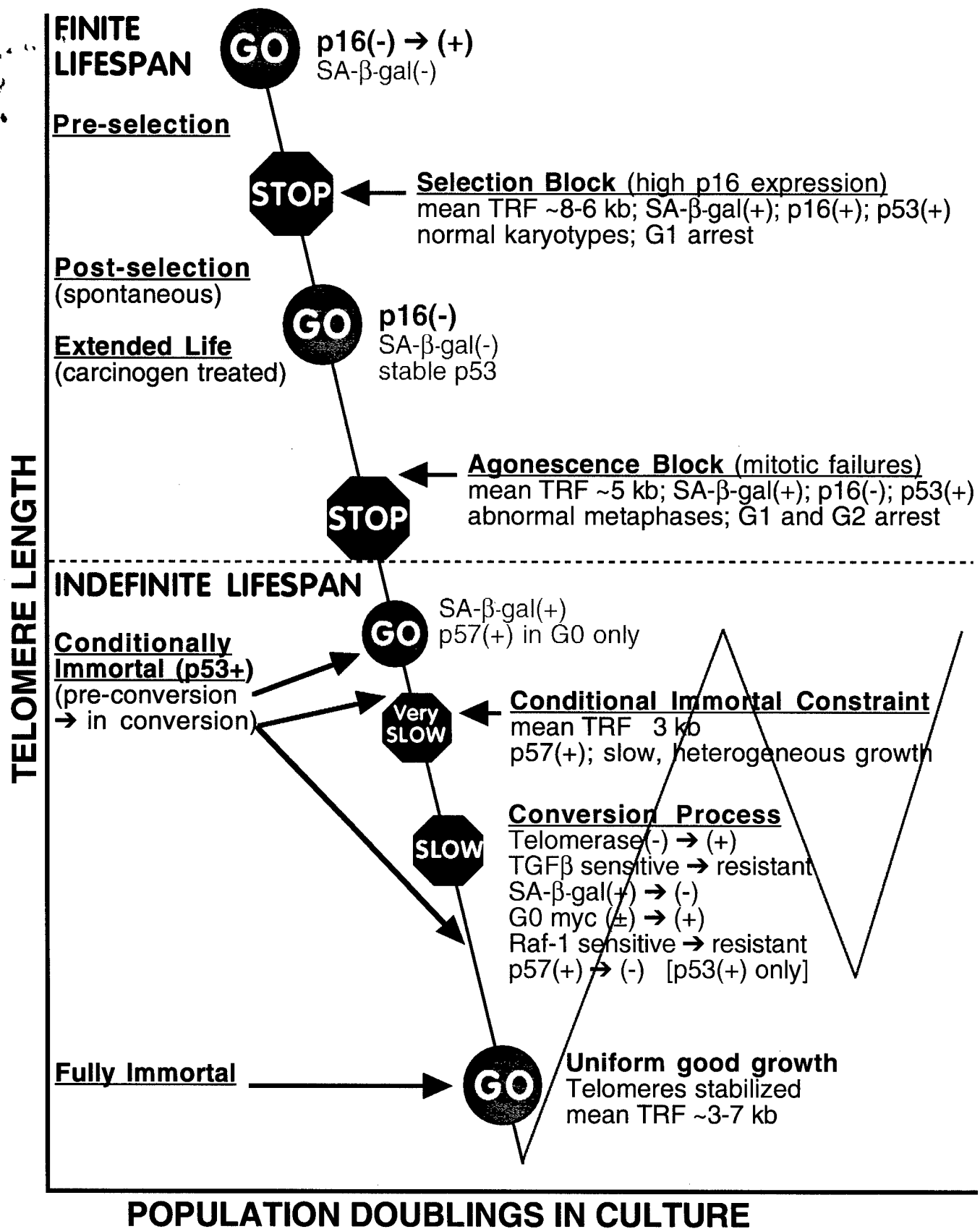
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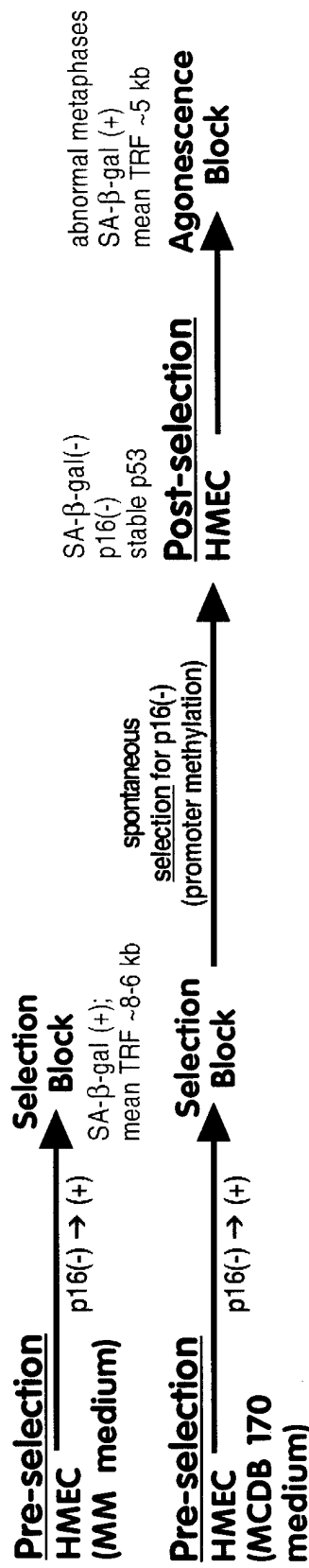
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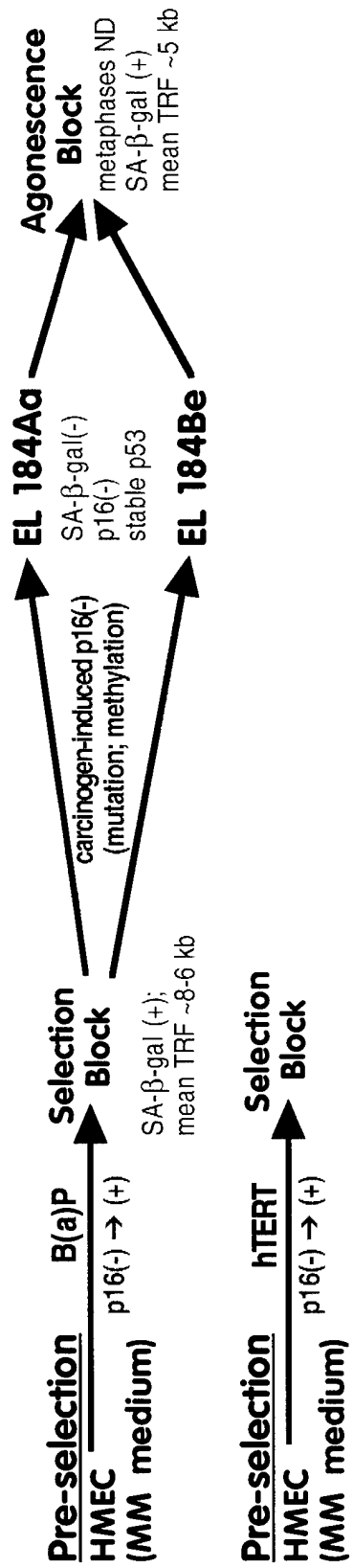


Finite Lifespan HMEC Culture Systems

(A) Untreated

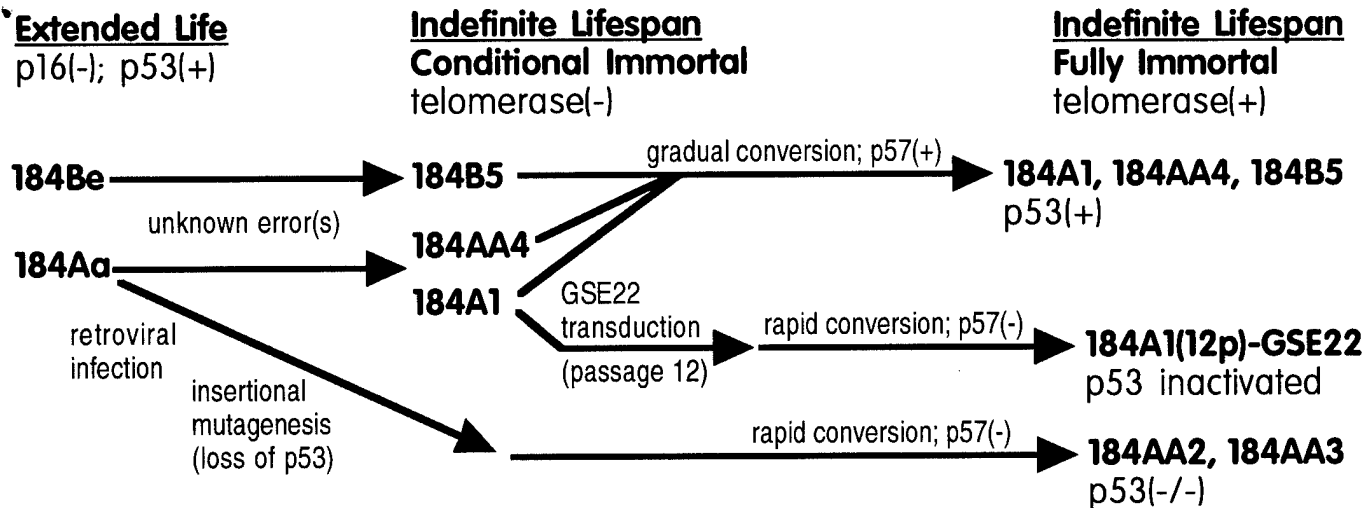


(B) Treated

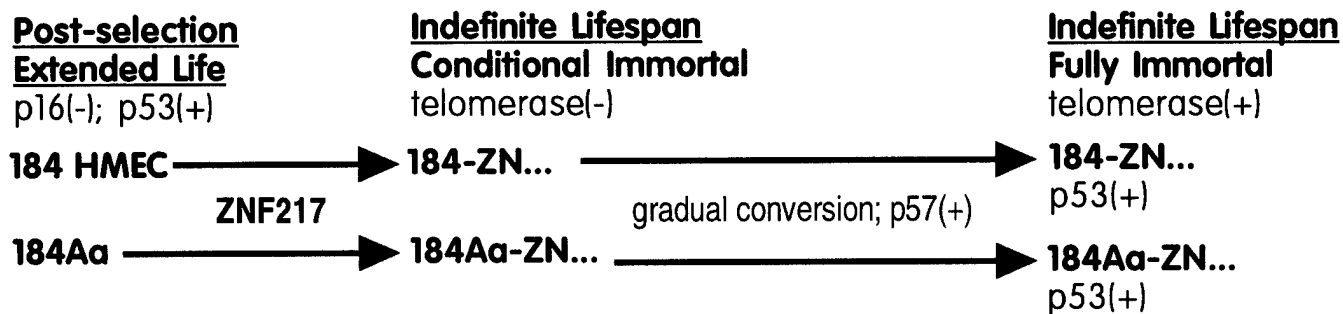


Indefinite Lifespan HMEC Culture Systems

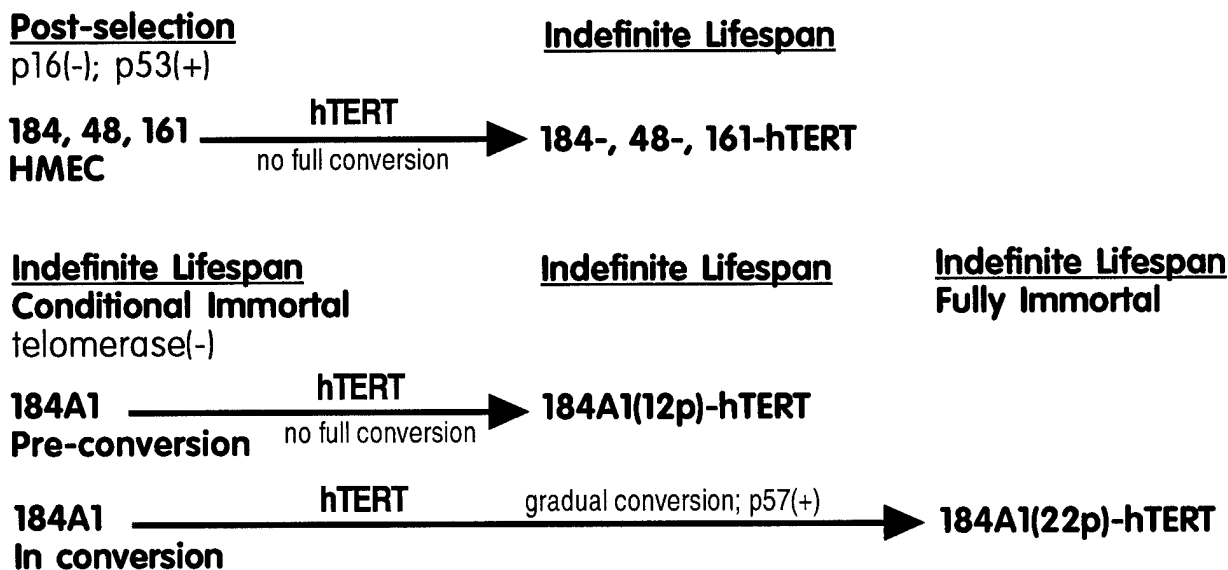
(A) Benzo(a)pyrene Exposed

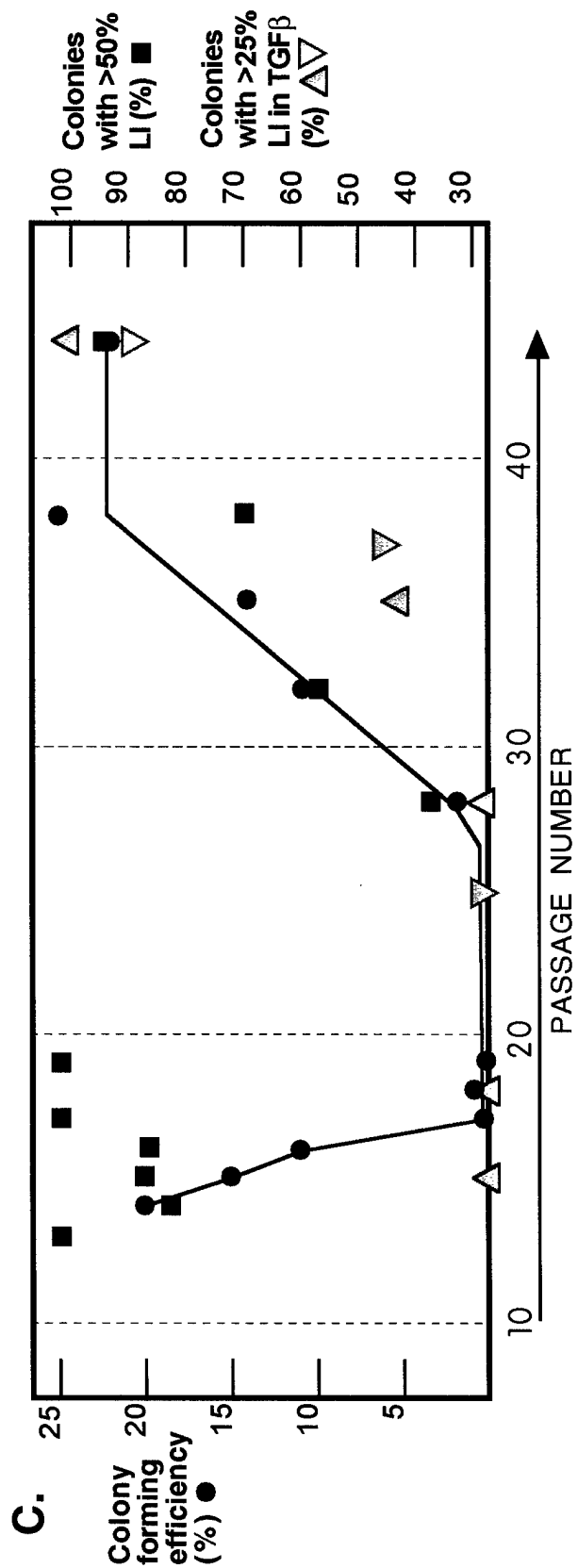
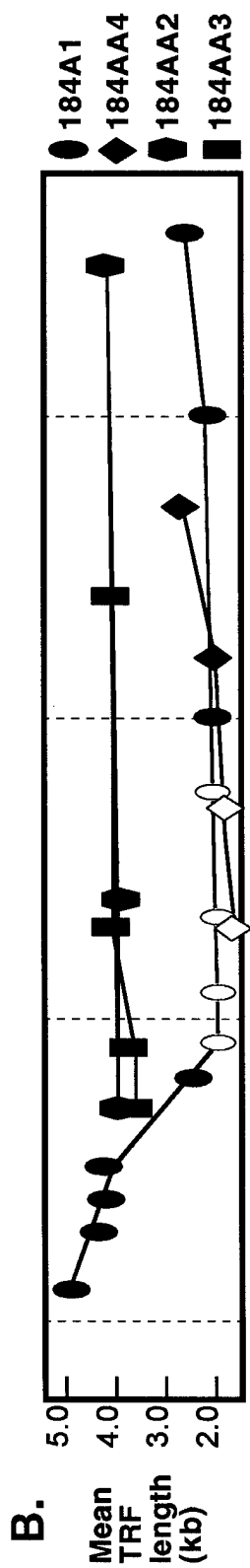
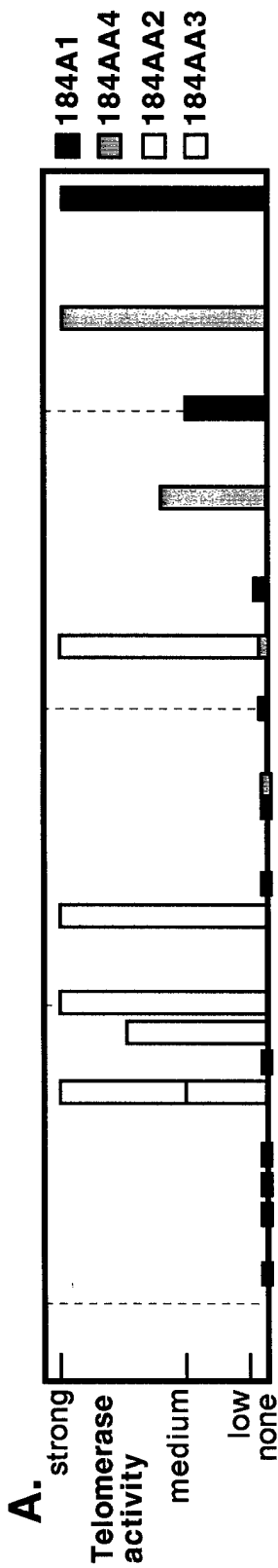


(B) ZNF217 Transduced



(C) hTERT Transduced





A. 13p

B. 16p

C. 66p

Go	1 hr	2 hr	4 hr	8 hr	12 hr	12 hr + TGF β
Go	1 hr	2 hr	4 hr	8 hr	12 hr	12 hr + TGF β
Go	1 hr	2 hr	4 hr	8 hr	12 hr	12 hr + TGF β

