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TITLE: The Role of p53 in Human Breast Cancer

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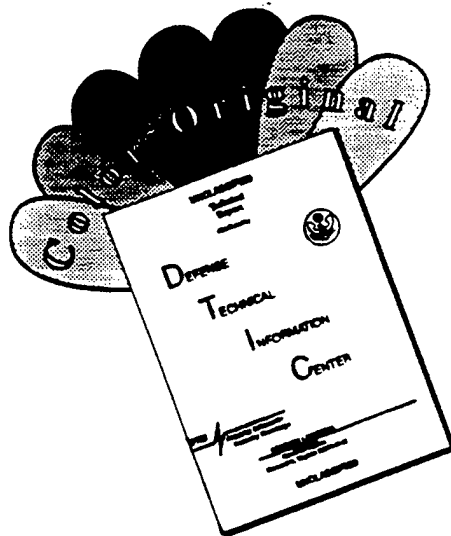
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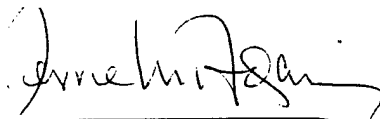
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Table of Contents

Table of Contents	page 1a
Introduction	page 1
Body	pages 1-17
References	pages 18-19
Conclusions	page 20

Introduction:

The principal objectives are to:

- 1) Transfect p53 mutants commonly observed in human breast cancer into normal human mammary epithelial cells obtained from different donors and isolate clones;
- 2) Characterize the clones for extension of lifespan and immortalization;
- 3) Determine in breast epithelial cells immortalized and expressing mutant p53 if expression of the mutant p53 is necessary for the maintenance of growth;
- 4) Determine if expression of any of the p53 mutants provide a selective growth advantage to breast epithelial cells prior to immortalization;
- 5) Determine downstream genomic targets of p53 that may be important in the development and progression of breast cancer.

Body:

This report will address the following:

- 1) The technical concerns noted from the first annual report will be addressed;
- 2) Relevant data collected directly pertaining to the grant specifications will be presented;
- 3) Significant new data stemming from ideas generated by findings from the original work indirectly involved with the grant specifications will be discussed.
- 4) Projects remaining to be completed and possible future directions for the study.

1) The first portion of this review is to address the technical issues of concern pointed out from the first annual report.

Abbreviations of commonly used phrases and chemicals within this field of study:

PDL - population doubling level, refers to the number of population doublings a cell strain/line has undergone while in culture. It is generally applied to estimate the age of the cells in culture in order to characterize lifespan *in vitro* for mortal cells and infinite proliferative capacity for immortal cell lines.

SSCP - single-stranded conformation polymorphism, shifts in electrophoretic mobility occur when there is sequence variation from the wild-type. Exons of interest are polymerase chain reaction (PCR) - amplified and α ^{32}P is incorporated into the reaction in order to obtain radio-labelled PCR products. Confirmation of the sequence variation is obtained by cloning the amplified fragments into M13 vectors and DNA sequencing.

RGC - ribosomal gene cluster, a DNA sequence that contains the 10bp p53 binding motif (5'-PuPuPuC(A/T)(T/A)(GpyPyPy-3') which is transactivated by wild-type p53, but not by mutant p53s.

CSPD - disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo{3.3.1.1}decan}-4-yl)phenyl phosphate = a direct chemiluminescent substrate for phosphatases.

AEBSF - (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochlorine) binds irreversibly to the protease active site and therefore acts as a protease inhibitor.

Technical Issues concerning figures, figure legends and text from the first report are addressed:

Figure 1a illustrates the difference observed between lipofectin transfection constructs that were under the control of the cytomegalovirus promoter (CMV) and defective retroviral infection constructs driven by the moloney murine leukemia virus (MMLV) promoter-enhancer sequences.

This has now been referred to in the text as well as the appropriate description of figure 1b.

To answer the reviewer's question concerning appropriate controls for growth comparison of the normal and temperature sensitive p53 mutant transfected cells, normal cells were grown concurrently with the transfected cells at 32°C as well as 37°C. Figure 4 graphically illustrates the results.

In regards to figure 3, the difference in signal for the DO-1 antibody between 32°C and 37°C may be due to mechanical error in loading. The major point of the figure is the fact that MAb 1620 which is essentially not present at 37°C, significantly increases in expression level at 32°C in conjunction with the expected conformational change to the wild-type p53 and the fact that not all the mutant MAb 240 disappears in these mortal cells suggests a possible time dependent relationship to the conformational change in relationship to the extended half-life of the mutant p53 protein product.

As the reviewer pointed out, figure 6 needs more clarification in order to compare expression levels at different PDL in culture. For clarification, 20 µg total protein was loaded for each sample immunoblotted. With this information, comparisons should now be possible.

The changes to these concerns are available for review in the enclosed reference which acknowledges DOD support of this fellowship.

Gollahon, L.S. and Shay, J.W. Immortalization of human mammary epithelial cells transfected with mutant p53 (273^{his}). *Oncogene* 1996; 12:715-725.

This section deals with comments about being more thorough in my introduction by supplying information on the native form of p53 in order to suggest inaccessibility of some of the p53 protein due to the possibility of heterodimerization. Cho et al. (1994) and Wang et al. (1994) both describe the native form of the p53 protein product. Basically, Cho et al. (1994) found that the regions most important to DNA-protein interactions were in the form of a loop-sheet-helix motif connected to a surrounding outer loop by a zinc finger. Most of the mutations involved in human cancers are

observed near the DNA-protein interface and decrease significantly as the residues become more distant from this interface. Within this loop-sheet-helix and outer loop (L3) 72% of documented p53 mutations can be found. Mechanisms of the inactivation of wild-type function may be divided into two categories, DNA-binding mutations or structural mutations. Examples of DNA binding mutants include 273^{arg} and 248^{arg}. Traits of p53 mutant 273 include no significant structural changes, no heat shock (hsc 70) protein association, reactivity to PAb 240 but not PAb 1620, a relatively stable core domain following proteolytic digestion and transactivation of the p53CON when fused to GAL4.

Structural p53 mutants such as 175^{arg} and 143^{val} are characterized by association with hsc 70 protein, binding to PAb 240 but not 1620, sensitivity to proteolytic digestion, fusion proteins with full length mutant plus the DNA binding domain of GAL4 do not demonstrate activation of transcription.

Cho et al (1994) then showed structurally that tetrameric binding was possible based on the following criteria; oligomerization in the COOH terminal binds to DNA with 4 copies of consensus pentamer sequences, forming a head-to-tail dimer. These dimers can interact with the H1 helices making protein-protein contacts. These contacts may also contribute to tetrameric association. The four core domain molecules occur on the same face with the COOH and H2 α helices extending through the major groove and ending up at the opposite face of the DNA. Carboxy termini of the four core domains are within 35-40Å that could be bridged by ~ 25-residue flexible linkers connecting the ends of the core domain to their oligomerization domains. Thus the biochemistry of the core domain-DNA complex suggests that intact p53 binds DNA as a tetramer. Wang et al (1994) confirmed these findings and also showed that in certain instances the tetramerization domain is the major determinant of the dominant negative phenotype leading to transformation by p53 mutants. They proposed a model where amino acids 315-360 could form mixed tetramers with wild-type p53. These mixed oligomers would be stable but would lack

transactivating functions thus reducing the suppression function of the endogenous wild-type p53 in cells and enhance transformation by other oncogenes. Amino acids 80-320 might form a different type of mixed oligomer that might bind p53 response elements with sufficient affinity to compete with binding and tetramerization of wild-type p53.

These findings may be relevant to the ability of a particular p53 mutant to access and/or abrogate wild-type function by forming mixed tetrameric complexes. If a certain level of wild-type p53 needs to be ablated in order to establish a dominant negative phenotype or confer a selective growth advantage and/or extension of lifespan, the amount of accessible p53 may be determined by the particular mutant p53, tetrameric binding of mutant p53 or a combination of both.

Relevant data collected directly pertaining to the grant specifications

2) Current data:

A. To address specific aim 1, p53 mutants 143^{ala}, 175^{his}, 248^{trp}, 273^{his} were infected into HME cell strains 15, (reduction mammoplasty); 17, (reduction mammoplasty); 73, (normal adjacent from a mastectomy) using recombinant retroviral vectors. This brings the total number of human mammary epithelial cell strains introduced to mutant p53s to five. Numbers of clones obtained and extension of lifespan data are shown in Table 1. Briefly, to date, the 143^{ala} mutant appears to give the cells a greater advantage for extension of lifespan in culture as well as the ability to produce more vigorously growing clones. Population doubling levels are illustrated in Figure 1. Due to results from a study by Klingelhutz et al (1996) in which they infected pre-selection mammary epithelial cells with HPV 16 E6/E7, E6 or E7 and observed activation of telomerase, I infected HME 73 organoids with p53 mutant 143^{ala} as a preliminary experiment. Currently, six clones are growing and one is being tested for telomerase activity. Due to the fact that the organoids are a heterogenous population of epithelial and stromal components, I was able to separate out the stromal population and expand them. These cells are also currently being tested for telomerase activity. So the potential remains for an immortal epithelial and possibly stromal clone containing p53 mutant 143^{ala}.

B. In order to better characterize the p53 mutants and to observe if mutants differ in their growth rates/capabilities, the non-small cell lung carcinoma line H1299 was utilized. This immortal, tumor derived cell line contains a homozygous 5' intragenic deletion of the p53 gene and produces no p53 mRNA or protein. Mutant 143^{ala} was introduced into the cells by both electroporation and lipofectin transfection. Mutants for p53 at codons 143^{ala}, 175^{his}, 248^{trp}, 273^{his} were also infected into the cells. This gave not only a basis for comparison of different introduction techniques, it also allowed comparison of the relative efficiency of the mutants under different promoters at 32°C and 37°C to observe whether promoters of different strengths would affect the

ability of the mutant to change to the wild-type conformation and stop cell growth in G1. This was tested by growth kinetics (Figure 2) as well as telomerase activity over a time course. It has been shown previously that in G1 arrest, telomerase activity significantly decreases with inhibition of cell growth (Holt et al, 1996). Western blot analysis was performed with antibodies reactive for p53, p21 and p16 (Oncogene Science) (Figures 3-5). Immunoprecipitations were performed to observe the transition from mutant to wild-type and back to mutant conformation (Figure 6). Interestingly, not every mutant p53 changed to the wild-type conformation. Also, some of the mutants which appeared to have high levels of mutant p53 present, did not show a significant change in growth rate from the parent line at 32°C. In addition, the parent line did not show a significant difference in comparison to growth rates at 37°C (Figure 7). Figure 8 shows a representative gel of telomerase activity as analyzed by the TRAP assay. Figure 9 shows representative PCR results demonstrating the presence of the mutants. Primers were generated to the regions flanking the promoter and the p53 gene at codon 273. In addition, telomere restriction fragment (TRF) analysis was performed and very interesting results ensued. Figure 10 demonstrates that the telomere length varies with particular clones. This finding was unexpected, but may be related to the paper by Hiyama et al (1995) in which they observed alterations in telomere length associated with loss of heterozygosity of p53 and pRb. This TRF analysis was performed four times and the TRF length variability was reproducible. This leads to the question of whether the mutant p53 is somehow regulating the telomere length or if insertion of the construct may have somehow disrupted a telomere length regulating gene or inserted near the end of the telomere and as a result caused premature shortening/ induced shortening to occur. To address this issue, fluorescent *in situ* hybridization (FISH) will be done on each of the clones showing altered telomere length as well as a parent control and a clone with the same telomere length as the parent line. Another experiment which will be performed is a luciferase assay. Plasmids containing a luciferase reporter gene and the p53 consensus sequence or RGC consensus sequence and a luciferase reporter gene and a β -GAL, luciferase reporter construct will be

transiently transfected into the clones to observe the transactivation activity of the different clones as well as the effect of different construct introduction techniques.

C. Another aspect of p53 presently under investigation focuses on the ability of mutant p53 to confer a selective growth advantage to the cells. In order to test this hypothesis, a secondary goat-antimouse IgG conjugated to fluorescein (FITC) will be used. At present, a cell mixture of 9:1 HME 31 and 32 : HME 31/32 (143) or HME 31/32 (175) or HME 31/32 (248) are growing in individual 6-well plates, 1 plate for each clone. Three of the wells will be counted and the remaining 3 will be immunostained with p53 PAb 240 (which only recognizes the mutant form) and nuclei counterstained with Hoechst 33258. Thus, selective growth advantage reflected over PDL and visual evidence of mixed cell populations will be documented. Mutant 273 could not be used in this portion of the study because it is not immuno-reactive with PAb 240. Another aspect of this experiment will be to mix HMS 73 normal and 143 mutant p53 containing cells as well as coculture with normal epithelial cells to observe any marked similarities or differences in comparison to the mutant p53 containing epithelial cells.

D. Mammary epithelial cells grow in serum free medium supplemented with growth factors and the stromal counterparts are fed medium containing 10% serum. In order to initiate the co-culture experiments, a medium formulation in which both cell types could grow was determined. The medium that was successful by supporting the growth of both epithelial and stromal cells consisted of fibroblast basal medium (FBM) from Clonetics supplemented with the mammary epithelial growth factors insulin, EGF, transferrin, hydrocortisone and bovine pituitary extract. We also determined that the stromal cells needed to be on the cell insert (Falcon) which enabled them to be easily trypsinized and we wanted the supernatant to "sit" on the epithelial cells. Optimal cell plating density was determined to be 10^5 epithelial cells and 5×10^5 stromal cells. The cells are allowed to proliferate until the control cells are 80% confluent at which time all the cells for that particular well plate are harvested.

3. Significant new data stemming from ideas generated by findings from the original work indirectly involved with the grant specifications

Within the last year, the field of telomerase re-activation has exploded. Since I am working for one of the premier researchers in this field, I decided to expand upon the original topics to include several areas of research on telomerase activation in breast cancer. These areas are in the breast cancer field and concern p53 and its role from the perspective of loss of function due to degradation by HPV 16 E6.

A. The first study concerned p53 levels in human mammary epithelial cells expressing wild-type p53 and mutant papillomavirus type 16 (HPV-16) E6 proteins and their relationship to reactivation of telomerase and immortalization. Since HME cells require only HPV 16 E6 to bypass the first blockade to immortalization, the question was what affect would mutant E6 proteins have in this cellular senescence pathway. To approach this question, we received mutant E6 constructs as a gift from K.Vousden and cloned them into a parental retroviral vector, pLXSN (gift of A.D. Miller). HME cells and human foreskin keratinocytes were grown as previously described and were infected ~20 PDL before cellular senescence. We found that only the expression of the wild-type HPV 16 E6 protein and none of the mutants resulted in the reactivation of telomerase and immortalization. These findings were consistent with the p53 degradation results *in vivo* but not with the *in vitro* results obtained by Crook et al. (1991). Neither the chimeric proteins (16/6 and 6/16) nor the N-terminal HPV-16 mutations {E6(Δ (-13) and E6(YYH)} caused extension of lifespan, reactivation of telomerase or immortalization of the HME cells. Our results indicate that the amount of residual p53 remaining in HME cells is significantly less than the amount observed in primary human foreskin keratinocytes. *In vitro* assays described by Crook et al. (1991) showed all exogenous p53 degraded upon the addition of wild-type HPV 16 E6. However, detection of the degradation of p53 in HME and keratinocyte cells resulted in a substantial amount of residual p53 left in the cells. The discrepancy between the HME and keratinocytes and the *in vitro* assays serves as a possible explanation to the relative susceptibilities of the different cell strains to the onset of

carcinogenesis. In the HME cells p53 is virtually undetectable which may be why only HPV E6 is sufficient to bypass M1 and eventually immortalize. The keratinocyte cells, which can have extended lifespan only in the presence of both HPV 16 E6/E7 has a substantial amount of p53 remaining in the E6 expressing cells. If the residual p53 is functional wild-type, its inactivation may eliminate the need for the second oncogene HPV 16 E7. For a detailed description of the HPV 16 constructs please see figure 1 of the enclosed article Holt SE, **Gollahon LS, et al.** p53 levels in human mammary epithelial cells expressing wild-type p53 and mutant papillomavirus type 16 (HPV-16) E6 proteins: relationship to reactivation of telomerase and immortalization. *Int J Oncology* 1996; 8:263-270.

B. The next project undertaken was to document the presence of telomerase in human breast cancer. This project was done as a collaboration with Dr. Eiso Hiyama, Department of General Medicine, Hiroshima University School of Medicine, Hiroshima 734 Japan. For a review of telomerase in human cancers see Shay JW, Wright WE. Telomerase in human cancer. *Curr Opin Oncology* 1996; 8:66-71.

Using the TRAP telomerase activity assay, we examined 140 breast cancers for telomerase activity, 4 phyllodes tumors, 38 noncancerous lesions (20 fibroadenomas, 17 fibrocystic disease and one gynecomastia), and 55 adjacent normal mammary tissues. Thirty-three fine-needle aspirated breast samples were also analyzed. Among the surgically resected samples 130/140 (93%) were telomerase positive. Activity was detected in 68% stage I, 73% of smaller cancers <20 mm and 81% of axillary node-negative. Telomerase was not detected in any of the 17 specimens diagnosed as fibrocystic disease. Low levels of telomerase activity was detected in 9/20, (45%) of fibroadenomas. For materials and methods see attached reference Hiyama E, **Gollahon LS**, Kataoka T, et al. Telomerase activity in human breast tumors. *JNCI* 1996; 88:116-122.

C. A study which has just been submitted for publication involved the "Detection of telomerase activity in breast masses by fine needle aspiration". This study was undertaken to determine whether telomerase activity could be reliably assessed in benign and malignant breast masses obtained by fine needle aspiration (FNA). Telomerase is an RNA-dependent DNA polymerase which compensates for the telomere shortening that occurs in its absence. Reactivation of telomerase is thought to be an important step in cellular immortalization, and recent studies have indicated that telomerase activity is frequently detected in primary human malignancies. The clinical implications of telomerase activity in human tumors are currently under investigation.

Eighty-nine samples (46 FNAs and 43 gross tissue biopsies) from 44 patients with breast masses were analyzed prospectively for the presence of telomerase activity by the Telomere Repeat Amplification Protocol (TRAP). All samples were obtained directly from the excised mass at the time of specimen removal in the operating room. Telomerase activity was detected in 17/19 (90%) of class IV and V FNA samples and 15/18 (83%) of the invasive breast cancer biopsies. Telomerase was also detected in 9/16 (56%) FNAs and 8/15 (53%) tissue biopsies from 16 fibroadenomas. Other benign proliferative lesions (n=6) did not have detectable telomerase activity in either FNA or tissue specimens. FNA-TRAP results correlated with the gross tissue specimen TRAP results in 95% of all cases. The FNA-TRAP assay for telomerase detection is a highly sensitive and accurate method for the detection of telomerase activity in breast masses. Future application of these techniques should facilitate evaluation of telomerase as a tumor marker in the clinical management of breast and other solid malignancies. For further details see attached manuscript: **Gollahon LS, Pearson SA et al. Detection of telomerase activity in breast masses by fine needle aspiration. Annals of Surgery 1996; submitted.**

D. Some very interesting studies have been published which deal with the issue of the 2-stage mechanism of cellular senescence (Shay et al, 1993). We have postulated that cells have a limited lifespan and as they approach the end of their replicative capacity, their telomeres shorten until a critical length is attained at which time cellular senescence genes such as p53 and pRb are activated signalling the end of normal proliferative capacity (M1). Some cells are able to bypass M1 generally by abrogating p53 and/or pRb function. They then enter an extension of lifespan in culture until a second cellular senescence checkpoint (M2) is triggered at which time the majority of the cells die. It is the rare cell which can bypass this M2 checkpoint, presumably by the reactivation of the ribonucleoprotein telomerase which stabilizes the ends of the telomeres by adding $(T_2AG_3)_n$ repeats. We have observed that human mammary epithelial cells do not appear to use pRb in their cellular senescence pathway. Two studies have emerged which may shed light on this observation and we are conducting experiments to corroborate their findings as well as add evidence of our own. The first study by Foster et al (1996) shows that mammary epithelial cells may have an M0 stage in culture which is equated to their "self-selection" process. Primary tissue samples obtained from surgical procedures are subjected to mechanical and enzymatic dispersion in order to try to establish *in vitro* cultures. Foster et al.(1996) have shown that this small period of time preceeding the self-selection process which resembles crisis may in fact be the M0 stage for mammary epithelial cells at which time those cells able to abrogate pRb function become established and grow for up to 60-70 PDLs in culture. The following proliferative period would actually be M1 or extension of lifespan until p53 is activated or mutated. Then following would be a period of crisis at which time the rare cell might reactivate telomerase and immortalize. They demonstrated this hypothesis by infecting with recombinant retroviral vectors HME cells obtained from reduction mammaplasty with HPV 16 E6, E7 or E6/E7. They observed that HPV 16 E6/E7 and HPV E7 but not HPV E6 bypassed the M0 proliferative block. This suggests that pRb but not p53 plays a role in the M0 block. Levels of p53 and the p53 dependent cell cycle inhibitor protein p21 were not

upregulated and pRb was observed to exist in a hyperphosphorylated form in early passages changing to a hypophosphorylated form in later passages.

Concurrently, a study was published by Klingelutz et al. (1996) that described the activation of telomerase by retroviral infection of HPV 16 E6/E7 or E6 alone in human cervical keratinocytes and human mammary epithelial cells. HPV 16 E7 did not activate telomerase in either cell strain. Mammary stromal cells showed no telomerase activation or extended lifespan from HPV 16 E6 infection. HPV 16 E6/E7 and E6 did activate telomerase in some but not all of the epithelial cell cultures tested. This study taken together with the Foster et al. (1996) paper appeared to be in direct conflict with each other. A direct comparison between the findings of the two articles could not be performed as Foster et al. (1996) did not analyze for the presence of telomerase. In order to better understand the role of p53 in the activation potential of telomerase, experiments to test the chronology of events are being conducted. Initially a number of both mammary epithelial and stromal organoid cultures are being established in order to observe differences in the p53 and pRb expression levels before self-selection. These cultures were transduced with HPV 16 E6/E7, E6 or E7 recombinant retroviral vectors under the MMLV promoter-enhancer sequences before the second passage *in vitro*. Control cultures and sister cultures for protein harvest grew concurrently. At the time of the second passage, samples were taken for TRAP analysis and protein extraction. Figure 11 is a representative protein blot of both pRb and p53 from a series of pre-selection cultures. Preliminary telomerase activity analysis indicates that certain organoids/primary cultures exhibit telomerase activity initially, possibly due to the presence stem cells within the population (Figure 12). For those cultures which had telomerase activity initially, telomerase activity disappeared and then reappeared within two population doublings of HPV 16 E6/E7 infection (Figure 13). Also, some of the organoids which did not have telomerase activity reactivated within 2 - 3 population doublings of infection. There were also cultures that either turned telomerase on 10 - 15 population doublings after infection or never reactivated telomerase. Basically, these findings indicate that it is most probably the genetics of the individual or the cell

type that determines the likelihood of telomerase reactivation. Western blots, more cell culture and telomerase characterization are currently underway.

E. Studies have shown that companion breast stromal tissues provide significant influence on tumor development in breast epithelial tissues. Interactions between the breast cancer derived cell line MCF7 and five different mammary and lymph-node derived fibroblast lines were studied in coculture (Adam et al., 1994). Charcoal-treated fetal calf serum (which eliminates all known estrogens) was used in parallel with standard fetal calf serum to compare growth of the cells with and without the presence of steroids. The results showed that almost 70% of the proliferation of MCF7 cells depended upon the presence of steroids in the media and upon the duration of time allowed for growth. The presence of steroid hormones such as in the growth media was required for proliferation of the cancer-derived epithelial cells. The cells tended to grow more quickly in the first few days of incubation and slowed by the fifth day.

This study also showed that the presence and growth patterns of cocultured fibroblast cells significantly affected the proliferation of the MCF7 cells. In general, the epithelial cells grew more slowly as the fibroblast cells approaching confluency proliferated slower. However, different fibroblast lines exhibited different stimulatory or inhibitory effects on the growth of the epithelial cells. While the different fibroblast cell lines afforded different growth patterns in the epithelial cell line, no significant patterns in predicting which fibroblasts would provide stimulus or inhibition for epithelial growth was found.

Stromal cells derived from tumors were stimulated into growth by the presence of the tumor derived epithelial cells, in contrast to the fibroblasts derived from "non-invasive" tumoral or non-pathological sources, which were not stimulated by the epithelial cells.

The *in vivo* transplantation of mouse fetal mammary mesenchyme into adult mammary tissue induced adult mammary epithelium to grow (Kanazawa et al. 1992). The resulting hyperplastic tissue was more sensitive to carcinogens and mammary virus than its "normal"

predecessor.

Growth factors have been shown to enhance proliferation and sustain mammary epithelial cells in culture. Hepatocyte growth factor (HGF) has been determined to be a paracrine cytokine produced by stromal (or mesenchymal) cells to communicate with adjacent epithelial cells, stimulating motility, proliferation, and morphogenesis (Rosen et al. 1994). However, expression of HGF by stromal cells has been shown to be down-regulated in the presence of epithelial cells, possibly indicating a negative feedback response. Hepatocyte growth factor (HGF, or Scatter Factor) and Keratinocyte growth factor (KGF) were used in studies looking at the growth and receptor complement of human mammary epithelial cells (Wilson et al. 1994, Imagawa et al. 1994). Both growth factors were found to maintain epithelial cell viability and stimulate growth in a manner similar to epidermal growth factor (EGF). However, the combination of the growth factors did not have an additive effect. HGF has also been described as a stimulator of intestinal epithelial cell motility (Dignass et al, 1994). In addition to stimulating proliferation, scatter factor was shown to regulate a seven-fold increase in migration of epithelial cells across an induced "wound" in a cultured monolayer. Serum free media was applied to the cells before and after "wounding" the monolayer to ensure cellular migration rather than proliferation. Keratinocyte growth factor (KGF), like HGF has been shown to maintain and stimulate proliferation of mammary epithelial cells. However, KGF has not been shown to regulate mobility of epithelial cells in culture (Dignass et al 1994).

The goal of this project is to observe the interactions and effects of "normal" human mammary stromal cells on their corresponding human mammary epithelial cells, from the same patient, in a coculture environment. Cellular proliferation and migration of mammary epithelial cells in the presence of stromal fibroblasts and growth factors will be also be examined as well as proliferation and migration of immortal mammary epithelial and tumor derived mammary epithelial cells. In addition to standard coculture, the effects of HGF, KGF and EGF will be studied.

The first part of the study was to establish base-line control measurements. These include

standard growth patterns and population doubling times of HME and HMS cells in culture, and in coculture. Controls for these experiments include the HME and HMS cells in culture alone and with an empty coculture membrane present. Figure 15 illustrate the results of this portion.

The second part of the study will establish control measurements for migration of HME cells in coculture with HMS cells. This will be accomplished using specially machined cloning rings to plate defined clusters of normal, immortal or tumor derived HME cells. Once the clusters of cells are in place, the rings were removed, and cocultures of HMS cells added along with mitomycin C (to inhibit mitosis). Migration of the HME cells were observed over time. Controls for these experiments include growth of the HME clusters in culture alone, or with an empty coculture membrane. Alternatively, Boyden's assays for cellular migration may be employed (Sherratt et al, 1993). This assay uses an elevated porous membrane on which the HME cells would be "plated". Stromal cells would be plated on the bottom of the assay chamber. Migration of the epithelial cells (in the presence of mitomycin C) through the porous membrane would be observed and monitored. The third part of this study looked at the proliferation and migration of HME cells in coculture with HMS cells and in the presence of HGF, KGF and EGF. Culture conditions similar to those described were established but include the growth factors in the media. The growth factors will be applied individually to gather preliminary data and later in combination to observe any additive effects. Controls include omitting growth factors and/or coculture conditions.

4. Projects remaining to be completed and possible future directions for the study

A. Tasks completed:

- 1) transfect p53 mutants commonly observed in human breast cancer into normal human mammary epithelial cells obtained from different donors and isolate clones.
- 2) characterize the clones for extension of lifespan and immortalization.
- 3) determine in breast epithelial cells immortalized and expressing mutant p53 if expression of the

mutant p53 is necessary for the maintenance of growth.

B. Tasks currently underway:

1) determine if expression of any of the p53 mutants provide a selective growth advantage to breast epithelial cells prior to immortalization.

C. Tasks to be initiated:

2) determine downstream genomic targets of p53 that may be important in the development and progression of breast cancer. This specific aim will be approached using not only normal mammary epithelial cell strains, but also the spontaneously immortalized Li-Fraumeni cell line and the immortalized mammary epithelial cell line 32(273) that contains the p53 mutant 273. Side-by-side comparisons of the differences in the products obtained through the casting technique may give insight into important gene(s) that when inactivated, allows the M1 cellular senescence mechanism to be overcome progressing the cell toward cancer formation.

D. Studies supported by this DOD grant published/submitted during the second period:

- Holt SE, **Gollahon LS**, Willingham T, et al. P53 levels in mammary epithelial cells expressing wild-type and mutant human papillomavirus type 16 (HPV-16) E⁶ proteins: relationship to reactivation of telomerase and immortalization. *Int J Oncology* 1995; 8:263-270.
- **Gollahon LS** and Shay JW. Immortalization of human mammary epithelial cells transfected with mutant p53 (273^{his}). *Oncogene* 1996; 12:715-725.
- Hiyama E, **Gollahon L**, Kataoka T, et al: Telomerase activity in human breast tumors. *J. Natl Cancer Inst* 1996; 88:116-122.
- **Gollahon LS**, Pearson AS, O'Neal NA, et al. Detection of telomerase activity in breast masses by fine needle aspiration. *Annals of Surgery*, 1996 submitted.

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Conclusions

To date, the tasks completed include transfecting p53 mutants that occur frequently in human breast cancer into normal human breast epithelial cells obtained from different donors. Clones have been isolated and have been characterized for extension of lifespan and immortalization. It has also been determined that breast epithelial cells immortalized and expressing mutant p53 require the mutant p53 for maintenance of cell proliferation. Experiments in progress include the task to determine if expression of p53 mutants provides a selective growth advantage to breast epithelial cells prior to immortalization. A more technically difficult and final remaining task is the determination of downstream genomic targets of p53 that may be important in the development and progression of breast cancer. Three manuscripts have been published during the initial two years of this project and one additional manuscript is in review.

Figure Legends/Results

Table 1. Number of clones obtained from each p53 mutant and extension of lifespan observed.

Cell strain / senescence		P53 mut 143	EL	P53 mut 175	EL	P53 mut 248	EL	P53 mut 273	EL	pZip Neo	EL
HME 15	50	8	8	6	3	3	0	5	1	6	0
HME 17	30	8	8	6	5	4	1	4	1	3	0
HME 73	40	6	6	4	3	2	0	1	0	3	0

Table 1 summarizes the cell strains infected with p53 mutants 143, 175, 248 and 273. The lifespan in culture has been determined for each cell strain from primary tissues to senescence. A maximum of 8 clones was pulled for each mutant when available. EL = the extension of lifespan initiated by the presence of the mutant p53. The criterion for this extension was 10 or more PDLs past cellular senescence characterized in culture. No immortalization events have been observed to date. As shown by the vector alone, no extension of lifespan was observed without the presence of the mutant p53. The results with these strains reflects that observed previously (Gollahon and Shay, 1996).

Figure 1. Extension of lifespan for the mutants infected into cell strains HME 15, 17 and 73. P53 mutant 143 appears to extend the lifespan of the HME cells in culture more efficiently than the other p53 mutants. However, there does not appear to be a significant difference within HME 17 between p53 mutants. Clonal density was also greater for the 143 mutant infected cells for all 3 cell strains. Although clones were isolated from the vector there was no observed extension of lifespan.

Figure 2. Growth kinetics of the 1299 mutant 143 clones. Representative clones from each integration method were cultured for up to 17 days and counted at time intervals of 1, 3, 6, 9, 13 and 17 days. A. Shows the growth curves of the clones at 37°C. At 37°C there is no significant

difference in growth rates compared to the parent cell line 1299. B. Growth curves for the clones at 32°C. At 32°C, the clones show variable rates of growth. Possible mechanisms for these differences are being examined.

Figure 3: Western blots for p53 levels in 1299(143) clones. 20 µg total protein was loaded for all the samples and probed with PAB DO-1 (Oncogene Science). A. Clones with different rates of growth at 32°C. Control parent 1299 cells are null for p53 protein product. HME 32 are normal mammary epithelial cells and 32(273) is the immortal clone obtained from insertion of the p53 mutant 273. B. Western blot of p53 levels over time in culture at both 32 °C and 37 °C. C. P53 levels of the different 1299 (143) clones at 37 °C. Clone references are as follows EC/L=electroporated; LL=lipofectin transfection; LC=transfection using calcium phosphate. In B and C it appears that LC is a revertant and may have lost the construct. It is currently being tested for the presence of the p53 construct.

Figure 4: Western blots of the 1299 clones observing levels of p16 and p21. Clones LL-1 and EC-2 were selected for testing based on cell growth and morphology at 37 °C and 32°C. EC-2 showed no morphological changes at either temperature and growth rate at 32°C paralleled that of the control 1299 cells. LL-1 showed larger, vacuolated, slow growing cells at 32°C of the type associated with senescence. These were the clones selected for experiments to determine biological differences in the effects of the same mutant introduced by different methods A. Shows levels of expression for these clones at 32°C and 37°C. The results would indicate that p16 is undetectable in clone LL-1 by the highly sensitive Tropix® Western blotting method. Alternatively, EC-2 appears to have comparable levels at both temperatures. B. The same clones as in A looking at levels of p21. At 37°C there is no detectable signal for either LL-1 or EC-2. However, C. Shows an overexposure at 37°C for all the clones and there appears to be very low levels present for all the clones. This may be indicative of the p53 independent pathways of p21 that are now starting to receive attention. D. Shows relatively high levels of p21 at 32 °C which may be confirming what is observed in C. If the p53 mutant 143 is changing to a wild-type conformation, then perhaps this wild-type p53 is affecting the levels of p21 directly. This question is currently under investigation.

Figure 5. 1299 cells were infected with p53 mutant 143 under the control of the MMLV promoter. This western is a representative clone showing levels of expression at 32 °C and 37 °C. As observed, there does not appear to be any significant difference in signal between temperatures.

20 μ G of total protein was loaded and probed with PAb DO-1 (Oncogene Science). The levels of expression may be dependent upon the mechanism of introduction of the construct, i.e. infection vs. Lipofectin or calcium phosphate or electroporation transfection. This is currently being investigated.

Figure 6. Preliminary immunoprecipitation of 1299(143) clone EC-2 using antibodies PAb DO-1 (which recognizes both mutant and wild-type forms of p53), PAb 240 (recognizes only mutant p53), PAb 1620 (recognizes wild-type p53 conformation only). At 37 °C, both DO-1 and 240 show detectable levels of p53 whereas 1620 does not recognize any. 32 °C shows DO-1 strong reactivity while both 240 and 1620 have lower levels. Samples were harvested after 24 hours at 32 °C.

Figure 7. A. Bar graph depicting the differences in growth rate between 32°C and 37°C. Also, the differences in growth rates between individual clones at both temperatures can be observed. X-axis indicates days in culture as well as the temperature, Y-axis shows the population doublings in culture. * indicates clones cultured at 37°C. Day 17 in culture all the clones were >100% confluent in the wells and therefore an accurate PDL greater than 10 could not be determined. Clones LL-1 and EC-2 were excluded as they were individually determined and are illustrated in part B. B. The 2 clones which had significantly different growth rates and morphologies are LL-1 and EC-2. As illustrated by the line graph, EC-2 grew with rates comparable to those of the parent cell line 1299. LL-1 however, grew more slowly and exhibited a morphology more reminiscent of cellular senescence. Both clones have the construct present as evidenced by PCR. 7C. Comparison of the remaining clones at 37°C after 6 days in culture.

Figure 8. TRAP gel of 1299(143) clones LL-1 and EC-2 which exhibit different growth rates and morphologies at 32 °C. This initial gel was performed using 100 cell equivalents. Rudimentary quantitations were conducted using the relative telomerase activity versus the intensity of ITAS. Initially, the LL-1 clone shows a 10 fold decrease in telomerase activity at 32 °C at 7,9 and 14 days in culture. In contrast, LL-1 at 37 °C and clone EC-2 at both temperatures do not show any significant differences in the intensity of telomerase signal. This experiment is currently being redone with a new extraction method that allows for >70% of the endogenous telomerase activity to be removed. This will more accurately show any differences in telomerase activity for individual clones. Whether this difference that we are seeing is due to the promoters driving the mutant p53 gene, the number of copies inserted with the genome of the target cells, where the constructs have

integrated into the genome or the method of introduction, i.e. electroporation, lipofectin or calcium phosphate transfection, is currently being investigated.

Figure 9. PCR analysis was performed on each of the clones to verify the integration of the p53 mutant 143 construct. Primers were generated to the regions flanking the promoter and the p53 gene at codon 273. For a review of the method and primer sequences, see Gollahon and Shay (1996). PCR confirmed the presence of the mutant p53 gene in each of the clones. A. Shows the control line along with a mock transfected sample and clones EC-2 and LL-1. The fragment generated is 347 bp. The ladder used is 100 bp. B. The plasmid construct and the remaining clones were PCR amplified to verify the presence of the mutant p53 gene.

Figure 10. A representative TRF gel of the 1299 (143) clones which shows the differences in TRF length in comparison to the control cell population. DNA was extracted using established techniques (Maniatis), run on an 0.8% agarose gel overnight, dried, treated, then probed with a $(T_2AG^3)_4$ oligomer incorporated with γ - ^{32}P overnight. After rinsing, the gel was exposed to a Molecular Dynamics Phosphorimage cassette overnight and the developed. HindIII and 1Kb ladders were used as marker lanes. Note the drop in TRF signal for clones EL-1, EL-2 < LC-2, EC-1, EC-2. These clones are presently in culture to determine if this phenomenon has stabilized or if the telomeres are continuing to shorten and if so, what effect this might have on the biology and/or telomerase activity of the cells.

Figure 11. Western blot for p53 and Rb from organoids and very early passage primary mammary epithelial and stromal cell cultures. A. Mammary epithelial cells appear to increase the levels of p53 as they approach senescence (HME 31 senescence near PDL 45, HME 32 at PDL 30). With the introduction of HPV-16 E6E7, the levels of p53 decrease as expected with the degradation of p53 by E6. It appears that this effect may be gradual and perhaps occur after 10 or more PDLs. B. The western of Rb is not conclusive at this time and is being redone. Initially it appears however, that there are baseline levels of Rb which remain constant throughout the culture of the mortal cells. The introduction of HPV-16 E6E7 may also be time dependent.

Figures 12 and 13. These figures illustrate the reactivation of telomerase after introduction of the HPV-16 E6E7 genes.

Figure 13 indicates that the reactivation may be strain specific due to the fact that we have infected HME 32 (normal mammary epithelial cells) at primary passage and 10 PDLs before senescence and

observe telomerase reactivation in both cases. In contrast, HME 50 (Li-Fraumeni cells) will only reactivate telomerase if it is infected within 5 PDLs of being established in culture while HME 51 (cells obtained from a patient with cancer involving the BrCa-1 locus) only immortalized with HPV 16 E6E7 when infected at later passages.

Figure 14. Reactivation of telomerase in HME 51 and 52. The PD of HME 51 after transfection with HPV 16 E6E7 (PD29) indicates the number of population doublings undergone from the time of transfection. Thus, ~ 30 PDLs later, an immortalization event is observed. HMS 52 which initially had strong telomerase, quickly lost this signal with progressive subculturing and was not observed to reactivate telomerase. These results taken together, initially suggest that it is not specifically E6 that is reactivating the telomerase, but most probably the genetics of each individual strain as well as the biology of the cells/tissue in culture at the time of transfection that determines success of immortalization. We are currently investigating this question.

Figure 14. Proliferation study of HME 50-9 in co-culture with HMS 50. Medium parameters were already established. This experiment determined optimum cell densities and effects of different densities on growth rates of human mammary epithelial and stromal cell types. A. Cultures grown individually to obtain an average of cell cycle turnover and cell proliferation. B-D. Graphs indicating the growth potential and affects of cells at different densities plated. From this initial experiment, it appears as though stromal cell growth may be dependent on plating density whereas. The epithelial cells grew ~ 2PDL greater than controls in the presence of the stromal cells. Likewise for those cultures at comparable densities to the controls, the stromal cells also appear to grow better in the presence of the epithelial cells. There may be a synergistic effect. Epithelial cells are now being grown in the presence of lung fibroblasts and the stromal cells are growing in the presence of cervical epithelial cells to observe whether cells from different tissue types reproduces the effect shown here.

Figure 1

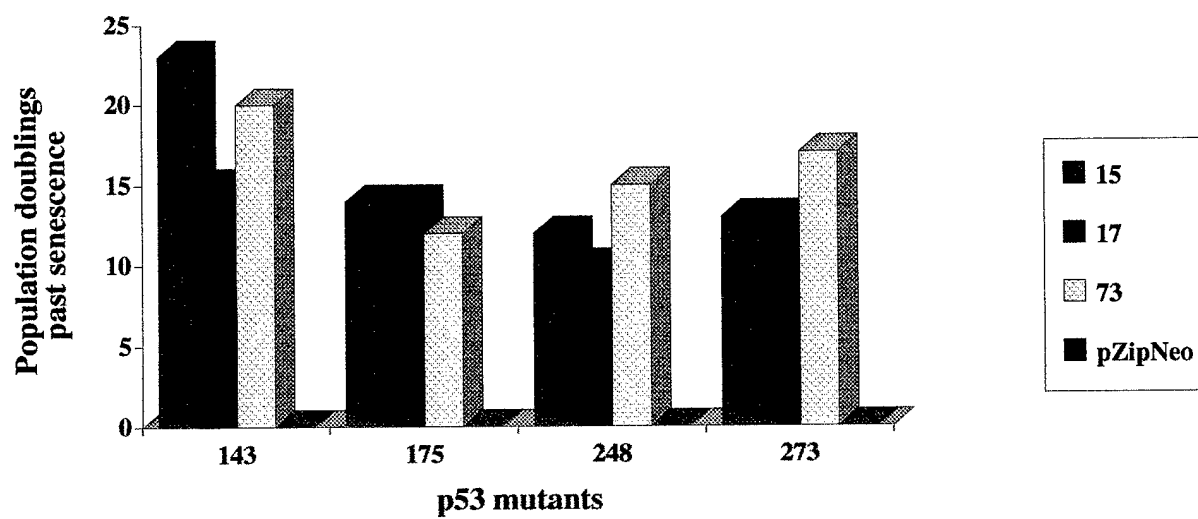
Extension of Lifespan for p53 mutants in HME cell strains

Figure 2A.

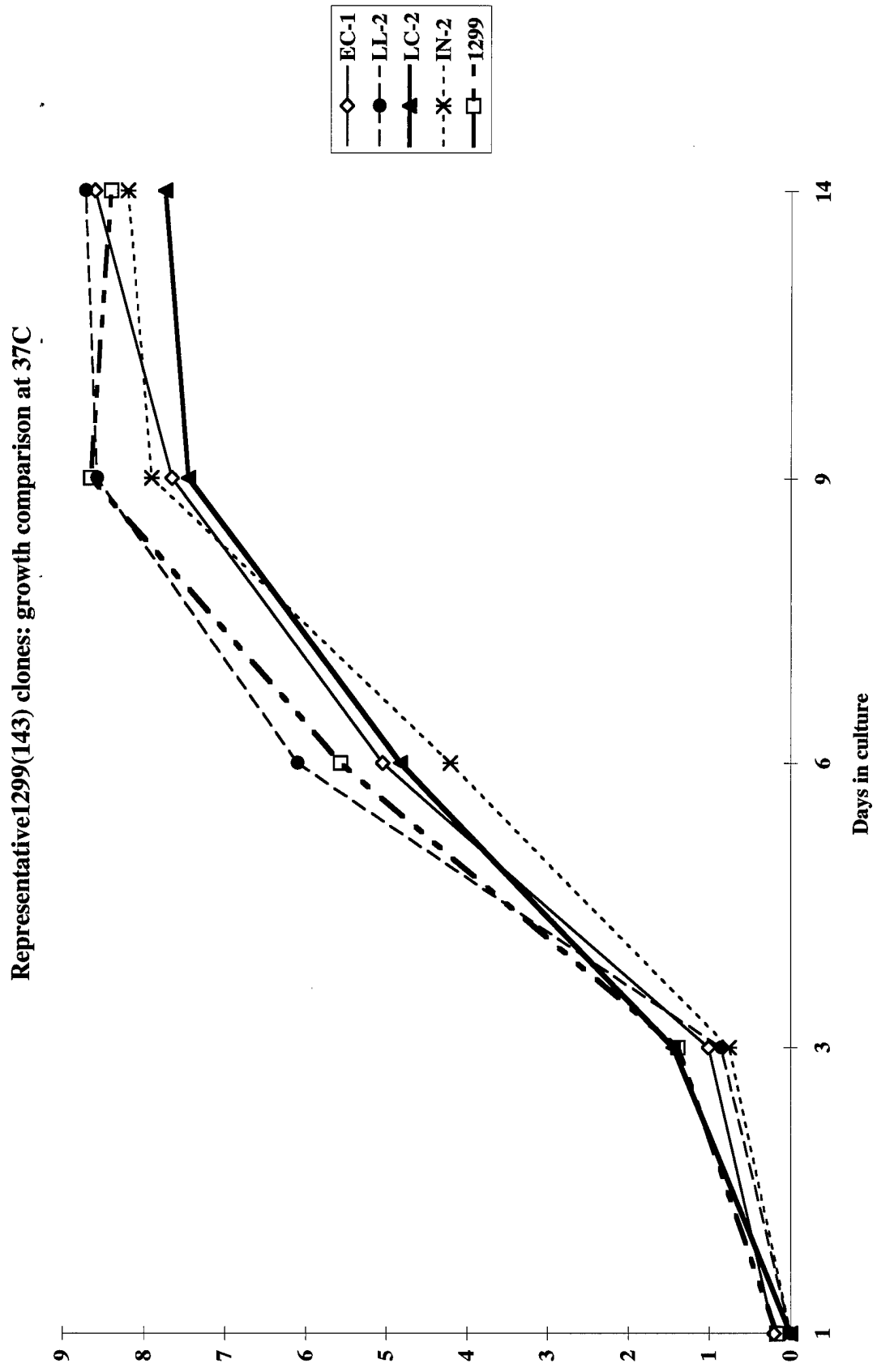


Figure 2B.

Representative 1299(143) clones: a growth comparison at 32C

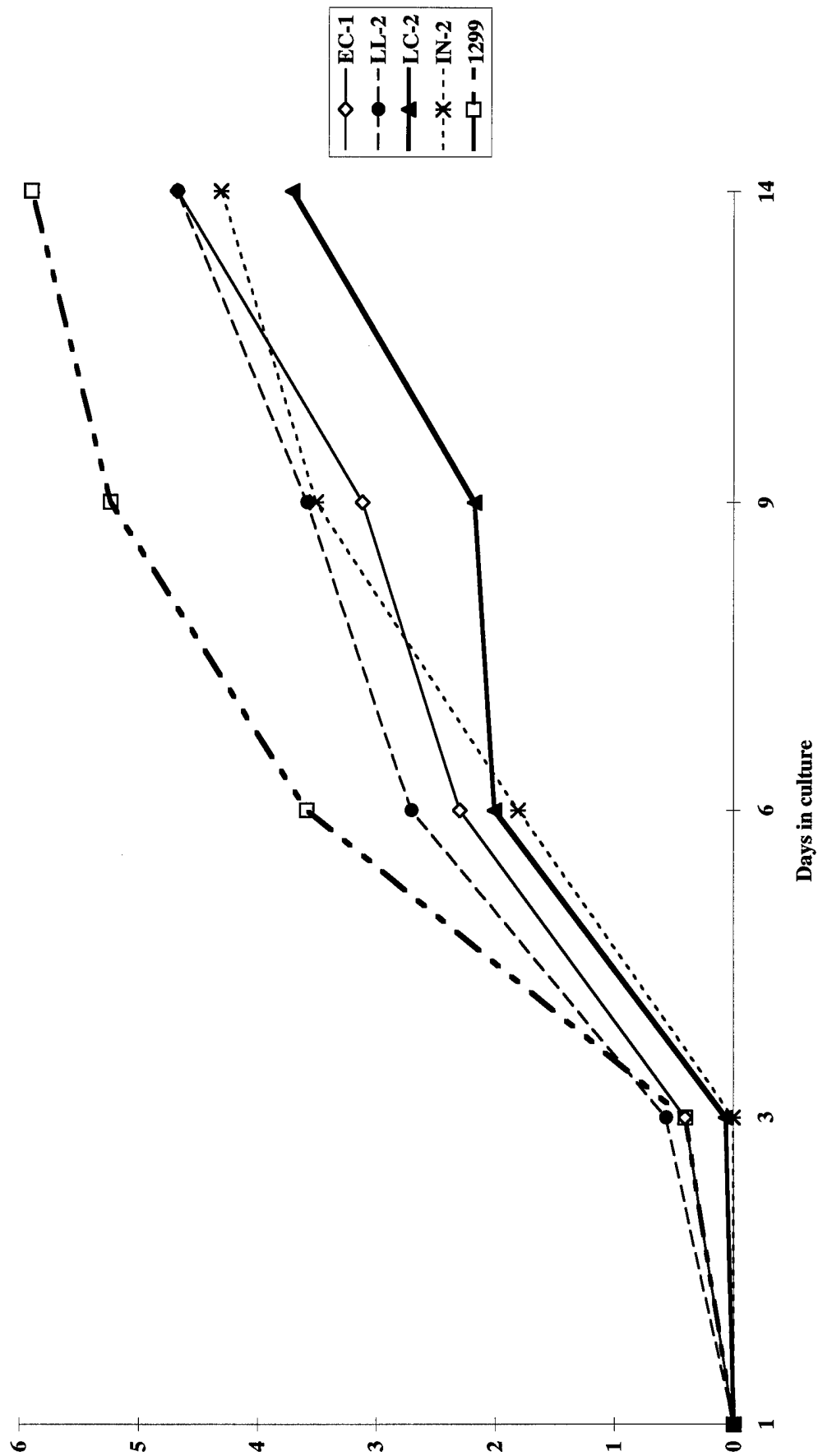


FIGURE 3A



FIGURE 3B



FIGURE 3C

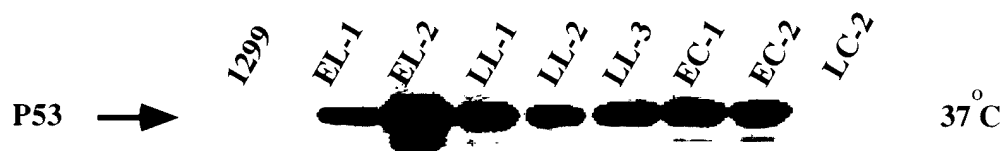


FIGURE 4A

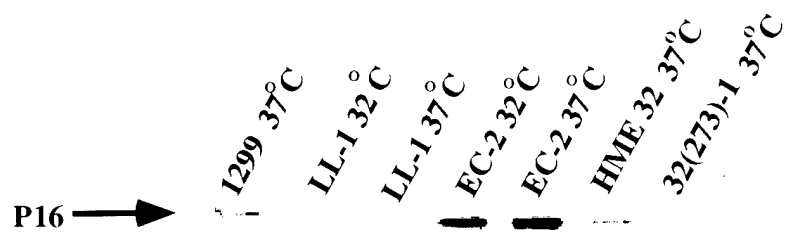


FIGURE 4B

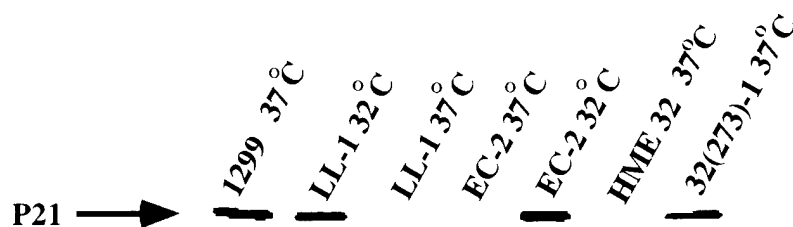


FIGURE 4C

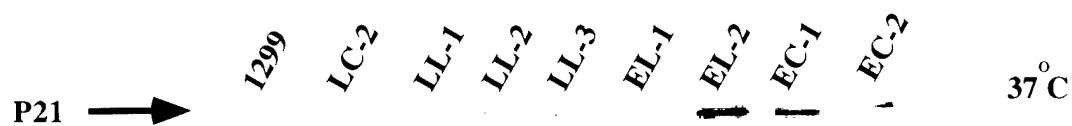


FIGURE 4D

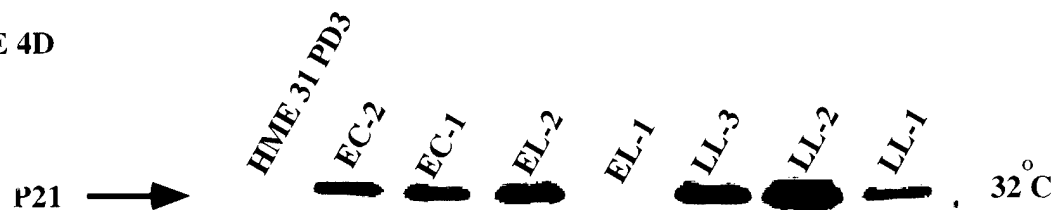


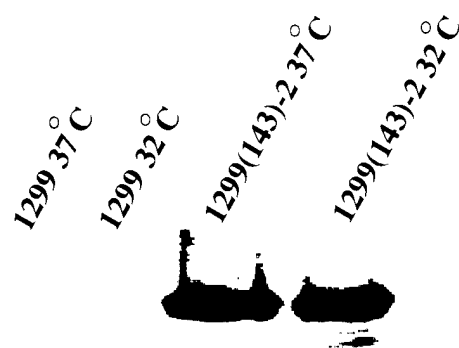
FIGURE 5

Figure 6. Immunoprecipitation of 1299(143) clone EC-2 with antibodies to p53

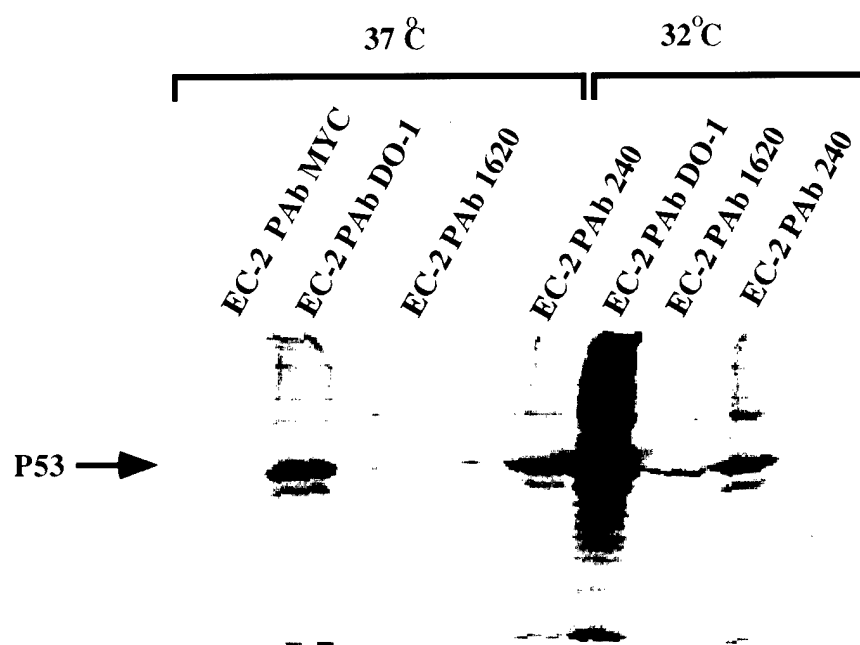
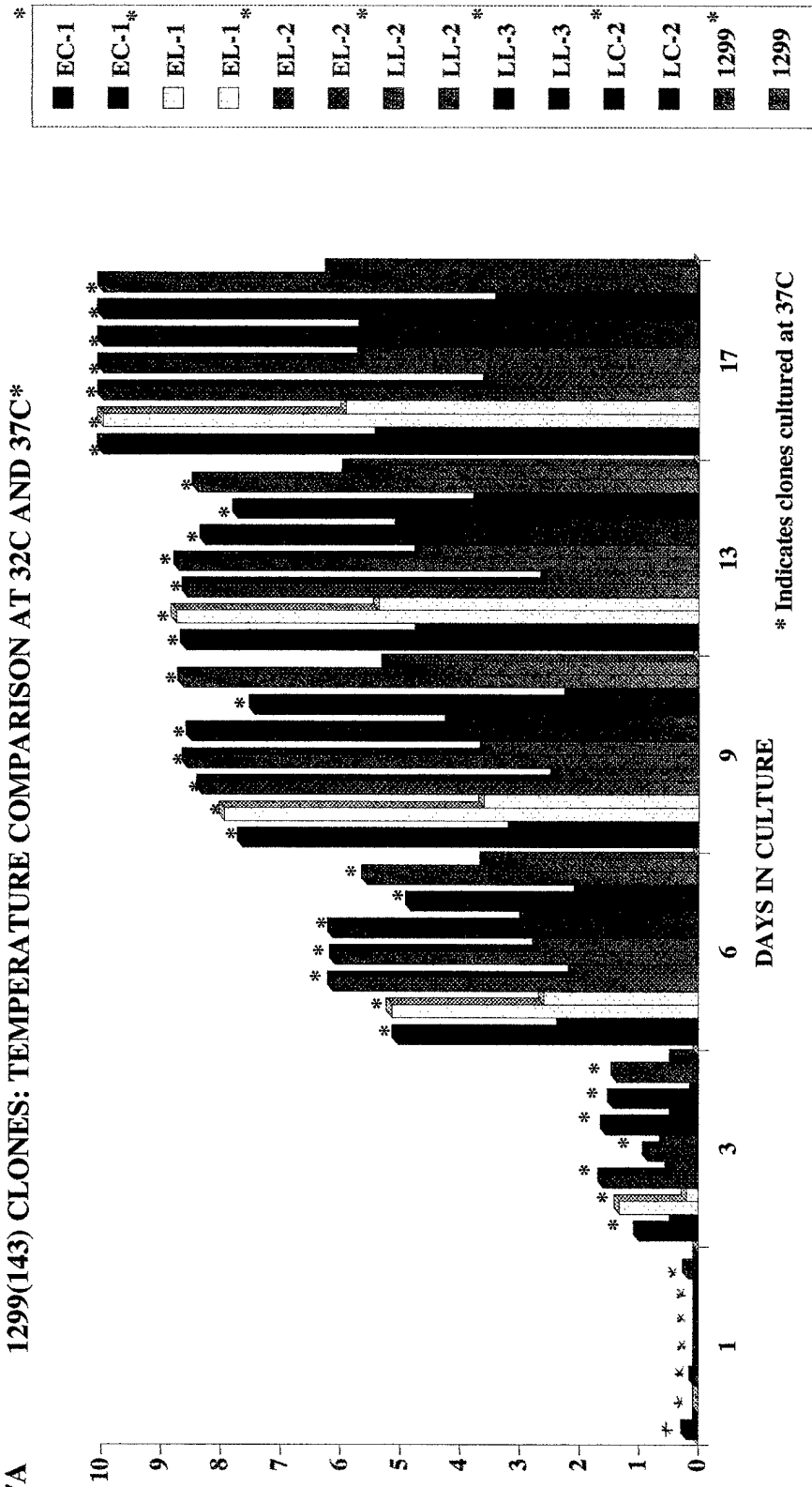


FIGURE 7A 1299(143) CLONES: TEMPERATURE COMPARISON AT 32C AND 37C*



* Indicates clones cultured at 37C

Figure 7B
Growth comparison between clones of 1299 (143) series

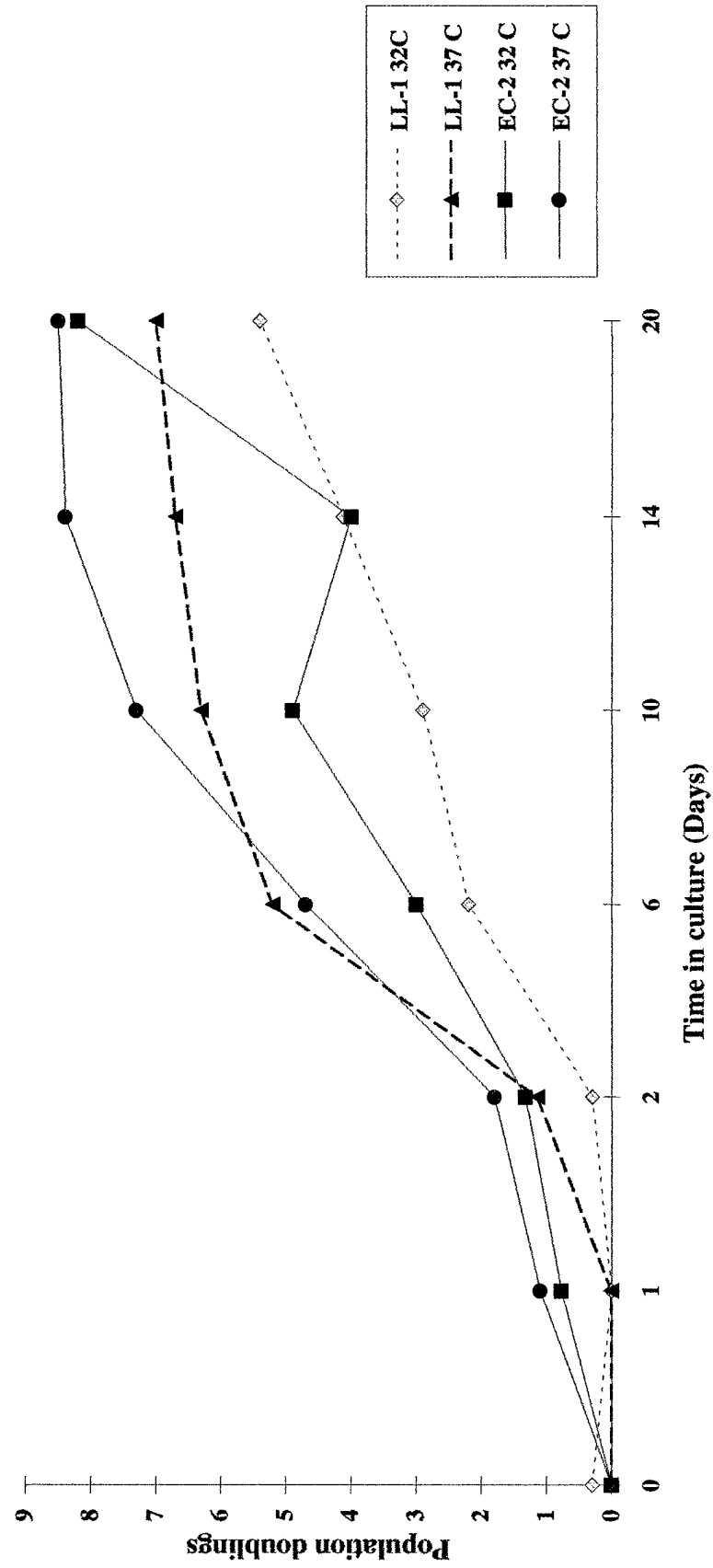


Figure 7C

1299(143) CLONES AT 37 C

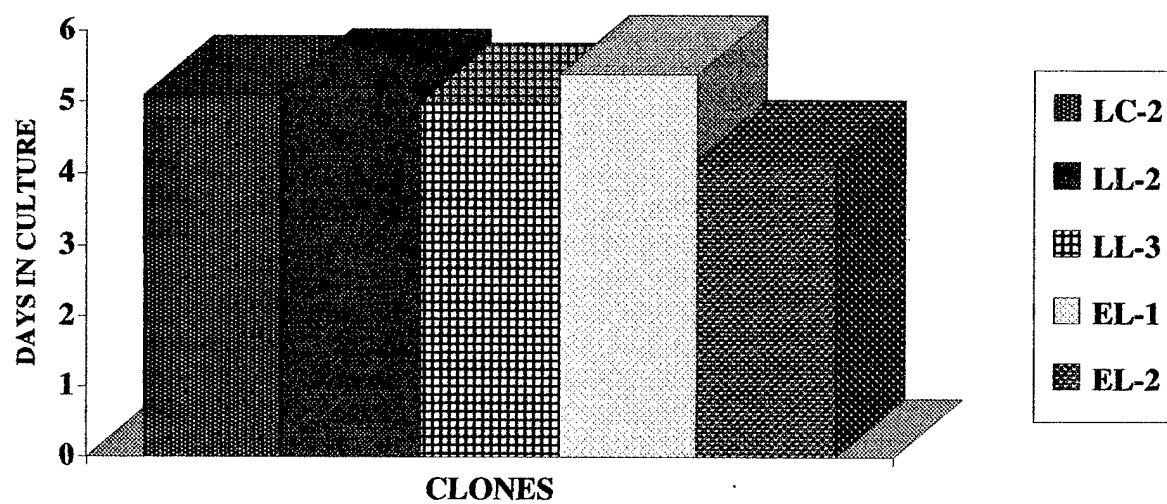


Figure 8

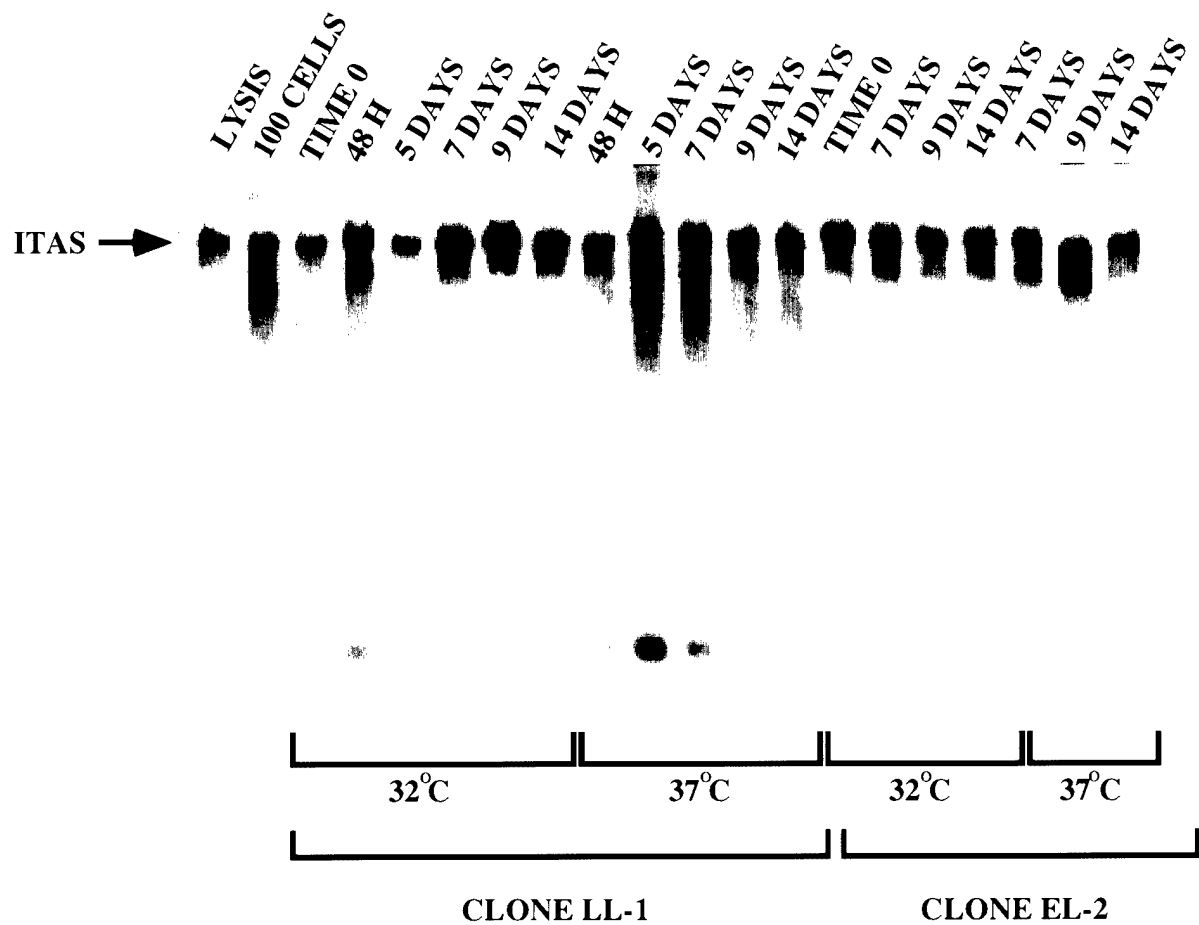


Figure 9. PCR analysis of the successful integration of the mutant p53 gene into 1299 cells.

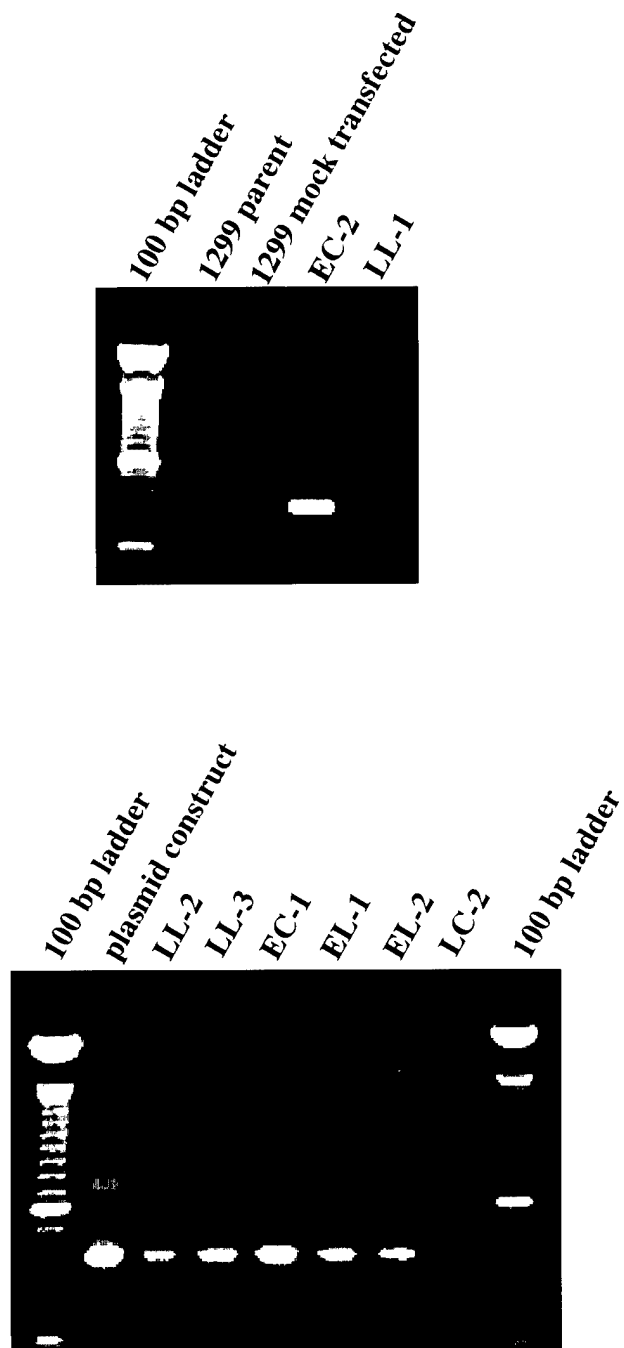


Figure 10. TRF analysis of 1299(143) clones

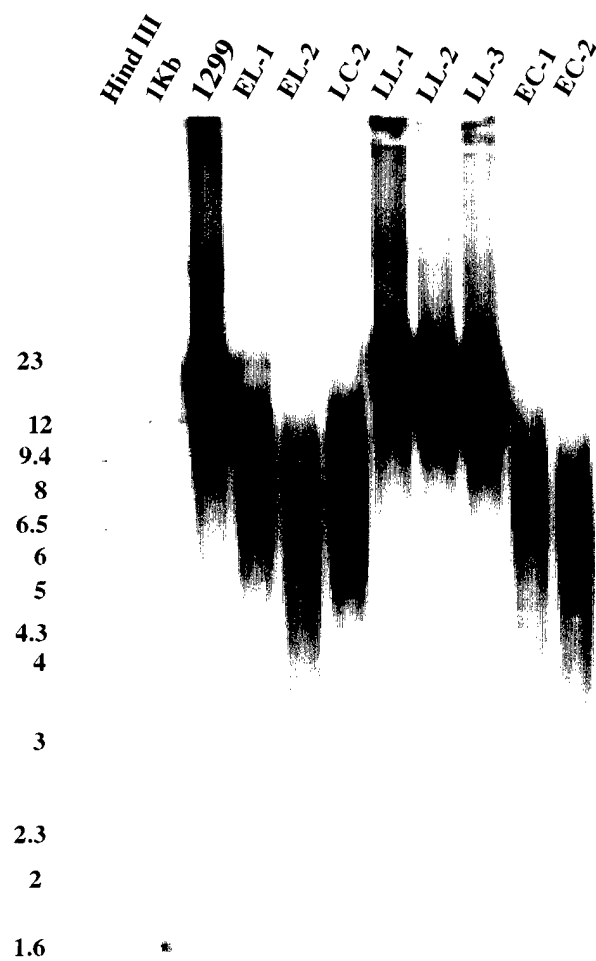


Figure 11

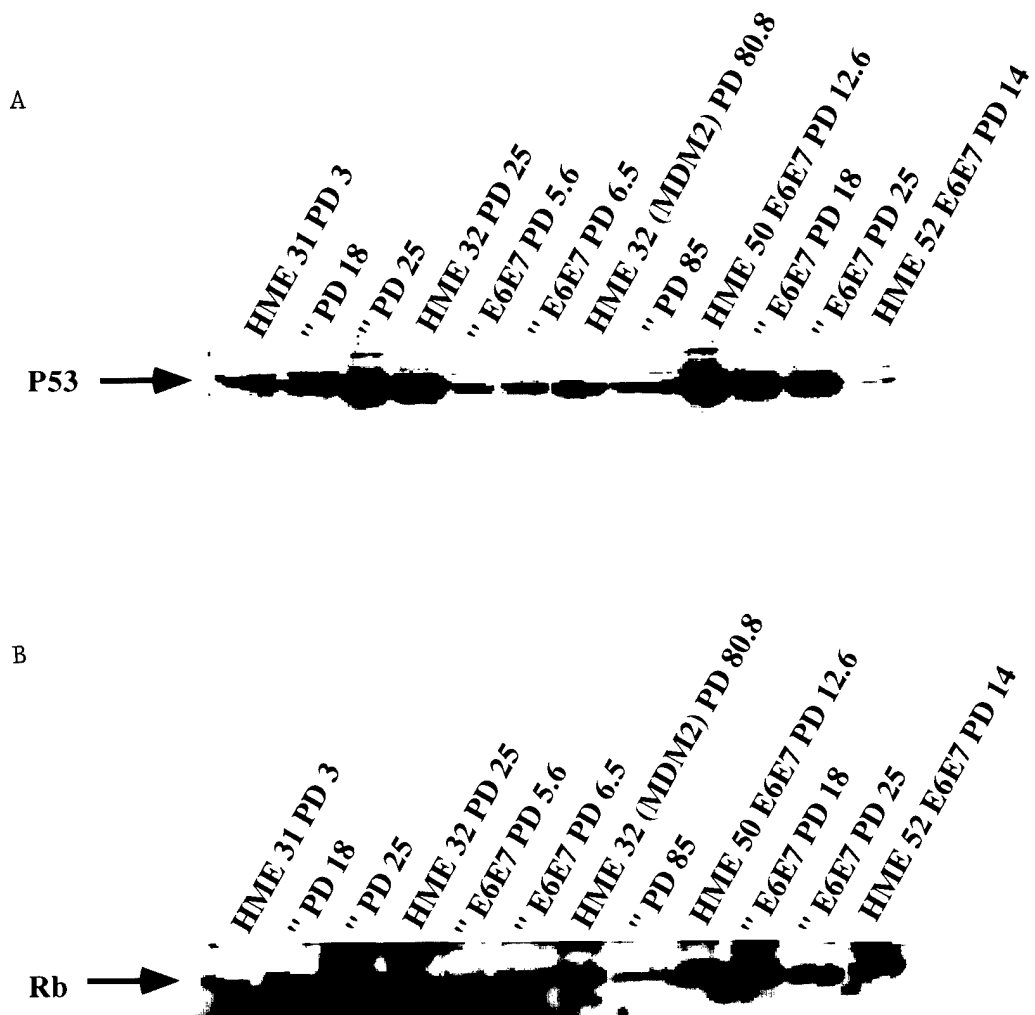


Figure 12

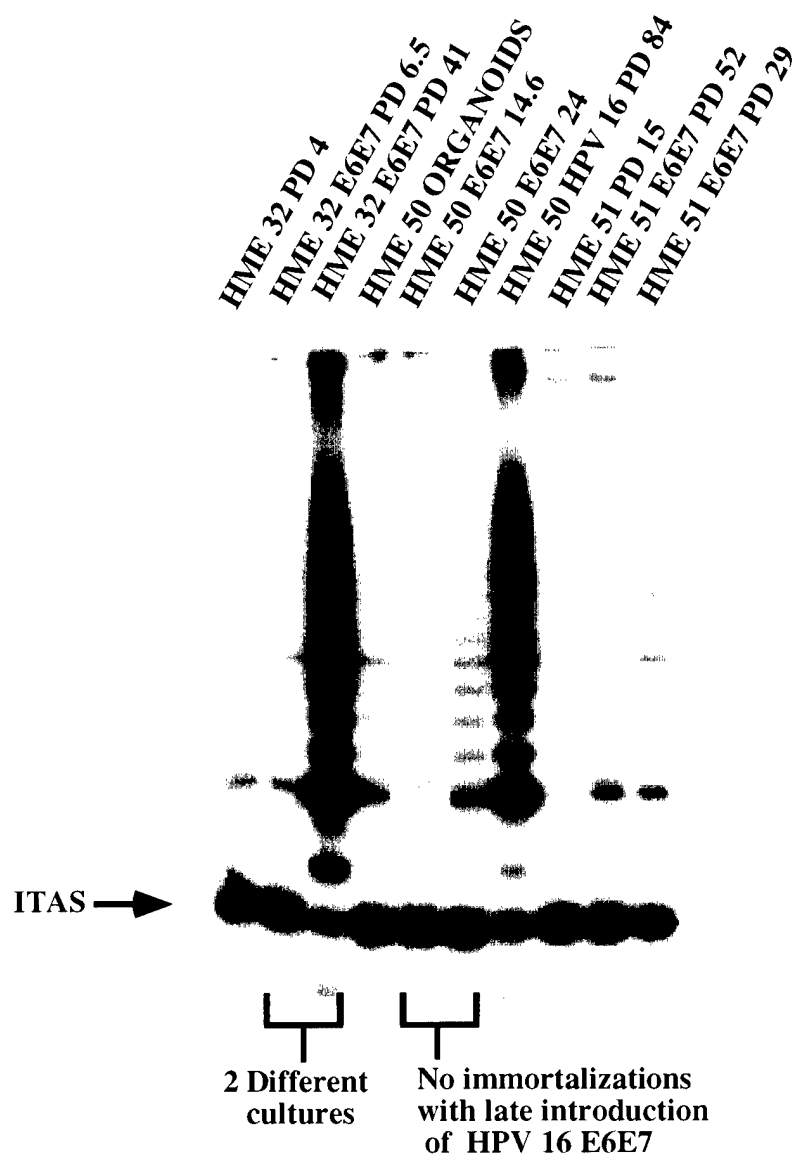


Figure 13

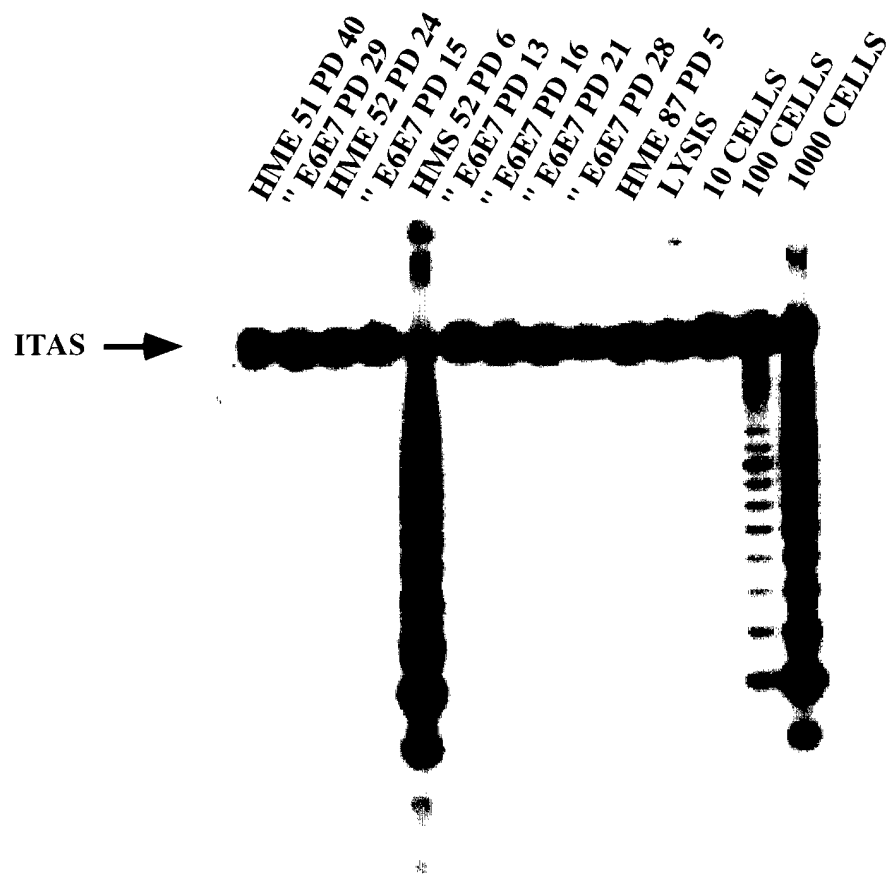
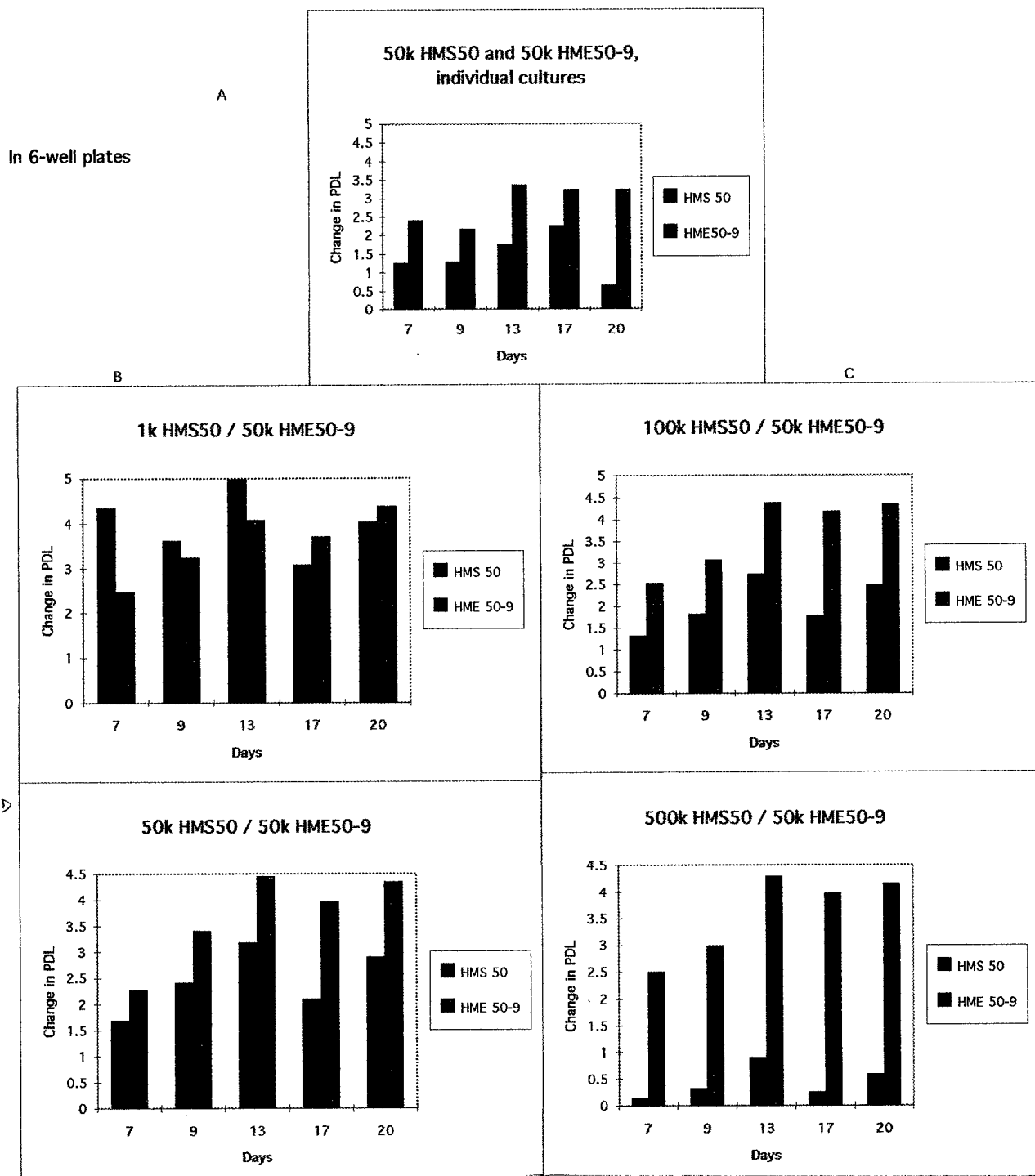


Figure 14

Proliferation Study of HME50-9 in Coculture with HMS50



**Detection of Telomerase Activity in Breast Masses
by Fine Needle Aspiration**

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Running title: FNA and telomerase in breast cancer

Mini-Abstract

Telomerase, an RNA-dependent DNA polymerase critical to the process of cellular immortalization, is reactivated in many human cancers. This study demonstrates that telomerase activity can be accurately determined using a combination of fine needle aspiration and the telomere repeat amplification protocol, allowing for expanded study of telomerase as a molecular marker in cancer.

Abstract

Objective: This study was undertaken to determine whether telomerase activity could be reliably assessed in benign and malignant breast masses obtained by fine needle aspiration (FNA).

Summary Background Data: Telomerase is an RNA-dependent DNA polymerase which compensates for the telomere shortening that occurs in its absence. Reactivation of telomerase is thought to be an important step in cellular immortalization, and recent studies have indicated that telomerase activity is frequently detected in primary human malignancies. The clinical implications of telomerase activity in human tumors are currently under investigation.

Methods: Eighty-nine samples (46 FNAs and 43 gross tissue biopsies) from 44 patients with breast masses were analyzed prospectively for the presence of telomerase activity by the Telomere Repeat Amplification Protocol (TRAP). All samples were obtained directly from the excised mass at the time of specimen removal in the operating room.

Results: Telomerase activity was detected in 17/19 (90%) of FNA samples and 15/18 (83%) of the invasive breast cancer tissue biopsies. Telomerase was also detected in 9/16 (56%) FNAs and 8/15 (53%) tissue biopsies from 16 fibroadenomas. Other benign proliferative lesions (n=5) did not have detectable telomerase activity in either FNA or tissue specimens. FNA-TRAP results correlated with the gross tissue specimen TRAP results in 95% of all cases.

Conclusion: The FNA-TRAP assay for telomerase detection is a highly sensitive and accurate method for the detection of telomerase activity in breast masses. Future application of these techniques should facilitate evaluation of telomerase as a tumor marker in the clinical management of breast and other solid malignancies.

Introduction

Telomeres are specialized structures at the ends of eukaryotic chromosomes that stabilize and protect the chromosome (1). In normal somatic cells, telomeres become progressively shorter with each cell division, and this loss of telomere length is considered to be one mechanism by which limited replicative ability (cellular senescence) and ultimately cell mortality, is signalled (2,3).

Telomerase is an RNA-dependent DNA polymerase that stabilizes telomere length by synthesizing and adding the hexameric repeats (TTAGGG) to telomeres (4). Thus, it has been postulated that the synthesis of DNA at the chromosome ends by telomerase may be required to sustain the indefinite proliferation of most malignant tumors (2,4,5). Recent studies have indicated that telomerase activity can be detected in tissues and cells by a highly sensitive PCR-based assay called the Telomere Repeat Amplification Protocol (TRAP) (6,7). Using the TRAP assay, it has been demonstrated that telomerase activity is present in a high percentage of primary human malignancies (8,9). Numerous reports have documented telomerase activity in a high percentage of a variety of tumors including those of the breast (10), lung (11), colon (12, 13), kidney (14), ovaries (15), liver (16), brain (17) and prostate (18), while in cleared margins around tumors telomerase activity is only rarely detected (9). Telomerase activity is absent in adult tissues, with the exception of male germline and embryonal cells (19), proliferating cells of renewal tissues (20, 21), basal epidermal

cells (22, 23), intestinal crypt cells (24) and activated lymphocytes (25, 26).

These findings have stimulated much discussion and investigation of the potential diagnostic, prognostic and therapeutic implications for telomerase or telomerase antagonists in the management of cancer. In this study, we sought to determine whether telomerase activity can be detected in breast neoplasms using standard fine needle aspiration (FNA) techniques. We examined both benign and malignant breast masses to evaluate the potential diagnostic use of FNA-TRAP for breast masses. Our data indicate that telomerase activity can be reliably assessed by FNA-TRAP. Further, FNA-based TRAP is a highly sensitive test for malignant breast lesions.

Materials and Methods

Tissue Samples

Forty-four female patients, age 14 to 82 (mean 45) and one male patient, underwent excisional biopsy of palpable breast masses. From these biopsies, 89 samples were obtained for telomerase analysis, including 46 fine-needle aspirates and 43 gross tissue samples. The FNA samples were obtained directly from the excised mass at the time of specimen removal in the operating room. The gross tissue samples were obtained immediately after the specimen was cut for frozen section. In some cases, FNA was obtained from more than one mass excised from the same patient. Additionally, gross tissue samples could not always be obtained for study due to small size of the breast mass and thus, the possibility of interfering

with histologic diagnosis in these smaller lesions.

Histologic Analysis

Invasive breast cancers were characterized using staging standards recognized by the American Joint Commission on Cancer. Rating scales for fibroadenomas were developed for four histologic categories: epithelial cellularity, stromal prominence, stromal cellularity and lymphocytic infiltration, and these rating scales are delineated below.

Histologic Rating Scale for Epithelial Cellularity:

Rating 1: very few epithelial cells - less than 10% of the cut surface occupied by epithelial cells; Rating 2: few epithelial cells - 10-20% of the cut surface epithelial cells; Rating 3: moderate epithelial cells - 20-30% of the cut surface epithelial cells; Rating 4: marked epithelial cells - greater than 30% of the cut surface epithelial cells.

Histologic Rating Scale for Stromal Prominence:

Rating 1: very little stroma - <30% of the cut surface occupied by stroma; Rating 2: mild stromal component - 30-40% of the cut surface stroma; Rating 3: moderate stromal component - 40-50% of the cut surface stroma; Rating 4: marked stromal component - >50% of the cut surface stroma.

Histologic Rating Scale for Stromal Cellularity:

Rating 1: very few stromal cells (hyalinized fibroadenoma) - < 10 stromal cells per 100X field; Rating 2: mild stromal cellularity - 10-50 stromal cells per 100X field; Rating 3: moderate stromal cellularity - 50-100 stromal cells

per 100X field; Rating 4: marked stromal cellularity - > 100 cells per 100X field.

Histologic Rating Scale for Lymphocyte Infiltration:

Rating 1: rare lymphocyte in the cut surface of the specimen; Rating 2: between 1 and 10 small aggregates of lymphocytes seen in the cut surface of the specimen; Rating 3: many lymphocytes seen diffusely throughout the specimen, or more than 10 small aggregates seen in a cut surface of the specimen, or a single germinal center seen in the cut surface of the specimen; Rating 4: many germinal centers seen in a single section of the specimen.

Fibroadenomas were rated according to the above scale in a blinded fashion by one pathologist (HS).

Telomerase Assay

Lysis of the gross tumor samples was performed as previously described (7,8). Lysis of fine needle aspirates was performed as follows: FNAs were lysed as whole cell extracts in 50 µl of 0.5% CHAPS (Pierce Chemical Co., Rockford, IL) as per the normal lysis recipe but with the addition of 0.025U/µl of RNase inhibitor (5Prime→3Prime, Boulder, CO). New lysates were placed on ice up to 1 hour and the material was further broken by pipetting every 15 minutes. Extracts were then snap-frozen in liquid nitrogen and stored at -80°C until use. From the whole cell extracts of 60 µl,

5 μ l (8%) were held back to determine estimate cell numbers within the individual FNAs. Detection of telomerase signal was determined by using the TRAP-eze™ Kit (Oncor, Gaithersburg, MD). Methods and sensitivity have been described by Holt et. al. (28). Initial screens were performed as per the manufacturers instructions with the gross tumor samples. Due to the small amount of cellular material available from the FNAs, the TRAP assay was modified to utilize 5 - 10 μ l of sample. This was done by decreasing the water content in reference to the respective sample volume increase.

FNA cell estimations

Each FNA extract sample (5 μ l) was placed on a 100 mm petri dish containing 10 ml of a 0.08% agarose and 0.1 μ g/ml of ethidium bromide (Sigma Chemical Co., St. Louis, MO) mixture. The samples were allowed to absorb into the agarose/EtBr for 10 min before visualizing with UV. Quantitation of the fluorescent signal was performed using an IS1000 Gel Documentation Imaging System (Alpha Innotech, Corp, San Leandro, CA). This quantity was then compared to the DNA constant for HeLa cells (15 pg/cell) (27) and a rough approximation was made based on the ratio of DNA concentration to cell number.

Frozen Sections Assay

A volume of 20 μ l - 50 μ l of a modified 0.5% CHAPS lysis buffer containing 1.5% glycerol (Sigma Chemical Co.) and RNase inhibitor (5Prime→3Prime)

was added directly onto 5 μ m thick frozen tissue sections fixed in OCT embedding compound (Miles, Inc., Elkhart, IN). This volume was then rigorously pipetted to resuspend and lyse tissues prior to running the telomerase assays. The lysate was then transferred to a 1.5 ml microfuge tube and snap frozen in liquid nitrogen. 2 μ l were used per assay (Figure 1).

Phenol/Chloroform Extractions

In the presence of Taq polymerase or other nonspecific PCR inhibitors, as evidenced by a lack of the telomerase 6 bp ladder and the significant decrease or lack of the internal telomerase amplification standard (ITAS), a phenol and chloroform extraction method was performed. Briefly, sample extract was added to a cocktail consisting of TS primer, dNTPs, TRAP buffer and water, incubated at room temperature for 30 min to allow elongation of the telomerase products then heated to 100°C for 5 min to inactivate the telomerase. Tris-saturated phenol (USB, Cleveland, OH) vol:vol was added, the mixture centrifuged and the aqueous layer containing putative Taq or PCR inhibitors removed. This procedure was then repeated using a 24:1 chloroform: isoamyl alcohol solution (USB). Telomerase products were precipitated with 7M ammonium acetate and 2 volumes of 100% ethanol (USB) overnight. The samples were centrifuged at 14,000 rpm for 1 hr, rinsed with 70% ethanol, centrifuged a second time for 30 min - 1 hr and air-dried. At this point the TRAP reaction mixture was added with the corrected water volume and hotstart TRAP - PCR was performed without the 30 min incubation. Gels were run according to

previously published methods (7).

Results

The TRAP assay was performed on all tissue samples and each FNA obtained directly from that sample. The histology of these lesions included 20 invasive cancers (16 ductal, 1 lobular, 1 mixed ductal/lobular, 1 comedo, and 1 squamous carcinoma), 3 carcinomas in-situ, 1 phyllodes tumor, 16 fibroadenomas, and 8 other benign breast tissue conditions. Table 1 summarizes the pathology of these lesions (and the T & N staging for invasive cancers), the corresponding observed tissue and FNA telomerase activity as well as FNA cell numbers calculated by the relationship of DNA content to HeLa cell (15 pg/cell). Telomerase activity was detected in 15 of 18 (83%) tissue biopsies and 17 of 19 (90%) FNA samples from invasive breast cancers. Figure 2 shows a representative TRAP gel with RNase treated controls illustrating the sensitivity of the telomerase ribonucleoprotein to RNase pretreatment. Of the 17 cancer biopsies for which corresponding tissue and FNA samples were available, identical TRAP assay results were obtained in 16 cases for a 94% correlation (Table 1). All three of the carcinoma in-situ specimens (Figure 3) as well as the malignant phyllodes tumor had telomerase activity in the tissue and FNA sample for a 100% correlation. Fibroadenomas had detectable activity in 53% of tissue and 56% of FNA samples tested. The TRAP results of the tissue and FNA samples from these fibroadenomas correlated in 100% of cases. Of the other benign lesions, all 6 of the tissue biopsies were negative while one of 8 (12%) FNAs had telomerase activity.

Cell numbers from the FNA's showed a range of 8,000 cells to 250,000 cells. Total cell numbers lysed appeared to affect the intensity of the telomerase signal, but not its presence. This was confirmed by a telomerase positive signal from an FNA of only 8,000 cells (infiltrating ductal ca) and a negative signal from a sample containing 212,000 cells (FA).

Some samples contained Taq polymerase inhibitors which were manifested as individual TRAP assays having no or very reduced internal TRAP assay standard (ITAS) present and these were purified by phenol-chloroform extraction. Telomerase activity was initially detected in 10/18 (56%) gross samples and 6/19 (32%) FNA samples from invasive breast cancers. From the FAs, only 3/15 (20%) gross samples and 2/16 (13%) FNA samples were positive after the initial TRAP-PCR. After phenol-chloroform extraction to remove inhibitors, telomerase was detected in an additional 5 gross samples and 11 FNAs from the invasive cancers while fibroadenomas showed telomerase in 5 additional gross samples and 7 more FNA samples. Figure 4 shows the results before and after sample purification.

There was no observed correlation between "positive" and "negative" fibroadenomas when evaluated by age of the patient or size of the mass (Table 3). In addition, terminal restriction fragment analysis (TRF) of the telomere lengths did not correlate with detection of telomerase activity or with the strength of the telomerase signal observed in fibroadenomas (data not shown). In an attempt to assess whether histologic variations of fibroadenomas, such as "juvenile adenofibroma" (29) with a pattern of epithelial hyperplasia and stromal hypercellularity may explain this

activity, we examined a subset of the fibroadenomas in this study and graded their histologic characteristics. In this small sample, there was no correlation between histologic characteristics analyzed and telomerase activity (Table 3).

Overall, FNA-TRAP results correlated with the gross biopsy TRAP results in 95% of cases from all diagnostic categories.

Discussion

In this study, we have documented the validity and usefulness of FNA for the clinical detection of telomerase activity in breast neoplasms. FNA, combined with the highly sensitive TRAP assay for detection of the presence of telomerase in excisional breast biopsies was highly accurate. Our results revealed a 94% correlation of FNA with tissue sample activity in invasive breast cancers. Further, we confirmed previous reports of telomerase reactivation in a high percentage of breast cancers (8, 21). In this series 83% of gross and 90% of FNA samples of invasive breast carcinomas were positive for telomerase activity. Two of 19 cancer FNA's (10%) were negative for activity, one of which was an unusual squamous cell variant of breast carcinoma and the other an unremarkable infiltrating ductal adenocarcinoma.

A specific association of telomerase activity in malignant breast tissue is further supported in this study by demonstrating the absence of activity in benign breast conditions such as fibrocystic disease and epithelial hyperplasia. The one exception was a benign FNA positive for telomerase.

A possible explanation for this result may arise from the fact that this biopsy was from a palpable mass which histologically showed fat necrosis with a predominant lymphocytic infiltrate. Hematologic cells, and particularly lymphocytes, have been shown to contain weak telomerase activity (30).

Although there were only 3 carcinoma in-situ specimens, all were positive for telomerase activity. This raises an interesting question about how early during neoplastic progression telomerase is reactivated and whether it could be used to distinguish pre-invasive from invasive disease. Our data from these 3 cases suggests that telomerase reactivation may precede acquisition of an invasive phenotype.

One unexpected finding in this study is that approximately one half of the fibroadenomas assayed were positive for telomerase activity. Fibroadenomas are benign tumors with mixed glandular and mesenchymal components in which either fibrous tissue or epithelial tissue may predominate. Although the pathogenesis of this tumor is not completely understood, some evidence exists for a heterogeneous origin (31, 32). Whether or not telomerase positive fibroadenomas behave any differently from telomerase negative fibroadenomas is unknown. The question of whether there is a connection between fibroadenomas that are telomerase positive and progression to phyllodes tumors or carcinomas in-situ is currently under investigation. As early as 1979, it has been established that women who have had excision of a fibroadenoma are at increased risk for later development of breast cancer (33, 34). More recent

data indicates that the risk of developing breast cancer ranges from 1.7-fold (35, 36) to 7-fold (37) for women with recurring fibroadenomas. It will be interesting to see if an association with telomerase positive fibroadenomas and breast carcinoma is established in future studies.

Even though the exact contribution of telomerase to the development of cancer requires further elucidation (38), its potential as a prognostic and therapeutic strategy in the management of cancer is already being explored. A significant association of telomerase activity from tumors of node positive breast cancer patients versus node negative patients has been reported (5, 10). This suggests a potential use of telomerase in detecting earlier stage cancers. Now, with the ability to detect telomerase activity from palpable tumors by a standard clinical technique such as FNA and the availability of a commercially prepared telomerase assay kit, further clinical evaluation of this tumor marker should be easier.

The most promising use of FNA in detecting telomerase activity may be in future therapeutic strategies of cancer management. Exciting studies focusing on inhibition of human telomerase indicate that this can be achieved by peptide nucleic acids directed against the RNA component of the enzyme (39). The ability to detect telomerase activity by FNA may permit specific preoperative treatment with anti-telomerase therapies as well as monitoring of patients undergoing therapy to observe the efficacy of the treatment regime. Further elucidation of telomerase regulation, including other possible chromosome stabilizing pathways independent of telomerase activation, will ultimately determine the validity of telomerase

inhibition as a novel therapeutic strategy (40).

Telomerase is a relatively new component identified within the machinery of the cancer cell which when reactivated allows the cell to escape normal regulation of replication. This very basic process has widespread implications for understanding and intervening in the progression of neoplasia. This study introduces the detection of telomerase as a molecular marker in breast cancer by the technique of fine needle aspiration. This is a highly sensitive and accurate method for the detection of telomerase activity in breast masses. Future application of the FNA-TRAP assay for telomerase detection should facilitate evaluation of telomerase as a tumor marker in the clinical management of breast and other solid malignancies.

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Figure 1. Schematic representation of TRAP assay lysis process for frozen histological sections. Twenty to 50 μ l of 0.5% CHAPS lysis buffer containing 1.5% glycerol is placed on the cryosection and vigorously resuspended. The sample extract is then placed in a 1.5 ml microfuge tube and snap frozen. 2 μ l is used per assay as previously described (7).

Figure 2. Control TRAP gel containing representative breast lesions illustrating the sensitivity of the telomerase activity to RNase. 6 μ g total protein of the gross tumor sample was loaded whereas 10 μ l of sample extract from the fine needle aspirates was used per assay. The samples were PCR amplified for 30 cycles. FNA = fine needle aspiration; Gross = tumor sample; FCD = fibrocystic disease; FA = fibroadenoma; Ca = carcinoma; ITAS = internal telomerase assay standard; +,- indicates the presence or absence of RNase from the reaction cocktail.

Figure 3. Representative TRAP gel of telomerase activity detected from lobular carcinomas in situ. 2 μ l of the lysate obtained from the frozen section was used in the reaction mixture. The frozen sample was from a histological section 5 μ thick. The specimen was originally preserved in OCT embedding medium. LCIS = lobular carcinoma in situ; G = gross tumor sample; A = fine needle aspirate sample; Ca = carcinoma; ITAS = internal telomerase assay standard.

Figure 4. Representative TRAP gel showing the activity obtained in positive, Taq polymerase inhibited samples after phenol - chloroform extraction. Sample underwent an initial telomerase elongation incubation and subsequent phenol - chloroform extractions followed by ethanol precipitation. The purified product was then PCR amplified using the TRAP-PCR program. Note the total inhibition of the ITAS signal from the gross sample. In contrast, the FNA has a strong ITAS signal. This is most probably due to the small amount of material obtained from an FNA compared to the gross sample thus yielding a dilution effect. Samples were processed as previously described (7). Before, After = before and after the phenol - chloroform extraction procedure. Gross = tumor sample; FNA = fine needle aspiration; Ca = carcinoma; ITAS = internal telomerase assay standard.

Figure 1.

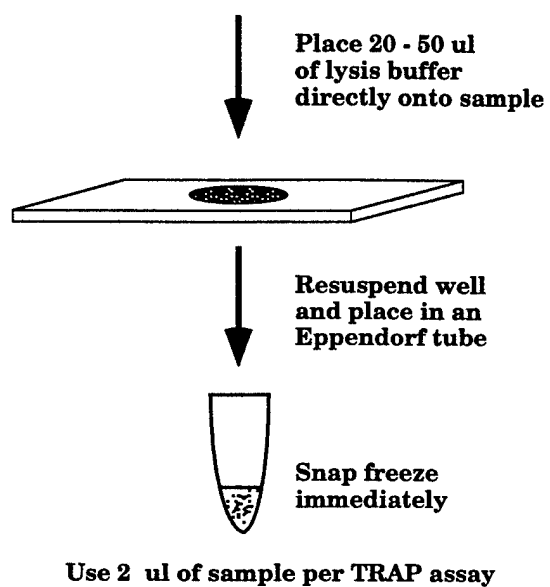


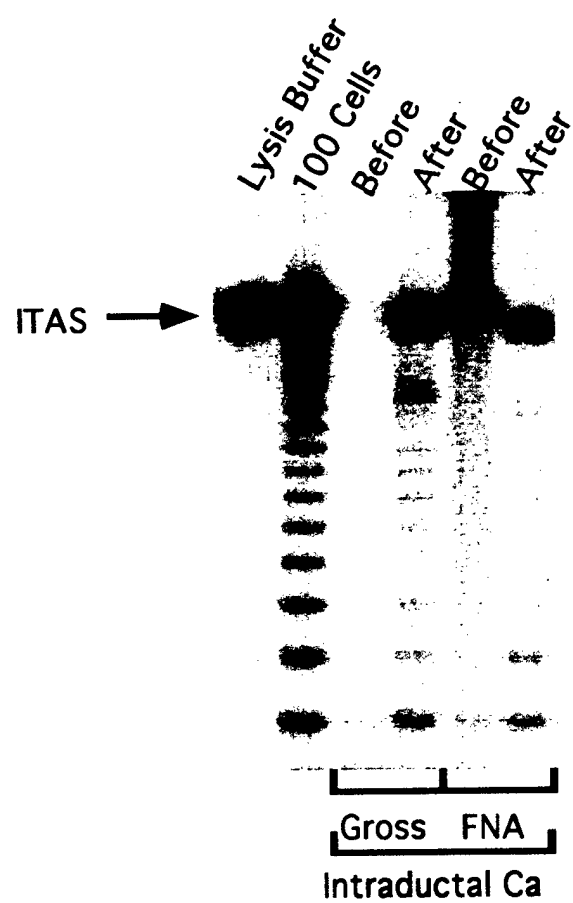
Figure 2



Figure 3



Figure 4





Immortalization of human mammary epithelial cells transfected with mutant p53 (273^{his})

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Normal human breast epithelial cells were transfected with expression vectors containing the p53 gene mutated at either codon 143, 175, 248 or 273, or by infection with a recombinant retroviral vector containing the p53 gene mutated at codons 143, 175, 248, or 273. The breast epithelial cells were monitored for extension of *in vitro* lifespan and immortalization. Expression of some, but not all, p53 mutants resulted in an extension of *in vitro* lifespan. Experiments with the p53 temperature sensitive mutant 143^{ts} revealed that at 32°C, the nonpermissive temperature, the growth of breast epithelial cells was inhibited. At 37°C, the mutant conformation, there was increased proliferation of cells, resulting in extension of *in vitro* lifespan. Breast epithelial cells expressing p53 mutant 273^{his} maintained DNA binding and transcriptional activities and one clone immortalized after a period of growth arrest (crisis). The progression of this immortalization event was characterized by the reactivation of telomerase using the telomeric repeat amplification protocol (TRAP), and terminal restriction fragment analysis (TRF). This is the first reported immortalization of human mammary epithelial cells transfected with a mutant 53.

Keywords: telomerase; telomeres; senescence; life'span

Introduction

Both normal human fibroblasts and epithelial cells maintained in cell culture undergo cellular senescence and do not spontaneously immortalize. A model involving a two stage mechanism for regulating cellular senescence or aging has been previously reported (Shay and Wright, 1989; Wright *et al.*, 1989; Wright and Shay, 1992a). In this model, the first stage or mortality stage 1 (M1) leads to normal cellular senescence. In order for cells to grow past this stage, the M1 mechanism must be overcome. This may be accomplished by interaction of mutant p53 with activated oncogenes such as Ha-ras (Eliyahu *et al.*, 1984) or by the activity of DNA tumor virus gene products such as human papilloma virus type 16 E6/E7, adenovirus 5 E1A/E1B or SV40 large T antigen (Linzer and Levine, 1979; Sarnow *et al.*, 1982; DeCaprio *et al.*, 1988; Huang *et al.*, 1988; Shay *et al.*, 1989; Werness *et al.*, 1990; Shay *et al.*, 1993a, b; Demers *et al.*, 1994), presumably by inactivation of the

tumor suppressor gene products pRb and p53. However, these DNA virus proteins fail to directly immortalize human cells. Cells overcoming M1 continue to proliferate in an 'extended lifespan' period until an independent second mortality stage (M2) mechanism is activated (crisis), and only if a critical M2 gene becomes inactivated can a rare cell escape crisis and become immortal.

The p53 tumor suppressor gene product and the retinoblastoma gene product (pRb) or a retinoblastoma-like activity appear to be important in regulating the M1 stage in most human cell types (Hara *et al.*, 1991; Shay *et al.*, 1991, 1993a,b; Gotz and Montenarh, 1995). In normal mammary epithelial cells the p53 levels remain constant throughout their lifespan in culture (this paper) in contrast to human diploid fibroblasts which showed an increase in levels of p53 as cells reached senescence (Kulju and Lehman, 1995). The pRb levels in HME cells, decrease significantly as the cells approach senescence (this paper). The p53 tumor suppressor gene is one of the most commonly mutated genes in human cancer (Harris and Hollstein, 1992), with approximately 50% of primary breast tumors containing alterations involving the p53 gene (Callahan and Campbell, 1989; Hollstein *et al.*, 1991; Harris and Hollstein, 1992; Moll *et al.*, 1992). Prompted by the findings of Band *et al.*, (1990, 1991), that human mammary epithelial cells may occasionally immortalize when expressing HPV 16 or HPV 18 plasmids defective in pRb binding but normal for the E6 function of p53 abrogation, we infected human mammary epithelial cells (HME) with defective retroviruses expressing HPV 16 E6, E7 or E6/E7 (Shay *et al.*, 1993a). The results showed that HME cells expressing either HPV 16 E6/E7 or E6 alone were capable of overcoming M1 and in some instances M2. Direct support for this was recently obtained when it was demonstrated that breast epithelial cells but not breast stromal cells obtained from a patient with Li-Fraumeni Syndrome (containing a germline mutation in p53) spontaneously immortalized in cell culture (Shay *et al.*, 1995). That breast epithelial cells immortalize more easily than stromal cells is also supported by the epidemiological findings that the annual incidence of epithelial cell carcinomas such as breast cancer, are at least 100 times higher than the annual incidence of soft tissue sarcomas (Pollock, 1992). Tissue specific lineages are likely to have different regulatory mechanisms which may influence their ease of escape from senescence followed by immortalization. This does not necessarily indicate that bypassing M1 is less frequent in fibroblasts, since Bond *et al.* (1994) have been able to successfully abrogate p53 function in

human diploid fibroblasts with the introduction of mutant p53 143^{ala}. This tissue specific lineage may help explain the apparently different roles that p53 and pRb have in cellular senescence for epithelial versus human mammary stromal cells (Shay *et al.*, 1993a,b).

Inactivation of M2 most likely involves recessive events consistent with the observations that a limited proliferative capacity is restored in hybrids between immortal and mortal cells (Pereira-Smith, 1987). Mutational inactivation of one allele followed by the elimination of the remaining wild-type allele by nondisjunctional or chromosomal conversions (Rew, 1994), selective growth advantage for the mutant (Harvey *et al.*, 1995) or possibly telomere shortening as theorized by Wynford-Thomas *et al.* (1995), are all likely mechanisms for escape from M2. However, the gene product regulating the M2 stage still remains to be determined. One possibility is that it may involve a gene in the telomerase repression pathway (Shay and Werbin, 1993). The ribonucleoprotein enzyme, telomerase, is involved in maintaining the stability of telomeres at the ends of chromosomes (Counter *et al.*, 1992). The end replication problem described by Watson (1972) would lead to progressive telomere shortening in normal cells since the mechanism of DNA replication in linear chromosomes is different for each of the two strands (e.g., leading and lagging). This progressive loss of telomeres (simple tandem repeats of the sequence TTAGGG) at the ends of human chromosomes may be a molecular mechanism that determines the time of onset of cellular senescence (Olovnikoff, 1973; Harley, 1990).

Telomerase, an enzyme expressed in germ line cells, stem cells and cancer cells (Greider and Blackburn, 1985, 1989; Morin, 1989; Counter *et al.*, 1994; Kim *et al.*, 1994; Hiyama *et al.*, 1995; Piatyzsek *et al.*, 1995) contains its own RNA template and thus extends the overhanging G-rich telomeric strand by direct polymerization of deoxynucleotides into tandem TTAGGG repeats. This extended G-rich strand is now used as the template for synthesizing the C-rich complementary strand. Telomerase therefore stabilizes the telomeric length in immortal and cancer cells by compensating for the end replication problem. The absence of telomerase in normal somatic cells results in loss of 50–200 bp cell from telomeres per round of replication (Harley *et al.*, 1990; Allsopp *et al.*, 1992). Bypassing M1 does not reactivate telomerase and telomeres continue to shorten during the period of extended lifespan (Counter *et al.*, 1992; Shay *et al.*, 1993c). The immortal cells that overcome M2 almost always re-express telomerase activity and are capable of maintaining stable telomere lengths (Counter *et al.*, 1992; Wright and Shay, 1992b; Shay *et al.*, 1993b,c). Escape from M2 may thus represent the abrogation in the repression pathway of telomerase activity in somatic cells (Counter *et al.*, 1992; Wright and Shay, 1992a; Shay *et al.*, 1993a,c; Piatyzsek, 1995).

In the present study we sought to determine if the M1 mechanism in HME cells could be directly overcome by the introduction of p53 mutants implicated in a variety of human cancers. While some of the p53 mutants inserted into normal human mammary epithelial cells resulted in extension of *in vitro* lifespan, one clone expressing p53 mutant 273^{his} immortalized.

Results

Cell culture

In culture, using defined medium supplemented with growth factors, normal human mammary epithelial cells (HME) vary in their proliferative capacities, ranging from 25–50 population doublings and then undergo morphological changes associated with finite lifespan (Shay *et al.*, 1993a; Van Der Haegen and Shay, 1993). Young HME cells initially display high proliferative capabilities, but as they approach M1 (the first stage of cellular senescence), the proliferation rate slows down until the cells cease to divide. Normally HME cells can remain in a senescent, growth arrested state for several months if nutrients are replaced frequently.

Transfection/infection of mutant p53 constructs

p53 'hot spot' mutants were introduced into normal HME 31 cells at PDL 28 and into HME 32 at PDL 15 either by lipofectin transfection of expression vectors, or by infection of recombinant retroviruses, containing p53 mutants. Since these cell strains were previously characterized by our laboratory for *in vitro* lifespan (Van Der Haegen and Shay, 1993), extension of lifespan is determined as a cell strain proliferating greater than 10 PDL past their oldest observed senescence point. Of 123 total clones obtained, 34 (28%) were able to bypass the M1 stage of cellular senescence and continue proliferating exhibiting an 'extended lifespan' (summarized in Table 1a). Table 1b shows the range of extended lifespan for those clones able to bypass M1 and the mean PDL of extended lifespan *in vitro*. Lipofectin transfection yielded more clones capable of extension of lifespan than did defective retroviral infections (Figure 1a). HME 31 cells had 24% of lipofectin transfected clones with extended lifespan in culture in comparison to 7.7% of infected clones (Figure 1a). Figure 1b summarizes the relative efficiencies of the promoters driving the mutant p53 constructs used in this study to overcome M1. Clones with extension of lifespan were more readily obtained via lipofectin transfection with a CMV promoter from HME 32 compared to HME 31 and one clone was able to bypass the M2 stage and immortalize (Figure 1c).

Table 2 summarizes the extension of lifespan of HME 31 and 32 for each mutant p53 introduced. HME cells were transfected with the expression plasmid (pRc/CMV) containing the p53 mutations at either codon 143^{ala}, 175^{his}, 248^{trp} or 273^{his} respectively, or infections with defective retroviral construct pZipneo SV(X) containing 143^{ala}, 175^{his}, 248^{trp}, or 273^{his} respectively. The two most frequently observed p53 mutations in human cancers, e.g., 248^{arg} and 273^{his} (Hollstein *et al.*, 1991), demonstrated extended lifespan in all clones isolated from HME 32 and in one instance an immortalized clone was obtained (HME 32 (273)-1). Introduction of the expression vectors without p53 mutants did not result in extension of lifespan or spontaneous immortalization in any instance (Table 2).

Analysis of mutant p53 proteins by Western blot

Western blot analysis of protein extracts from HME cells expressing transfected p53 mutants with mono-

clonal antibody PAb DO-1 revealed p53 overexpression in the 143, 175, 248 and some but not all of the 273 clones. Overexpression was not observed however in the immortalized clone, HME 32(273)-1, nor HME 32

(273)-6, another 273 clone that did not immortalize (Figure 2a). Figure 2b shows protein levels of p53 and pRb in young and senescent HME cells and reveals that pRb levels decrease in both senescent HME 31 and 32 cells and may explain the 100-fold greater frequency of immortalization of mammary epithelial cells versus stromal cells (Pollock, 1992). Western analysis of the MDM2 protein revealed no overexpression of this known p53 transcription target (data not shown). Experiments were then undertaken to determine if the overexpression of the temperature sensitive mutant p53 143^{ala} could repress cell growth when the wild-type conformation of p53 was expressed (32°C). While the cells continued to proliferate when the mutant conformation was expressed (37°C), the cells markedly decreased proliferation within 24 h when shifted to 32°C (wild-type p53 conformation) in both HME 31 and 32. This was evidenced by cell counts taken at 12, 24, 36 and 48 h after temperature shift to 32°C. For the clones containing the 143^{ala} p53 mutant, the decrease in the total cell count was 43% of the control cells. By 48 h in culture, cell counts were 28% of the control counts. Three weeks in culture revealed the mean percentage of HME 31 (143)-1 and HME 32 (143)-1 cells to be 13% of their respective controls. Thus overexpression of the wild-type p53 without DNA damage induction (Smith *et al.*, 1995) inhibits HME growth irrespective of the age of the cells.

Analysis of the temperature sensitive mutant p53 143^{ala}

To determine relative levels of mutant 143^{ala} p53 expression, immunoprecipitations were performed (Zhang *et al.*, 1992). HME 32 cells expressing p53 mutant 143^{ala} were immunoprecipitated with monoclonal antibodies PAb 240, PAb 1620 and PAb DO-1 at both the permissive and nonpermissive temperatures (Figure 3). While a strong signal using PAb 240 was observed, detection with PAb 1620 yielded weak signal at 37°C. However, at 32°C, there was a significant decrease in the expression of mutant p53 and correspondingly, an increase in signals exhibited by PAb DO-1 and PAb 1620.

Control HME 31 and HME 32 cells were cultured at both temperatures to determine growth rates as well as to observe morphological alterations. Figure 4 shows, as expected, normal cells at 32°C grow much slower. Photomicrographs representative of the morphologies observed from these cells are illustrated in Figure 5. Normal cells at 32°C and 37°C and p53 143^{ala} mutant transfected cells at 37°C share the same phenotype in culture whereas the mutant transfected cells at 32°C (wild-type conformation) become large and flat.

Analysis of immortalization of HME 32 with p53 mutant 273^{his}

HME 32 transfected with the 'hot spot' p53 mutant 273^{his} were able to bypass the M1 stage and after an extended crisis period also escaped from the M2 stage. Characterization of the immortalization process was followed by several techniques including terminal telomere restriction fragment (TRF) analysis, telomerase activity assays using the telomeric repeat amplifica-

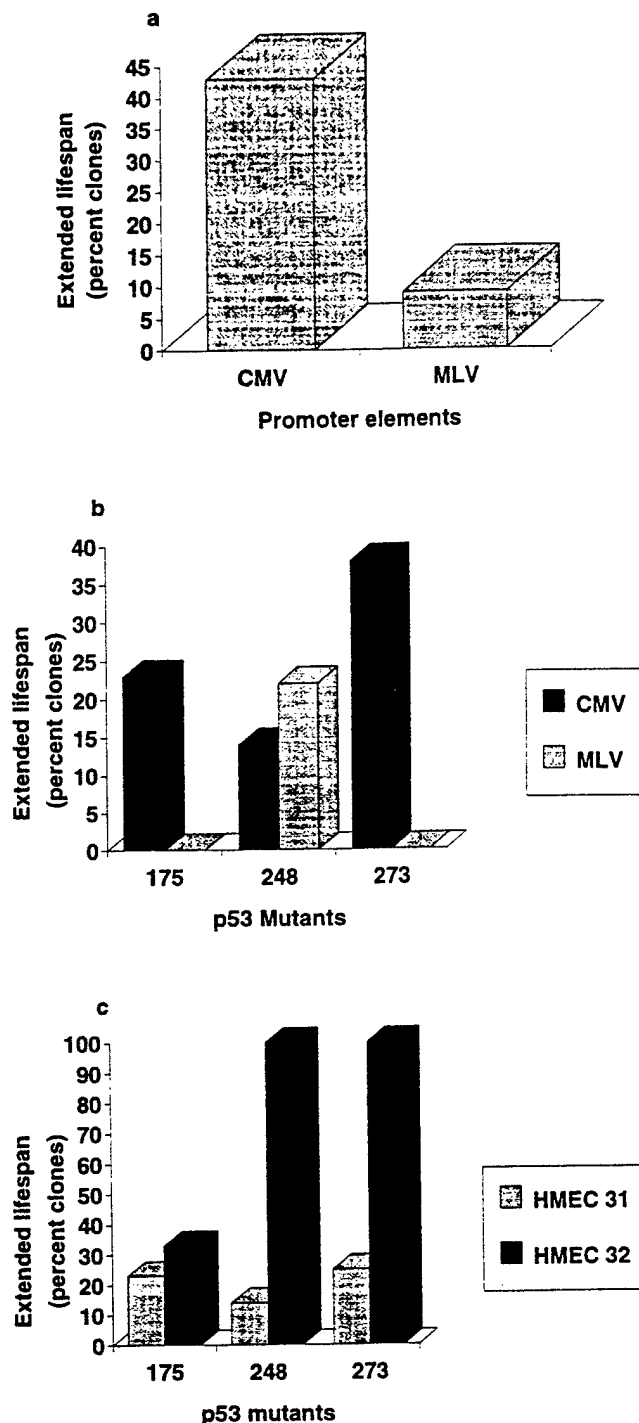


Figure 1 (a) The relative efficiency of the promoters used to introduce mutant p53 into the cells in this study to bypass M1 and induce extension of lifespan. CMV: cytomegalovirus promoter; MLV: Moloney murine leukemia virus promoter-enhancer sequences. (b) Comparison of the p53 mutants introduced via each vector and their ability to extend lifespan in culture. p53 mutants are: 175^{his}, 248^{trp}, 273^{his}. (c) Variability between cell strains is illustrated by comparing the efficiency of bypassing M1 and extending *in vitro* lifespan

tion protocol (TRAP), p53 DNA binding reporter assays, chromosome counts, number of population doublings in culture, as well as Western blot analysis with several p53 reactive antibodies. The presence of the original mutant p53 vector introduced was confirmed by PCR amplification of a 347 bp fragment from the CMV promoter region while the presence of the mutant within the genomic p53 gene was confirmed by SSCP (G Tomlinson, personal communication).

The frequency of immortalization was calculated by determining the total number of cells plated and collected using what is essentially a fluctuation analysis (Shay *et al.*, 1993a). From this number, the likelihood of the single HME 32 (273)-1 clone escaping from crisis (e.g. M2) was estimated to be 2×10^{-7} . This figure is consistent with the range previously determined for human fibroblasts (Shay *et al.*, 1993a) but lower than that for HME cells expressing human papillomavirus 16 E6/E7 or SV40 T antigen (Shay *et al.*, 1993a).

Western blot analysis showed that the mutant p53 273^{his} protein product was not overexpressed in the immortalized clone. Levels of p53 remained comparable at all PDLs examined (Figure 6).

In order to verify the expression of mutant p53 273^{his} protein products and the lack of wild-type p53 in the immortal cells, transient transfections were performed by cotransfection of a LacZ reporter construct containing either the p53 consensus sequence or RGC consensus sequence and a luciferase reporter gene. The

p53CON and ribosomal gene cluster (RGC) are DNA sequences that had been identified by their ability to bind wild-type p53 either alone (RGC) or as part of a nuclear complex (p53CON) (Kern *et al.*, 1992; Funk *et al.*, 1992). Chen *et al.* (1993) previously showed that transcriptional activation by 273^{his} of the p53con sequence is comparable to wild-type activity, while little transcriptional activity is observed with the RGC consensus sequence. Figure 7 illustrates that while wild-type HME transactivation is high for both p53CON and RGC, the immortalized HME cells containing the 273^{his} mutant exhibit minimal transactivational activation of the RGC consensus but strong activity to the p53CON sequence.

PCR amplification using primers custom synthesized for the pRc/CMV promoter sequence revealed the presence of the construct in the HME 32 (273)-1 clone and the pRc/CMV 273 positive control but not in the parental HME 32 cell strain (Figure 8).

TRF results are illustrated in Figure 9. Normal HME 32 have an average length of approximately 7–8 kb at population doubling 20 (PDL 20). The HME 32(273)-1 cells at PDL 60 show a significant decrease in average telomere length to approximately 3–4 kb (nearly 100 bp of the telomeres are lost per doubling). By PDL 80 there is no further shortening and telomere length has stabilized at about 2 kb. Additional TRF analysis at PDL 225 showed no change in the telomere length from that observed at PDL 80 and 100 (data not shown).

A PCR based assay for the measurement of telomerase activity (Kim *et al.*, 1994; Piatyszek *et al.*,

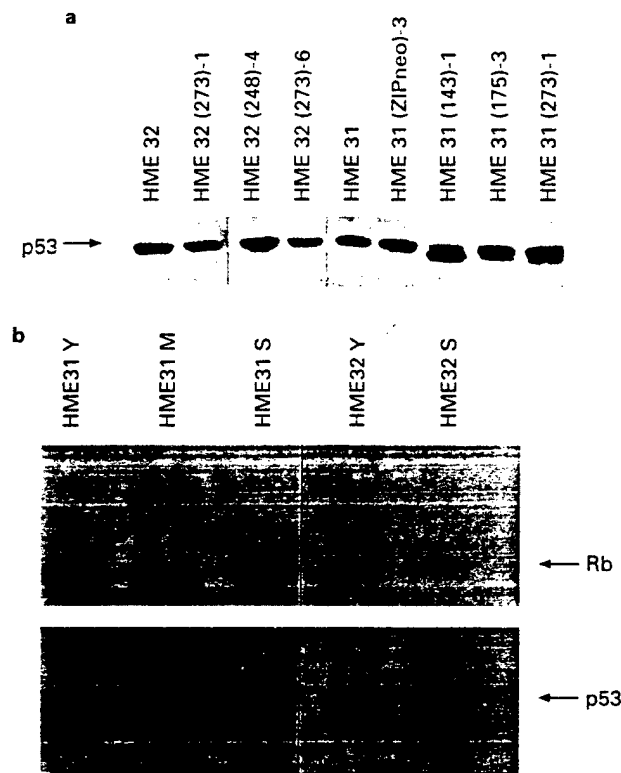


Figure 2 (a) p53 expression levels are illustrated from representative p53 mutant clones. Each lane contains 40 μ g of protein and p53 was detected using anti-p53 monoclonal antibody PAb 1801. All clones shown were lipofectin transfected for the p53 mutants. (b) Relative levels of endogenous p53 and pRb are shown for young and senescent normal HME, p53 levels were detected using PAb 1801. Monoclonal antibody MAb-1 was used to detect levels of pRb

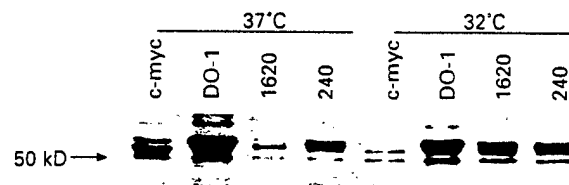


Figure 3 Immunoprecipitation results for the p53 conformation of the p53 temperature sensitive mutant 143^{tsa} p53 was immunoprecipitated with the indicated antibodies at 37°C and 32°C from HME 32 cells transfected with the 143^{tsa} p53 mutant as described in the Methods section. An antibody against c-myc was used as a negative control. kDa: molecular weight protein markers sizes

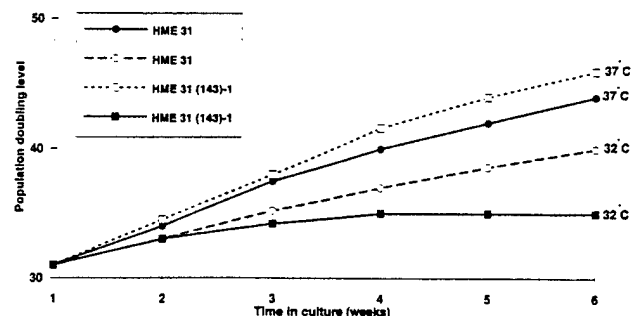


Figure 4 Growth of HME at 32°C and 37°C. Cultures at 32°C were approximately 3–4 PDL slower than those at 37°C. Proliferation was markedly decreased by 24h in culture after temperature shift to 32°C. By 3 weeks, cell counts in the transfected cells were 10% that of the control cells

1995) was performed (Figure 10). The parent cells at PDL 20, and precrisis HME 32(273)-1 cells PDL 41 had no detectable telomerase activity while from PDL 60 on, HME 32 (273)-1 did. This is consistent with a rare telomerase positive clonal population emerging around PDL 50-60 and quickly predominating the population of cells which were initially heterogeneous with a population of normal cells undergoing crisis and continuing to shorten their telomeres. Co-amplification of a genomic internal DNA standard (ITAS) indicated that there were not PCR inhibitors present in the extracts which could yield false negatives. Quantitation against this standard also revealed that there was no significant increase in the activity of telomerase with increasing PDL.

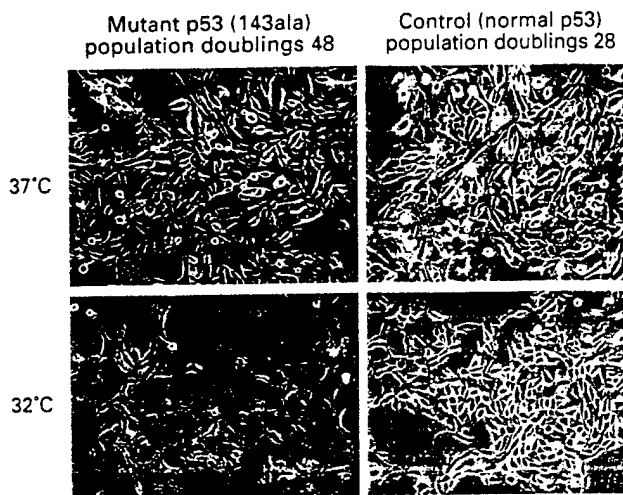


Figure 5 Photomicrographs representative of temperature effects on the conformation of the p53 mutant 143^{ala} introduced into HME cells. Morphologically there was no difference in cell appearance for control and 143^{ala} expressing cells at 37°C nor for the control cells at 32°C. At 32°C however, there was a marked change in the morphology toward a senescent-like phenotype. At 37°C, 143^{ala} has a mutant conformation and no DNA transactivation activity. At 32°C the mutant exists in a wild-type conformation, resulting in overexpression of wild-type p53, cell enlargement and inhibition of cell proliferation

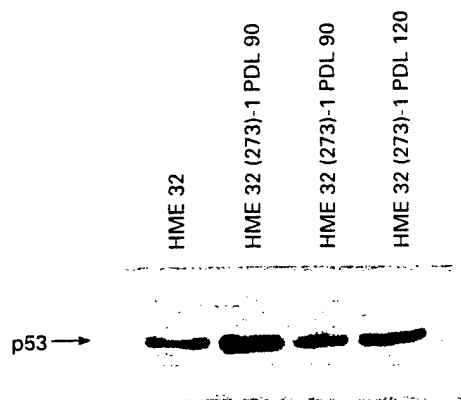


Figure 6 Western blot analysis of the p53 expression levels for HME 32 and HME 32 (273)-1 at different times in culture. Extracts were detected using the PAb DO-1 antibody (20 µg were loaded per lane). No significant increase in p53 expression was observed

Metaphase spreads were counted on HME 32(273)-1 cells after 150 PDLs in culture. Analysis consisted of counting 27 random metaphase spreads. Of the spreads counted, a range of 62-139 chromosomes was observed. The median chromosome spread value was 81. Eleven percent of the population was pseudotetraploid, 22% were triploid, with the remaining 67% aneuploid.

Discussion

The onset of breast cancer is generally associated with increased aging except in cases of Li-Fraumeni Syndrome and familial breast cancers (Li *et al.*, 1988; Malkin *et al.*, 1990; Kessler, 1992; Smith *et al.*, 1992; Malkin, 1993). Previously, it has been reported that abrogation of normal p53 function may be important in the immortalization of HME cells (Band *et al.*, 1991; Shay *et al.*, 1993a,b). The present study illustrates that

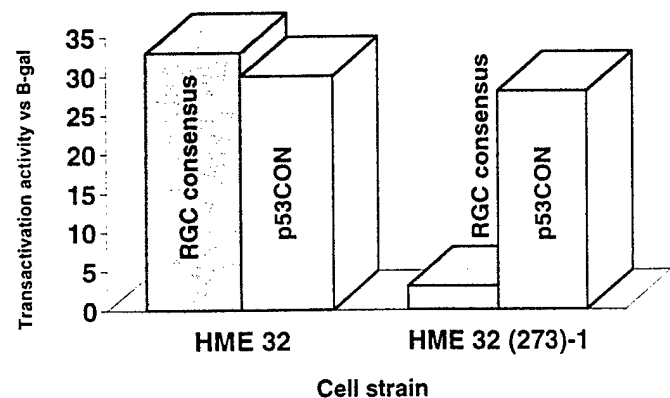


Figure 7 Luciferase activity analysis. The transactivation activity of HME 32 and HME 32 (273)-1 was standardized against β -galactosidase activity electroporated into both parental cells HME 32 and the clone HME 32 (273)-1. The transactivation activity refers to the luciferase activity as measured against basal β -gal activity in the control cells. Immortal cells were tested at PDL 250, normal cells at PDL 18

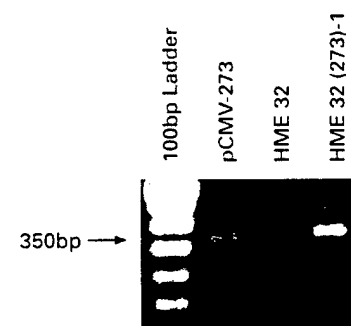


Figure 8 Polymerase chain reaction analysis to detect the presence of the pRc.CMV construct within the immortalized clone. DNA from the originally transfected construct was used as a positive control. Normal HME 32 cells were tested at PDL 18. As shown, the presence of the construct is confirmed in the clone but not the parental cell strain. A 347bp fragment of the CMV promoter region is the product from the PCR reaction

some but not all p53 mutants can neutralize the M1 mechanism of cellular senescence in human mammary epithelial cells (HME) in culture. Previous studies have shown that for most human cells to abrogate the M1 stage of cellular senescence both p53 and an Rb-like function must be overcome (Munger *et al.*, 1989; Woodworth *et al.*, 1989; Watanabe *et al.*, 1989; Band *et al.*, 1990). While HPV16 E6 only gives partial extension of lifespan in IMR90 fibroblasts as does HPV16 E7, combinations of the two result in the full extension of lifespan (Shay *et al.*, 1993a). Thus, for cells to reach the M2 stage and have the potential to immortalize, the cellular mechanisms involved in the M1 mechanism must be fully overcome. It has been shown that for HME cells, abrogation of only p53 is required to fully overcome the M1 stage. HPV16 E6 results in most HME cells reaching M2 and then a relatively rare cell in the population immortalizes (Shay *et al.*, 1993a). In addition, we recently reported the spontaneous immortalization of HME from a patient with Li-Fraumeni Syndrome (germline mutation in p53). Wazer *et al.* (1995) recently showed immortalization of distinct mammary epithelial cell types by HPV 16 E6 or E7 while Tsutsui *et al.* (1995) demonstrated extension of *in vitro* lifespan with the carcinogen aflatoxin B1. The present study extends these observations further to demonstrate that expres-

sion of mutant p53 into normal HME can also result in immortalization. The clone HME 32 (273)-1 was observed to have telomeres that continued to shorten with progressive subculturing as well as the reactivation of telomerase upon overcoming M2. PCR revealed the presence of the p53 mutant construct within the cells, SSCP showed the presence of the mutant allele (G Tomlinson, personal communication), while continuous subculturing has the cells currently at PDL 300. These observations taken together provide evidence for the immortalization of HME 32 by p53 mutant 273^{his}.

We observed different relative efficiencies of expression vectors carrying mutant p53 to extend lifespan of the HME cells. The CMV expression vectors containing p53 mutants were more efficient in extending lifespan and abrogating wild-type p53 function (as indicated by immunoblotting and immunoprecipitations) but less so than HPV16 E6 (which degrades p53) in which over 90% of the clones had extension of lifespan (Shay *et al.*, 1993a). The p53 mutants carried in the defective retroviral vectors were not only less effective in inducing extension of lifespan but also did not give rise to any immortal clones. Thus,

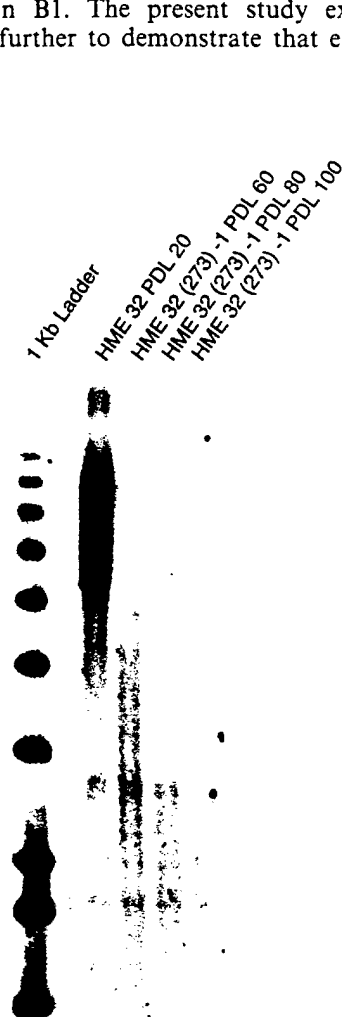


Figure 9 TRF analysis by Southern blot. Immortal cells at PDL 60, 80 and 100 were analysed for decreasing telomere length vs normal, parent cells at PDL 23. A 24-mer, (TTAGGG)₄, was used as a probe. Telomeres stabilized to approximately 2.5 kb in length

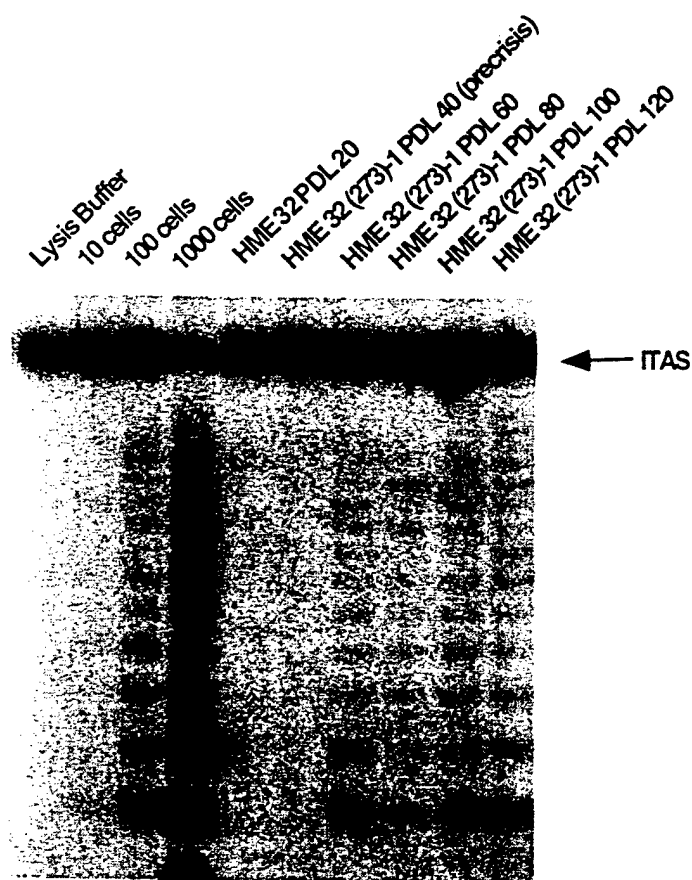


Figure 10 Telomerase activity analysed using the telomeric repeat amplification protocol (TPAP). Parental cells (PDL 20) and precisis cells had no detectable telomerase activity. Telomerase activity was first observed at about PDL 60 with no subsequent significant increase in telomerase activity with increasing passage as measured against the internal telomerase amplification standard (ITAS) as described in the Methods. Controls consisted of lysis buffer, dilutions of 10 cell, 100 cell and 1000 cell equivalents of a telomerase expressing cell line immortalized with HPV 16 E6 E7 mixed with normal cells from the original parent strain.

Table 1a Clones obtained and percent extension of *in vitro* lifespan for mutant p53s introduced into HME 31 and HME 32

Cell strain	Lipofectin transfections: no. of clones with extended lifespan/total no. clones	Retroviral infections: no. of clones with extended lifespan/total no. clones
HME 31	11/45 (24%)	4/52 (7.7%)
HME 32	18/23 (78%)	1/3 (33%)
Total % extended life	29/68 (43%)	5/55 (9%)

The total number of clones obtained from both methods used to introduce mutant p53 constructs into normal HME cell strains in this study. Lipofectin transfections plasmid construct products were under control of the CMV promoter. Defective retroviral infection plasmid products were controlled by the Moloney MLV-LTR. CMV = cytomegalovirus; MLV-LTR = murine leukemia virus, long term repeat

Table 1b Mean and range of the extended lifespan *in vitro* for those clones with mutant p53 which bypassed M1

Mutant p53s and vectors	Cell strain	No. of clones	Range of lifespan (PDL)	Extended lifespan (PDL)	Mean lifespan (PDL)
vector-pCMV	HME 31	4	46.3–51.2	none	48
143ala pCMV	HME 31	ND	ND	ND	ND
175his pCMV	HME 31	7	62.5–67.3	17.3	64.2
248trp pCMV	HME 31	1	63.1	13.1	63.1
273his pCMV	HME 31	3	61.6–72.7	22.7	68.8
pZipNeoSV(X)	HME 31	6	47.2–50.2	none	49.3
143ala pZipneo	HME 31	2	65.7–70.2	20.2	68.0
175his pZipneo	HME 31	0	none	none	none
248trp pZipneo	HME 31	2	63.3–67.3	17.3	65.3
273his pZipneo	HME 31	0	none	none	none
vector-pCMV	HME 32	3	24.6–28.3	none	26.1
143ala pCMV	HME 32	3	42.0–55.1	22.1	48.3
173his pCMV	HME 32	1	44.6	14.6	44.6
248trp pCMV	HME 32	6	44.1–58.2	28.2	47.5
273his pCMV	HME 32	8	42.7–59*	29.4*	54.6*
pZipNeoSV(X)	HME 32	2	28.6–28.7	none	28.65
143ala pZipNeo	HME 32	1	43.3	13.3	43.3

ND = not done. * The immortal clone HME 32 (273)-1 was not used in calculating these averages

Table 2 Clones obtained for each mutant p53 introduced into HME 31 and HME 32

	CMV 143	CMV 175	CMV 248	CMV 273	MLV 143	MLV 175	MLV 248	MLV 273	2neo	pZip.Neo SV(X)
HME 31	ND	7/30 23%	1/7 14%	3/8 38%	3/12 25%	0/10	2/9 22%	0/2	0/4	0/6
HME 32	3/6 50%	1/3 33%	6/6 100%	8/8 100%	1/3 33%	ND	ND	ND	0/4	0/6

Comparison by percentage of the individual p53 mutants introduced and the number of clones obtained that were able to bypass M1 and achieve extended lifespan *in vitro*. Mutants: 143^{ala}; 175^{his}; 248^{trp}; 273^{his}. 2neo and pZipNeoSV(X) were control vectors tested for the transfections and infections respectively. ND = not done

sequestration or degradation of a critical amount, if not all, of wild-type p53, appears to be required for HME cells to completely overcome M1, thereby fully extend cellular lifespan to reach the M2 stage. For some of the p53 mutants, M1 was not completely bypassed and thus, no immortalization was observed. One explanation is that the relative strength of the promoters in the expression vectors used to carry the mutant p53 to the target cells may be important in determining the amount of wild-type p53 neutralized. Liu *et al.* (1995) showed that the oncogenic potential of the HPV 16 E7 protein is regulated by the level of E7 expression which in turn is dependent upon the promoter driving expression. Milner and Medcalf (1991) showed that p53 dominant negative mutant expression can overcome the wild-type. Wyllie *et al.* (1995) reported complete abrogation of p53 function in

thyroid cell lines whereas Williams *et al.* (1995) showed incomplete abrogation of wild-type p53 via dominant - negative effects for 4 mutant p53s introduced. Bond *et al.* (1994) showed the abrogation of M1 in normal diploid human fibroblasts using a retrovirally packaged 143^{ala} mutant. This study indicates that a required amount of p53 may need to be inactivated in order to obtain an extended lifespan. Another possible explanation may be that the manner in which p53 is abrogated is important. HPV16 E6, a viral oncoprotein, degrades the majority of p53 protein product via the ubiquitin pathway (Scheffner *et al.*, 1990). It is possible that HPV16 E6 may also have other functions besides binding p53 which could contribute in a small way to overcoming M1 completely. CMV expression vectors are considered strong promoters due to the characteristic of having better and/or more binding sites for

transcription factors Liu *et al.* (1995). Therefore, while the mutant p53 transfected into the cells may sequester the wild-type p53 product very quickly, there may be heterodimerization occurring which protects some of the wild-type p53 in the cell. This may explain why there is a lower frequency of extension of lifespan and immortalization compared to HPV16 E6. In contrast, p53 mutants in retroviral vectors such as pZipneo may not bind sufficient wild-type p53 product to fully extend lifespan. The present results seems to support this explanation in that those cells that were transfected with mutant p53 in the context of pCMV-Neo-Bam construct resulted in a higher frequency of extension of lifespan (13 of 52 clones, 25%) versus the recombinant retroviral constructs pZIPNeoSV(X) carrying the mutant p53 genes (4 of 48 clones, 8%).

In addition to the relative strength of the expression vectors and the amount of wild-type p53 function neutralized, factors such as the specific mutant p53 inserted into the cells and the genetics of the strain used may also be important. Cho *et al.* (1994) identified two major classes of p53 mutants. The first class consists of residues that interact with DNA. Missense mutations at these sites would abrogate p53 by eliminating critical DNA contacts. 273^{his} and 248^{trp}, two major 'hotspot' mutations in human cancer, are examples of this class. The second class of p53 mutants exhibits abnormal structure due to missense mutations at sites crucial to the conformational architecture of the core domain (Cho *et al.*, 1994). These 'structural' mutants abrogate DNA binding through their effect on the tertiary structure (Milner, 1995). Therefore, different p53 mutants may be more effective in abrogating wild-type p53 than others. In this study, the transfected CMV-p53 273^{his} mutant was the most efficient at ablation of p53 function to bypass M1 for *in vitro* extended lifespan.

Another factor which may be important is the recipient cell type and strain used. Milner and Medcalf (1991) indicated that preliminary screening of the cell lines is necessary since the internal milieu of the cell may also affect the conformation of p53. Finlay *et al.* (1989) showed that the phenotype of mutant p53 can be determined by the cell line used for target transfection of the mutant p53 allele. Forrester *et al.* (1995) reported that the effects of p53 mutants on wild-type p53 mediated transactivation are cell type dependent and that there is an enhanced activity with 273^{his} on wild-type p53. In the present series of experiments it appeared that HME 31 and HME 32 demonstrated differences in their receptiveness to both particular mutants and mechanisms of introduction of mutant p53. For example, using HME 32, extension of lifespan via transfection was observed, whereas no clones exhibited extension of lifespan by infection. HME 31 however, appeared to be equally receptive for either method in extending *in vitro* lifespan. Both HME 31 and 32 have been previously reported to contain wild-type p53 (Shay *et al.*, 1993a,b).

It has been previously reported (Shay *et al.*, 1992; Shay *et al.*, 1993a) that the clones generally emerging from M2 and continuing to proliferate indefinitely are those that maintain a chromosome complement near diploid. Cell hybrid studies have indicated that overcoming the M2 mechanism is likely to be a recessive event and thus requires the loss of two

alleles. Experimentally, only a small fraction of fibroblast clones expressing T-antigen or HPV16 E6/E7 ever immortalize whereas a slightly higher fraction of HME cells do. We proposed that since a greater percentage of fibroblasts tend to become tetraploid upon expression of T-antigen or HPV16 E6/E7, cells would have to eliminate/alter/mutate several additional alleles of a critical gene regulating the M2 stage prior to immortalization. However, HME cells expressing T-antigen or HPV16 E6/E7 or E6 alone maintain a population of both pseudodiploid and pseudotetraploid cells within most clones and the cells that eventually overcome the M2 stage and continue proliferating almost always have a near diploid chromosome constellation (Shay *et al.*, 1993a). In contrast, metaphase spread analysis of the immortal clone HME 32 (273)-1, showed a mean chromosome number of 81 with a range from 67-139. Eysfjord *et al.* (1995) showed that p53 mutations lead to genomic instability in primary breast tumor cells. Since this immortalized cell line appears to be established through a dominant-negative p53 mediated event, perhaps a similar mechanism is occurring, one which may be related to the loss/mutation of p53 and/or particular alleles carrying putative tumor suppressor genes with selection for the mutated allele. This may indicate that while HPV 16 degrades p53, there are no missense or other mutations present. Therefore direct mutations of p53 may play a role in genomic instability. The question of nonrandom chromosomal event(s) in this cell line is now being addressed.

In conclusion, a better understanding of the molecular events required for normal human cells to escape the protective blocks against a critical step in the development of cancer (e.g. immortalization) are beginning to emerge. Cells have to overcome two independent cellular protective mechanisms (M1 and M2) to become immortal. While alterations mutations in p53 appear to be important in overcoming the first block, little is presently known about the second block although there is a strong correlation with re-expression of telomerase activity indicating that the M2 mechanism may be associated with the regulatory pathway in the repression of telomerase activity. In addition, we have observed that if the M1 mechanism is not completely overcome or neutralized then the cells only partially escape the M1 blockade and do not appear to reach M2 thus decreasing the likelihood of cellular immortalization. These results help explain why differing methods used to abrogate wild-type p53 function are not equally efficient at immortalizing cells and reaffirms that the 'guardian of the cell', p53 (Lane, 1992), is a formidable protective protein. For example, individuals inheriting germline mutations in one p53 allele do not generally develop cancer for several decades. In addition, breast epithelial cells from patients with Li-Fraumeni syndrome require extensive *in vitro* culture prior to spontaneously immortalizing (Shay *et al.*, 1995). These results indicate that perhaps other events such as loss of karyotypic stability and the genetic background of the tissue and cell type appear also to be important in the loss of the remaining wild-type p53 allele leading to the escape from M2. The present studies provide a clearer understanding of the molecular events required for immortalization.

Materials and methods

Cell lines and cell culture

Normal mammary epithelial cells (HME) 31 were obtained from a patient undergoing breast cancer surgery. HME 32 was obtained from a patient undergoing reduction mammoplasty. Cells were cultured as previously described (Van Der Haegen and Shay, 1993). Briefly, serum-free medium was utilized, consisting of a modified basal medium MCDB 170 (MEBM, Clonetics, San Diego, CA) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, CA); 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 25 µg/ml gentamicin, 10 ng/ml transferrin (Sigma, St. Louis, MO); and 10 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA). Medium was changed every other day. Primary cultures were originated from organoids and grew predominantly as epithelioid cell populations. Following a 'self-selection' process HME 31 could be subcultured for an additional 45–50 doublings while HME 32 could only be subcultured for an additional 25–30 doublings. These cell strains have been previously characterized by our laboratory (Van Der Haegen and Shay, 1993).

Recombinant retroviral vectors

Retroviral vectors consisted of the parent vector pZip-NeoSV(X) or pZipNeoSV(X) containing the genes for 143^{ala} mutant p53; 175^{his} mutant p53; 248^{trp} mutant p53; 273^{his} mutant p53 under the transcriptional regulation of the Moloney murine leukemia virus promoter-enhancer sequences (MLVLR). These vectors also contained the gene conferring neomycin resistance under the transcriptional regulation of the SV40 promoter. The mutant p53 vectors were all kindly provided by Curtis Harris (National Cancer Institute, Bethesda, MD). Recombinant viruses were generated in the amphotrophic packaging line PA317 according to previously described procedures (Shay *et al.*, 1993 a,b,c). Viruses produced from the PA317 cells were used to infect HME cell strains 31 at PDL 22 and 32 at PDL 18, similar to those described by Halbert *et al.* (1991, 1992). The cells were selected on G418 (50–100 µg/ml) and clones isolated. Each HME clone was subcultured and continuously passaged at 2×10^5 cells/T75 flask and tested for escape from crisis at a total population size of $1-2 \times 10^6$.

Transfections

HME 31 and 32 cells were Lipofectin (Gibco/BRL, Gaithersburg, MD) transfected at the same aforementioned PDLs with pRc:CMV constructs containing single p53 genes mutated at codons 143^{ala}, 175^{his}, 248^{trp} and 273^{his} respectively. These pCMV-NeoBam constructs were kindly provided by Dr John Minna (Simmons Cancer Center, UT Southwestern Medical Center, Dallas, TX). The neomycin resistant gene was included as a dominant selectable marker. The transfections were performed under the following conditions optimized for HME cells; 10–20 µg of plasmid DNA was incubated with 150–200 µl of Lipofectin at room temperature for 15–45 min. This newly formed complex was then added to 4 ml of Opti-MEM II transfection medium (Gibco/BRL, Gaithersburg, MD) and incubated at 37°C for 6–10 h. After incubation this medium was removed and replaced with fresh MEBM. Cells were allowed to recover for 24–48 h, then trypsinized, counted, replated at various densities with G418 added. After approximately 2 weeks, individual colonies were ring clone isolated and cultured as described above.

Transient transactivation analysis

Transient transfections were performed by electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, CA) as previously described (Chen *et al.*, 1993). At 70–80% confluency HME 32 and HME 32(273)-1 cells approximately 2×10^7 cells (0.5 ml) were mixed with 60 µg of DNA (20 µl) containing 10 µg pCMV-LacZ expression plasmid plus 10 µg of reporter plasmid supplemented with sonicated salmon sperm DNA. Cells were incubated at room temperature for 5 min followed by electroporation at 960 µF, 240 and 260 volts. Transfected cells were immediately re-suspended with pre-warmed medium and incubated under normal conditions. Within 12–24 hours, the medium was changed. Forty-eight to 72 h post-transfection, cell extracts were prepared and luciferase activity measured as previously described (Funk *et al.*, 1992). Luciferase activity was assessed and normalized for differences in transfection efficiency as determined by a spectrophotometric β-galactosidase assay.

Gel electrophoresis and immunoblotting

Cell extracts were prepared as per Gillespie and Hudspeth (1991), using a modified buffer lacking β-mercaptoethanol until time of denaturation. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Samples were separated on 10% SDS-PAGE gels with 4% stacking gels using a mini-gel apparatus (Bio-Rad Mini Protean II System, Richmond, CA).

Immunoblotting, incubation and developing procedures followed the protocol for chemiluminescence detection of proteins as modified by Gillespie and Hudspeth (1991). Briefly, after electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL) and incubated with a monoclonal, primary antibody [either anti-p53 clone Pab 1801 or DO-1 recognizing wild-type and mutant conformations (Oncogene Science, Cambridge, MA)], followed by a goat-antimouse IgG secondary antibody conjugated to alkaline phosphatase. pRb was detected using MAb-1 for the pRb gene product (Triton Diagnostics, Alameda, CA). Chemiluminescent reagents, nitro-block and CSPD (Tropix, Bedford, MA), were used for signal detection and blots were exposed on Fuji X-ray film.

PCR amplification of pRc/CMV

The conditions for PCR amplification of sequences from within the CMV promoter of pRc/CMV are as follows: primers; 5'-ATAGTAATCAATTACGGGGTCATT-3', 5'-TATCGCTACTGATTATGCATCTAC-3'. These primers amplify a 347 base pair fragment which contains codons 260→607. Cocktail for the 50 µl reaction contained 1.5 mM MgCl₂, 2.5 U of Taq polymerase (Gibco/BRL), 200 mM dNTP's, 50 pM of 94°C, 1 min; (94°C, 30 s; 55°C, 30 s; 72°C, 30 s) × 40; 72°C, 1 min. The PCR product was then run on an 0.8% agarose gel for 45 min at 85V, stained with 0.05 µg/ml ethidium bromide and photographed.

Telomere length measurements

DNA from normal HME 32 and HME 32(273)-1 immortalized cells at PDLs 60, 80 and 100 was digested with HinfI. 10 µg of each sample was run on a 0.8% agarose gel overnight at 70 volts constant. Gels were dried under vacuum at 50°C for 45 min, soaked in 0.5 M sodium hydroxide/1.5 M sodium chloride for 15 min, neutralized in 0.5 M Tris pH 8.0/1.5 M sodium chloride for 15 min. Gels were then prehybridized in 5×SSC (standard saline/citrate), 5×Denhardt's, 0.5 mM sodium pyrophosphate, 10 mM disodium hydrogen phosphate at 37°C for 4–6 h. A second incubation in fresh solution with the ³²P end-labeled

telomeric probe (TTAGGG)_n for 12 h followed. After washing three times in 0.1×SSC at room temperature (7 min each), the gel was either exposed to X-ray film or analysed on a phosphorimaging device (PhosphorImager, Molecular Dynamics, Sunnyvale, CA).

Telomerase assays

A one tube PCR-based telomerase assay was performed as originally described (Kim *et al.*, 1994) with some modifications (Wright *et al.*, 1995). The assay was performed in two steps: (1) Telomerase mediated extension of an oligonucleotide primer (TS), which serves as a substrate for telomerase; and (2) PCR amplification of telomerase activity product (an incremental 6 nt ssDNA ladder) with the oligonucleotide primer CX in a competitive amplification reaction with a 150 bp fragment encoding aa 97–132 of rat myogenin as an internal telomerase amplification standard (ITAS).

Details of the method are as follows: For the cells in culture, 10⁵ cells were pelleted in culture medium. The supernatant was removed and the dry pellet stored at –80°C. The cells were lysed with 20 µl of ice cold lysis buffer consisting of 0.5% CHAPS, 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 10% glycerol, 5 mM beta-mercaptoethanol, 0.1 mM AEBSF and left on ice for 30 min. The lysate was centrifuged at 14 000 r.p.m. for 20 min at 4°C, and 160 µl of supernatant was collected into an Eppendorf tube making sure that no traces of the pellet were withdrawn; flashfrozen in an EtOH-dry ice bath and then stored at –80°C. Generally 2 µl of each lysate was analysed containing the equivalent of approximately 1000 cells. In some instances the concentration of the protein in the extract was measured using the BCA protein assay kit (Pierce Chemical Company, Rockford, IL) and an aliquot of the extract containing approximately 6 µg of protein used for each telomerase assay.

Specificity of the processive 6 nt ladder is demonstrated by RNase treatment. For RNase controls, 5 µl of extract is incubated in 1 µg of RNase (5'–3', Boulder, CO) for 20 min at 37°C. A 2 µl aliquot of extract is then assayed in 50 µl of reaction mixture containing 50 µM each dNTP, 344 nM of TS primer (5'-AATCCGTCGAGCAGAGTT-3'), and 0.5 µM of T4 gene 32 protein (USB, Cleveland, OH), [α32P]dCTP, 5 attograms ITAS and 2 units of Taq polymerase (Gibco/BRL, Gaithersburg, MD) in a 0.5 ml tube which contained the CX primer (5'-CCCTTACCCTTACCCTTACCCTAA-3') at the bottom sequestered by a wax barrier (Ampliwax™, Perkin-Elmer, Foster City, CA). After 30 min of incubation at room temperature for telomerase mediated extension of the TS primer, the reaction mixture is heated to 90°C for 90 s for inactivation of telomerase, and then subjected to 31 PCR cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s. The PCR products were electrophoresed on a 10% acrylamide gel as previously described (Kim *et al.*, 1994). Since human telomerase is processive, during the initial 30 min of incubation, in the presence of the TS primer, varying

numbers of hexameric repeats are added to the primer and when subsequently amplified yield a 6 bp DNA incremental ladder. Extracts from the samples not containing telomerase do not extend the TS primer (Kim *et al.*, 1994).

Metaphase spread analysis

Methods for obtaining metaphase spreads were described previously (Aldaz *et al.*, 1989). Cultures were incubated with 0.01 µg/ml Colcemid (Gibco/BRL, Gaithersburg, MD) in fresh medium for 4 h. After collection by trypsinization, cells were incubated for 1 h at 37°C in a 0.067 M KCl hypotonic solution then fixed in 3:1 methanol:glacial acetic acid, rinsed and spun 2× for 5 min each at 1200 r.p.m. After resuspension in 1–2 ml of fix, pellets were dropped onto precleaned microscope slides and stained with Giemsa Stain (Sigma Chemical Company, St. Louis, MO). Chromosomes were counted from >25 randomly chosen metaphase spreads.

Fluctuation analysis

The frequency of escape from crisis (e.g. immortalization frequency) was estimated using an approach based on what is essentially a fluctuation analysis previously described (Shay *et al.*, 1993a). Clones were expanded several population doublings before crisis into multiple series in several sizes of culture dishes at a constant cell density. Each series was subsequently maintained as a separate culture, so that at the end of the experiment the fraction of each series that gave rise to an immortal cell line could be determined. Using different size dishes permitted series to be set up which contained a different number of cells per dish while maintaining a constant culture environment (cells/cm²). Cultures were split at or just prior to confluence. Once cells reached crisis, they were split (at least once every 3 weeks) until only a few surviving cells remained or the culture had immortalized. Immortalization was expressed as the number of immortal lines per number of culture series. Frequency is expressed as the probability of obtaining an immortal cell line based on the total number of independent immortalization events and dividing by the total number of cells plated.

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Notes

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Telomerase Activity in Human Breast Tumors

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Background: The activity of the ribonucleoprotein enzyme telomerase is not detected in normal somatic cells; thus, with each cell division, the ends of chromosomes consisting of the telomeric repeats TTAGGG progressively erode. The current model gaining support is that telomerase activity in germline and immortal cells maintains telomere length and thus compensates for the "end-replication problem." **Purpose:** Our objective was to determine when telomerase activity is reactivated in the progression to malignant breast cancer and if knowledge of telomerase activity may be an indicator for the diagnosis and potential treatment of breast cancer. **Methods:** Using a polymerase chain reaction-based telomerase activity assay, we examined telomerase activity in 140 breast cancer specimens (from 140 patients), four phyllodes tumors (from four patients), 38 noncancerous lesions (20 fibroadenomas, 17 fibrocystic diseases, one gynecomastia; from 38 patients), and 55 adjacent noncancerous mammary tissues (from 55 of the 140 breast cancer patients). In addition, 33 fine-needle-aspirated breast samples (from 33 patients) were analyzed. **Results:** Among surgically resected samples, telomerase activity was detected in 130 (93%) of 140 breast cancers. Telomerase activity was detected in 68% of stage I primary breast cancers, in 73% of cancers smaller than 20 mm, and in 81% of axillary lymph node-negative cancers. Moreover, the activity was detected in more than 95% of advanced stage tumors but in only two (4%) of 55 adjacent noncancerous tissues. While telomerase activity was not detected in any of 17 specimens of fibrocystic disease, surprisingly low levels of telomerase activity were detected in nine (45%) of 20 fibroadenomas. Among

samples obtained by fine-needle aspiration, 14 (100%) of 14 patients whose fine-needle-aspirated specimen contained telomerase activity and who subsequently underwent surgery were confirmed to have breast cancer. Multivariate analysis of 125 specimens from patients for whom data were available on age at surgery, stage of disease, tumor size, lymph node status tumor histology, and menopausal status indicated that stage classification exhibited the strongest association with telomerase activity (for stage I versus stages II-IV: odds ratio = 1.0 versus 73.4; 95% confidence interval = 2.0-959.0; $P = .02$). **Conclusion:** Telomerase activity was detected in more than 95% of advanced stage breast cancers. It was absent in 19%-32% of less advanced cancers. Since a determination of any association between telomerase activity and patient survival is not possible at the present time, it remains to be determined whether lack of telomerase activity predicts for favorable outcome. [*J Natl Cancer Inst* 1996;88:116-22]

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See "Notes" section following "References."

Breast cancer is the most common malignancy in U.S. women and the second leading cause of cancer death, exceeded only by lung cancer in the United States (1,2). In Japan, the incidence of breast cancer is lower than in the United States, although it is gradually increasing (2). During the last decade, intense clinical and biological research efforts have been initiated to determine the causes of breast cancer. A number of factors, both endogenous and exogenous, that increase the risk of breast cancer development have been identified (2-4). During the previous decade, screening for early detection of breast cancer has increased dramatically (2,5). The increased use of mammography has led to early detection of nonpalpable breast cancers, and fine-needle aspiration has been shown to be a useful procedure for the early and accurate diagnosis of these cancers. Tumor size, lymph node status, and histopathologic findings are considered to be good prognostic indicators in breast cancer (6,7). Moreover, several molecular changes, such as amplification or overexpression of the *c-erbB-2* gene (also known as *ERBB2*) (8), overexpression of the epidermal growth factor receptor (9), DNA aneuploidy (10), estrogen and progesterone receptor status (11), and the diminished expression of *BRCA1* (12), appear to be involved in breast cancer development. However, the molecular events underlying the development of human breast cancer still need to be elucidated.

Telomeres are specialized structures containing unique (TTAGGG)_n repeats at the ends of eukaryotic chromosomes; these repeats are thought to be important in the protection and replication of chromosomes (13,14). Lagging strand DNA synthesis at the very end of linear chromosomes cannot be completed (referred to as the "end-replication problem") and this situation results in the progressive shortening of telomeric repeats with each division (15,16). Telomerase contains an RNA component that has a template region complementary to (TTAGGG)_n repeats that permits the *de novo* synthesis of TTAGGG telomeric DNA onto chromosomal ends (17-19). While germline cells expressing telomerase activity maintain telomeric repeats, in somatic cells, progressive erosion of telomeres with each cell division is likely due to the repression of telomerase activity during development (20-23). Although the reactivation of telomerase alone may be insufficient for cells to proliferate indefinitely, its expression and the stabilization of telomeres appear to be concomitant with the attainment of immortality in cancer cells (24,25). Previously, we (26) hypothesized that telomerase reactivation had an important role in the etiology of breast cancer (26). To add experimental support to this hypothesis and to evaluate the clinical usefulness of detecting telomerase activity in clinical specimens, we examined telomerase activity in a variety of different types of breast cancer and noncancerous tissue specimens.

Recently, a highly sensitive polymerase chain reaction (PCR)-based telomerase assay called the TRAP (Telomeric Repeat Amplification Protocol) assay was developed for the detection of telomerase activity (25). By this method, telomerase activity has been found in most tumor tissues examined (covering a large variety of tumor types). Among these tumors, telomerase activity was detected in 94% of neuroblastoma (27), 80% of lung cancer (28), 93% of colorectal cancer (29), 85% of hepatocellular carcinoma (30), and 85% of gastric

cancer (31). In addition, in cultured cells, 98 of 100 immortal and none of 22 mortal cell populations expressed telomerase activity (25). Thus, telomerase activity appears to be repressed in somatic cells and tissues, but it is reactivated in most immortal cells and human cancers. The results of these studies suggest that telomerase activation occurs during the development of various malignant tumors and that, in almost all instances, telomerase activity may ultimately be required to maintain tumor growth. Although it is not known at what stage in cancer development telomerase is reactivated, clinical interest is now focused on whether the detection of telomerase activity may be a useful diagnostic tool in clinical specimens, especially in cytologic materials.

In the present study, we measured telomerase activity and telomere length in benign and malignant breast disease samples in order to evaluate a putative role of telomerase in breast carcinogenesis. In addition, we examined telomerase activity in fine-needle-aspirated samples to determine if telomerase activity can be detected in these specimens and if this activity can be used as a diagnostic or prognostic indicator of breast cancer (which may increase the value of cytologic diagnosis).

Patients and Methods

Tissue Samples

A total of 140 breast cancer tissues (from 140 patients) and 55 adjacent noncancerous breast tissue samples (from 55 of the 140 breast cancer patients) were obtained at the time of surgery. In addition, four phyllodes tumors (one malignant, one borderline, and two benign tumors), 20 fibroadenomas, 17 specimens of fibrocystic disease, and one gynecomastia sample were obtained from 38 separate patients. Among these samples, 64 breast cancers, five fibroadenomas, two cases of fibrocystic disease (mastopathy), and one malignant phyllode tumor were obtained from patients who underwent surgery at Parkland Memorial Hospital, Dallas, TX. The remaining tissues were obtained from patients who underwent surgery at Hiroshima University Hospital, Hiroshima, Japan. Tumor sizes were determined after surgery, and tumor samples were stored at -80 °C until use. Institutional guidelines for the use of patient materials were followed in both the United States and Japan. Written informed consent was obtained from all patients. The breast cancers of patients in both the United States and Japan were staged according to the International Union Against Cancer tumor-node-metastasis (UICC-TNM) classification (32).

After we received written informed consent, fine-needle-aspirated samples were obtained from 33 women who underwent cytologic examination for diagnosis of their breast tumors. The fine-needle aspiration was performed as follows: The needle, attached to a syringe, was inserted into the breast tumor. The tumor cells were obtained by applying mild suction. The needle was moved back and forth several times, and then the negative pressure was released. Fine-needle aspiration was performed twice on most samples. One aspirate was examined cytologically, while the other was deposited in a phosphate-buffered saline (PBS) solution. This latter sample was washed two times with PBS, and the number of cells was counted with a hemocytometer. The remainder of the sample was centrifuged at 1500g for 5 minutes, and the pellet was stored at -80 °C until use.

Telomerase Assay

Extracts of tissue specimens and assays of telomerase activity were done as described earlier (25,33). Briefly, frozen breast tissue samples of 50-100 mg were homogenized in 200 µL of 3-[(3-cholamidopropyl)dimethyl-ammonio]-1 propanesulfonate (CHAPS) lysis buffer. After 25 minutes of incubation on ice, the lysates were centrifuged at 16 000g for 20 minutes at 4 °C, and the supernatant was rapidly frozen in liquid nitrogen and stored at -80 °C. The concentration of protein was measured by use of the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL), and an aliquot of extract containing 6 µg of protein was used

for each TRAP assay unless otherwise indicated. For fine-needle-aspirated samples, aliquots corresponding to an extract derived from approximately 10^3 cells were used for the TRAP assay. For ribonuclease (RNase) treatment, 5 μ L of extract was incubated with 1 μ g RNase (Boehringer Mannheim Corp., Indianapolis, IN) for 20 minutes at 37 °C. Assay tubes were prepared by sequestering 0.1 μ g of CX primer (5'-CCCTTACCCTTACCCTTACCCTAA-3') under a wax barrier (HotStart 50 PCR tube; Molecular Bio-Products, San Diego, CA). Each extract was assayed in 50 μ L of reaction mixture containing 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 68 mM KCl, 0.05% Tween 20, 1 mM ethylene glycol-bis (β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 50 μ M deoxynucleoside triphosphates, 150 kilobecquerels [³²P]deoxycytidine triphosphate, 0.1 μ g of TS oligonucleotide (5'-AATCCGTCGAGCAGAGTT-3'), 0.5 μ M T4 gene 32 protein (United States Biochemical Corp., Cleveland, OH), and 2 U of *Taq* DNA polymerase (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD, or Wako Chemicals, Osaka, Japan). Each reaction mixture contained 5×10^{-18} g (5 attograms) of an Internal Telomerase Assay Standard (ITAS) for quantitative estimation of the levels of telomerase activity and the identification of false-negative tumor samples that contain *Taq* polymerase inhibitors (34). ITAS is a 150-base-pair DNA standard, which is coamplified with telomerase-elongated products and is sufficiently long that it does not interfere with the visualization of the telomerase ladder. After 30 minutes of incubation at room temperature for telomerase-mediated extension from annealed TS oligonucleotides, the reaction mixture was heated at 90 °C for 90 seconds and then subjected to 31 PCR cycles at 94 °C for 40 seconds, 50 °C for 40 seconds, and 72 °C for 50 seconds. The PCR product was electrophoresed on a 10% polyacrylamide gel. To estimate telomerase activity in tissue samples, we compared the intensity of the TRAP assay-generated DNA ladder with that of the ITAS signal using the Bioimage Analyzer (BAS 2000; Fuji, Tokyo, Japan) or the Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Southern Blot Analysis

Genomic DNA was isolated from 22 adjacent noncancerous tissues and 60 tumor tissues of untreated breast cancer patients and 33 specimens of benign breast disease tissue (one gynecomastia, 15 fibrocystic disease tissues, 14 fibroadenomas, and three phyllodes tumors) surgically obtained in Japan as previously described (35-37). For the analysis of length of terminal restriction fragments, 2 μ g of DNA was digested to completion with 10 U of *Hinf*I, electrophoresed on 0.8% agarose gels, and then blotted onto nitrocellulose filters. The filters were hybridized to a ³²P-end-labeled (TTAGGG)_n probe, washed, and then autoradiographed as previously reported (35-37). We estimated the mean length of terminal restriction fragments at the peak position of hybridization signal. To confirm complete *Hinf*I digestion, we rehybridized the same filters with a ³²P-labeled β -globin or a K-ras probe. To exclude the possible effect of DNA degradation, we analyzed the integrity of undigested DNA by gel electrophoresis.

Statistical Analysis

For statistical analysis, we divided tumor samples into one of two groups: tumors with undetectable telomerase activity and tumors with telomerase activity. To compare these groups, we analyzed clinical data by univariate analysis. All *P* values refer to either a chi-squared test with Yates correction or Fisher's exact test for tables and the Mann-Whitney test for nonparametric data, where appropriate. All *P* values resulted from two-sided statistical tests. In addition, multivariate analysis using the multiple logistic regression model was used to identify which, if any, other status of cancer indicators was associated with telomerase expression. This analysis was performed using the CARE Software system (Hiroshima University, Japan).

Results

Telomerase Activity in Breast Cancers Compared With Clinical Parameters

Telomerase activity was detected in 130 (93%) of 140 breast cancer tissues analyzed (Table 1), whereas telomerase activity was detected in only two (4%) of 55 adjacent noncancerous breast tissues and even then at low levels (data not shown). His-

Table 1. Comparison of tumor stage and size and lymph node metastasis with telomerase activity in breast cancer

	No. of specimens	Telomerase activity		
		Negative	Positive	% positive
Stage				
I	22	7	15	68
II	52	1	51	98
III	46	2	44	96
IV	7	0	7	100
Unknown	13	0	13	100
Tumor size, mm				
<20	30	8	22	73
≥20	104	2	102	98
Unknown	6	0	6	100
Lymph node metastasis				
Negative	37	7	30	81
Positive	88	3	85	97
Unknown	15	0	15	100
Total	140	10	130	93

tologically, there were no obvious differences in the ratio of tumor cells to stromal cells in the tumors with and without detectable telomerase activity, and the reproducibility of the TRAP assay was confirmed by sampling multiple, different sites of some of the tumors (data not shown). The frequency of tumors detected with telomerase activity differed markedly between stage I tumors (68%; 15 of 22) and other advanced stage tumors (>95%) ($P < .0001$, chi-squared test). Among 88 primary tumors resected from patients with lymph node metastasis, 85 tumors (97%) showed telomerase activity, whereas 30 (81%) of 37 of the tumors without lymph node metastasis had detectable telomerase activity ($P = .01$, Fisher's exact test) (Fig. 1). Tumor sizes also revealed an association with telomerase activity. Tumors without detectable telomerase activity were significantly smaller than those with observed activity ($P = .003$, Mann-Whitney test). These findings demonstrated that, whereas almost all advanced breast cancers (>95%) had telomerase activity, a statistically significant fraction of early breast cancers lacked detectable telomerase activity. In addition, 34 (100%) of 34 tumors from premenopausal patients had detectable telomerase activity, whereas 96 (91%) of 106 tumors diagnosed in perimenopausal or postmenopausal patients had detectable telomerase activity ($P = .045$, Fisher's exact test). Three (3%) of the 102 advanced tumors (stage II, III, or IV) examined were considered noninformative because the internal standard (ITAS) (34) was not amplified during the PCR reaction. Multivariate analysis of 125 specimens from patients for whom the age at surgery, stage of disease, tumor sizes, lymph node status, histology, and menopausal status were known revealed that stage classification exhibited the strongest association with the expression of telomerase activity (stage I versus stage II-IV odds ratio = 1.0 versus 73.4; 95% confidence interval = 2.0-959.0; $P = .02$).

The presence of telomerase activity was also examined in eight metastatic lesions obtained from seven patients with breast cancer: five lymph node metastases, two lung metastases, and one liver metastasis. All metastatic lesions showed high levels of telomerase activity. From these cases, four primary tumors

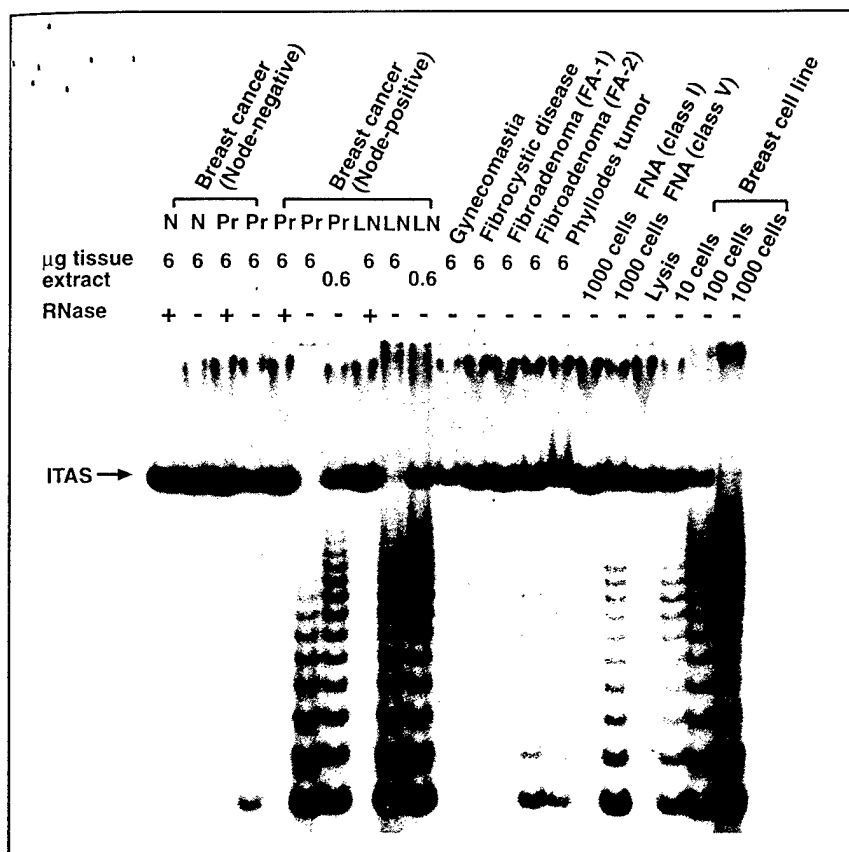


Fig. 1. Telomerase activity in breast cancer, benign breast diseases, and fine-needle aspiration samples. From each tissue sample, an aliquot of the extract containing 6 or 0.6 µg of protein with (+) or without (–) ribonuclease (RNase) pretreatment was used in each TRAP (Telomeric Repeat Amplification Protocol) assay. In samples taken by fine-needle aspiration (FNA), 10³ cell equivalents were used for the TRAP assay. Extracts of a human breast cell line having telomerase activity were used as a positive control. Telomerase activity was detected after electrophoresis of the enzyme reaction products and autoradiography as a 6 nucleotide repeat ladder. An Internal Telomerase Assay Standard (ITAS) was used to identify noninformative specimens due to inhibitors of *Taq* polymerase affecting the TRAP assay. Primary breast cancer (Pr), lymph node metastasis (LN), fibroadenoma (FA-2), and phyllodes tumor (borderline) showed telomerase activity, whereas adjacent noncancerous breast tissue (N), gynecomastia, and fibroadenoma (FA-1) did not have detectable telomerase activity. The telomerase activity detected in the lymph node metastasis was stronger than that detected in the primary tumor. Samples (cytology: class I) obtained by FNA did not have detectable activity, whereas telomerase activity was detected in cytology class V samples.

were available; all were telomerase positive. The levels of telomerase activity observed in metastatic lesions were equivalent to or higher than those observed in analyzed primary lesions.

Telomerase Activity in Other Breast Diseases

We examined 20 fibroadenomas, 17 fibrocystic disease specimens, and one gynecomastia specimen (Table 2). In addition, we examined four phyllodes tumors (two benign, one borderline, and one malignant). Telomerase activity was undetectable in all specimens diagnosed as fibrocystic disease or in the gynecomastia sample. However, nine (45%) of 20 fibroadenomas had detectable telomerase activity. In the fibroadenoma specimens, the intensities of the telomerase signals were relatively weak (Fig. 1). There were no obvious differences in the age at surgery, tumor sizes, and histologic findings between telomerase-negative and telomerase-positive fibroadenomas (data not shown). In the phyllodes tumors, two benign tumors

did not have detectable telomerase activity, whereas the one borderline tumor exhibited a low level of activity (Fig. 1) and the malignant phyllodes tumor exhibited a high level of telomerase activity.

Length of Terminal Restriction Fragments in Breast Cancer Tissues, Adjacent Breast Tissues, and Tissue Samples From Cases of Benign Breast Disease

Telomere lengths were examined for 22 adjacent noncancerous tissues and 60 primary breast cancer tissues, including two tumors without detectable telomerase activity (Fig. 2, B). The terminal restriction fragment lengths of all adjacent tissues ranged between 8 and 15 kilobase pairs (kbp), whereas those of 60 breast cancer tissues varied between 3.4 and 27 kbp. We classified samples as shortened terminal restriction fragments when the lengths were shorter than 8 kbp and as elongated when the lengths were longer than 15 kbp. Among these primary cancers, the terminal restriction fragment lengths were shorter than 8 kbp in 13 tumors (22%) and longer than 15 kbp in seven tumors (12%). There was no apparent difference between altered length of terminal restriction fragments and tumor stage, tumor size, or lymph node status (data not shown). However, all seven tumors with elongated terminal restriction fragments and 11 of 13 tumors with shortened fragments showed strong telomerase signals. Of 40 tumors without altered lengths of terminal restriction fragments, telomerase activity was not detected in two tumors, whereas 26 tumors showed strong telomerase signals. In addition, there were 22 cases in which both breast cancer tissue and adjacent noncancerous tissue were available

Table 2. Telomerase activity in other breast lesions

	No. of specimens	Telomerase activity		
		Negative	Positive	% positive
Fibrocystic disease	17	17	0	0
Gynecomastia	1	1	0	0
Fibroadenoma	20	11	9	45
Phyllodes tumor	4	2	2*	50

*One of these tumors was borderline, and the other was malignant.

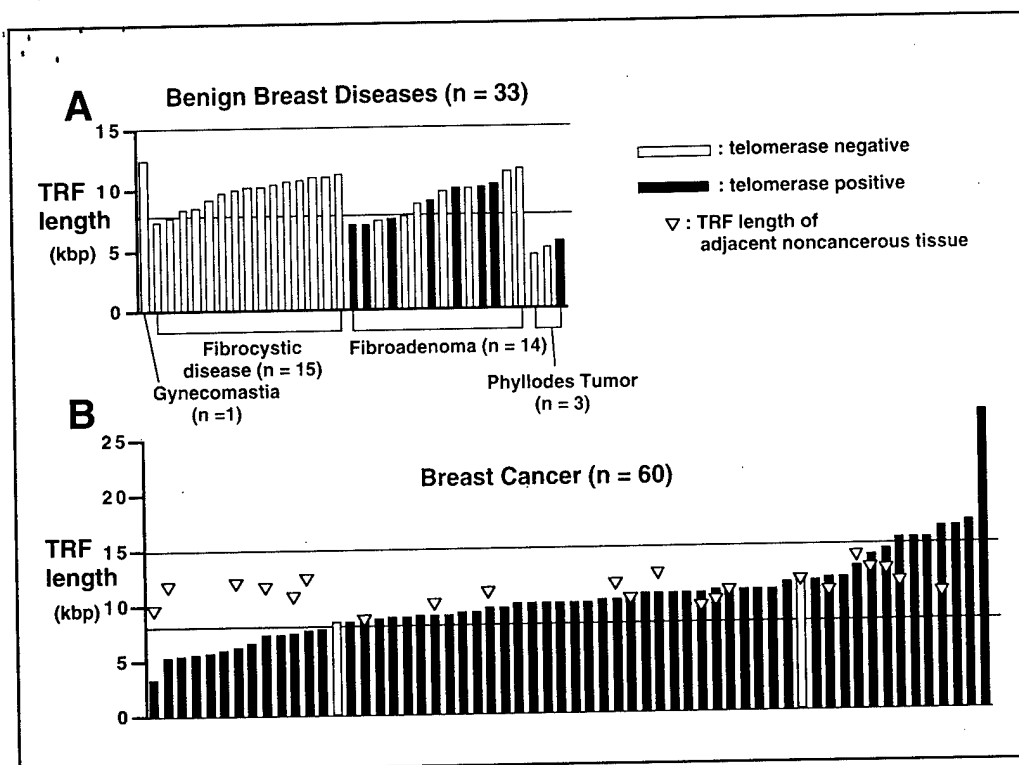


Fig. 2. Telomere lengths in benign breast diseases, breast cancer tissues, and adjacent noncancerous tissues. For each tissue sample, 2 μ g of genomic DNA was digested to completion with *HinfI* and was used in Southern blot hybridizations. A) Mean terminal restriction fragment (TRF) lengths in benign breast diseases (n = 33), including one gynecomastia, 15 fibrocystic disease tissues, 14 fibroadenomas, and three phyllodes tumors. The TRF lengths in most of these specimens were between 8 and 15 kilobase pairs (kbp), whereas all three (two benign and one borderline) phyllodes tumors showed reduced TRF lengths (4.8, 5.4, and 6.8 kbp, respectively). Seven of 14 fibroadenomas and one borderline phyllodes tumor had detectable telomerase activity. B) Mean TRF lengths in breast cancer tissues (n = 60) and in adjacent noncancerous tissues (n = 22). The mean TRF lengths in breast cancer ranged between 3.4 and 27 kbp, whereas those in adjacent noncancerous tissues ranged between 8 and 15 kbp. Among 60 samples examined for TRF length, 58 had telomerase activity. The two cancers without detectable telomerase activity had mean TRFs of 8.7 and 11 kbp, respectively.

for terminal restriction fragment determinations (Fig. 2, B). We also examined the telomere length of 14 fibrocystic disease tissues, 15 fibroadenomas, three phyllodes tumors, and one gynecomastia specimen (Fig. 2, A). The lengths of terminal restriction fragments in most of these tissues (except for phyllodes tumors) were in the normal range between 8 and 15 kbp. It is interesting that all three phyllodes tumors examined (two benign and one borderline) showed shortened lengths of terminal restriction fragments (<7 kbp).

Telomerase Activity in Fine-Needle-Aspirated Samples

Using extracts derived from 10^3 cells, we examined telomerase activity in 33 fine-needle-aspirated samples (Table 3). In 18 samples without detectable telomerase activity, 17 (94%) were diagnosed as class I, II, or III (suspected to be benign) by cytology. The one remaining sample without detectable telomerase activity was diagnosed as class IV; upon surgery, this tumor was determined to be an invasive breast cancer. One telomerase-negative, cytologically diagnosed class I tumor was subsequently surgically resected, and it was found to be a fibroadenoma. Among the 33 fine-needle-aspirated samples, 15 (45%) had telomerase activity (Fig. 1). Of the 15 telomerase-positive samples, 14 (93%) were diagnosed as class IV or V (suspected to be malignant) by cytology, and all were subsequently determined to be invasive breast cancer at surgery. The remaining one tumor was diagnosed as class III by cytology, and the patient has been followed without surgical resection. These findings indicate that fine-needle aspiration may be useful in the detection of telomerase activity as a diagnostic marker for breast cancer.

Table 3. Telomerase activity in breast samples obtained by fine-needle aspiration

Case patient No.	Age, y	Cytology	Telomerase activity*	Histology after surgery†
1	88	I	Negative	ND
2	32	I	Negative	ND
3	46	I	Negative	ND
4	44	I	Negative	ND
5	75	I	Negative	ND
6	29	I	Negative	ND
7	43	I	Negative	ND
8	43	I	Negative	Fibroadenoma
9	40	I	Negative	ND
10	55	II	Negative	ND
11	73	II	Negative	ND
12	19	II	Negative	ND
13	30	II	Negative	ND
14	71	II	Negative	ND
15	50	II	Negative	ND
16	50	III	Negative	ND
17	44	III	Negative	ND
18	78	IV	Negative	Invasive ductal carcinoma
19	48	III	Positive	ND
20	69	IV	Positive	Invasive ductal carcinoma
21	69	IV	Positive	Papillary carcinoma
22	51	V	Positive	Invasive ductal carcinoma
23	49	V	Positive	Invasive ductal carcinoma
24	53	V	Positive	Invasive ductal carcinoma
25	71	V	Positive	Invasive ductal carcinoma
26	49	V	Positive	Invasive ductal carcinoma
27	83	V	Positive	Invasive ductal carcinoma
28	77	V	Positive	Invasive lobular carcinoma
29	51	V	Positive	Invasive ductal carcinoma
30	63	V	Positive	Invasive ductal carcinoma
31	78	V	Positive	Invasive ductal carcinoma
32	77	V	Positive	Invasive ductal carcinoma
33	48	V	Positive	Invasive ductal carcinoma

*Telomerase activity estimated from extracts containing approximately 10^3 cells.

†ND = surgery was not done.

Discussion

In the present study, telomerase activity was detected in almost all malignant breast tumor samples (present in 95% of advanced stage breast cancer specimens but absent in 19%-32% of less advanced breast cancer specimens), whereas the activity was not detected in most normal breast tissues or in benign breast tumors analyzed (with the exception of fibroadenomas). Our results indicate that telomerase reactivation may be an important step in the progression of normal breast epithelial tissue to breast cancer, as previously hypothesized (26). The low levels of telomerase activity detected in two adjacent noncancerous breast tissues may have been caused by the presence of occult microinvasion.

In somatic cells without telomerase activity, telomeres progressively shorten and, after many cell divisions, undergo cellular senescence (21,22). In immortal cells, unlimited growth capacity appears to be acquired concomitantly with telomere stabilization, which is likely due to reactivation of telomerase activity (25). In recent studies, telomerase activity has been detected in approximately 85%-90% of tumor samples from many types of malignant tumors, including lung cancer (28), colorectal cancer (29), gastric cancer (31), and hepatocellular carcinoma (30). In the present study, 93% of the breast cancers analyzed showed telomerase activity. These results suggest that almost all human cancers consist of a population of cells that have acquired immortality.

Clinical stage, histology, tumor size, and lymph node status are known to be prognostic factors in breast cancer patients (6,7). The tumors without telomerase activity were at a significantly earlier clinical stage and were smaller than the tumors with telomerase activity. The incidence of lymph node metastasis in the patients with telomerase-negative primary tumors was lower than that in the patients with telomerase-positive primary tumors. The metastatic lesions of breast cancer samples were telomerase positive, and the activity in these cases appeared stronger than that in primary lesions. One explanation may be that telomerase activity is acquired during the progression of breast cancer to metastatic lesions. Alternatively, contrary to the more homogeneous population in metastatic lesions, primary breast tumors exhibit heterogeneous clusters of cells, where the telomerase-positive population is diluted by intercalating stromal and connective tissue cells (38).

Telomere length has been examined in many types of tumors (35-37). Altered lengths of telomeres occur in less than half of the breast cancers when compared with normal adjacent breast tissues of the same patients (39) as well as in the other types of tumors (36). In the present study, although almost all tumors have telomerase activity, altered telomere lengths were found in only 20 (33%) of 60 breast cancers when compared with normal breast tissues from the same respective patients. It is interesting that all tumors with elongated telomere length and most tumors with reduced telomere length exhibited high levels of telomerase activity. Contrary to the low frequency of these altered telomere lengths, the frequency of detectable telomerase activity was very high. Southern blot analysis measures the mean length of terminal restriction fragments of all cells contained in the tumor specimen—not only tumor cells but also stromal and con-

nective tissue cells. These results indicate that altered telomere length may be difficult to detect using Southern blot analysis until the majority of the cancer cells have telomeres at altered length. On this assumption, the tumors whose telomeres were stabilized at altered lengths have high levels of telomerase activity because such tumors may consist almost exclusively of cancer cells with telomerase activity. Thus, to identify whether the cancer cells have acquired immortality, telomerase activity is likely to be a better indicator than alterations of telomere length.

In cases of benign breast disease, telomerase activity was not detected in the fibrocystic disease and gynecomastia samples. Surprisingly, nine (45%) of 20 fibroadenomas analyzed exhibited low levels of telomerase activity. The mean terminal restriction fragment lengths in most fibroadenomas ranged between 8 and 15 kbp, which is similar to those of adjacent noncancerous breast tissues. That some fibroadenomas had telomerase activity cannot easily be explained at present because the pathogenesis of fibroadenoma is not well understood. Several reports (40-42) indicate that the origins of fibroadenoma are heterogeneous; our present results seem to support this theory. Fibroadenomas are benign tumors that are commonly diagnosed in young women. An analysis (43) demonstrated that fibroadenoma is associated with a long-term risk for breast cancer. It remains to be determined whether patients with telomerase-positive fibroadenomas are at greater risk of subsequently developing breast cancer than patients with telomerase-negative fibroadenomas. Thus, immortality may not always be acquired concomitantly with malignant transformation. In contrast, telomerase activity was not detected in either of two benign phyllodes tumors, whereas it was detected in a borderline tumor and in a malignant phyllodes tumor. Phyllodes tumors are relatively large, usually grow rapidly, and consist of an extremely hypercellular stroma accompanied by proliferation of breast ductal structures (44). The reduction in telomere length in benign phyllodes tumors is likely due to many cell divisions in the absence of sufficient telomerase activity. It is possible that telomerase may be reactivated in phyllodes tumors when they acquire malignant potential. However, additional studies are required to address this issue.

Among the fine-needle-aspirated breast samples, all telomerase-positive specimens were subsequently shown to be breast cancer except for one tumor that was diagnosed as class III, and this patient has been followed without surgical resection. In this study, telomerase activity was analyzed from an extract estimated to have been made from 10^3 aspirated cells. Since peripheral blood mononuclear cells are reported to exhibit low levels of telomerase activity when 10^4 or more cells are examined (28), we confirmed that telomerase activity could not be detected in 10^3 total blood cells, permitting us to eliminate the possible contributions of telomerase activity from positive peripheral blood cells. Clinically, aspiration cytology is being used more frequently to diagnose breast cancer before surgery. Fine-needle aspiration of the breast is a well-established technique for diagnosing breast lesions, and its specificity has been reported to be greater than 90% (45). Thus, detection of telomerase activity in fine-needle-aspirated samples in combination with morphologic examination may be useful in detecting

immortalized cancer cells not only in breast but also in other organs for which fine-needle aspiration or cytology is applicable.

In summary, telomerase activity in breast cancer may be useful in early diagnosis and in obtaining an accurate diagnosis. In the future, the development of telomerase inhibitors may lead to novel approaches for the treatment and management of breast cancer. However, since analysis of the relationship between telomerase activity and patient survival was not possible at present, it remains to be determined whether lack of telomerase activity predicts for favorable outcome in breast cancer patients.

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Notes

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p53 levels in human mammary epithelial cells expressing wild-type and mutant human papillomavirus type 16 (HPV-16) E6 proteins: relationship to reactivation of telomerase and immortalization

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Abstract. Human fibroblast cells must overcome both the M1 and the M2 stages of cellular senescence to immortalize, at which point cells almost always express telomerase activity. The human papillomavirus (HPV) oncoproteins, HPV-16 E6 and E7, can block the progression to senescence in fibroblasts by associations with p53 and pRb, respectively. Human mammary epithelial (HME) cells require only HPV-16 E6 to bypass M1, suggesting that pRb may not have a direct role in HME cells senescence. In the present report, we show that only wild-type HPV-16 E6 allows complete degradation of p53, immortalization and reactivation of telomerase activity in HME cells. These results suggest that the ability of HPV-16 wild-type and mutant E6 proteins to degrade p53 in intact HME cells and keratinocytes does not completely correlate with their ability to degrade p53 in a cell-free system. This discrepancy between *in vitro* and *in vivo* p53 degradation may be biologically significant and may provide insight into the susceptibility of certain human cells and tissues for reactivation of telomerase and immortalization.

Introduction

Human diploid fibroblasts and human mammary epithelial (HME) cells normally divide until they become senescent; that is, they proliferate for a limited number of cell divisions and then stop dividing. Cellular senescence in human cells can be divided into 2 components, mortality stage 1 (M1) and

mortality stage 2 (M2) (1). M1 in human fibroblast cells is controlled by the normal functions of the tumor suppressor proteins p53 and pRb (2). Viral oncoproteins that can sequester or degrade these proteins have the ability to allow cells to overcome M1, leading to an extended lifespan, or M2 (1). However, viral proteins do not allow cells to overcome M2, which occurs when few, if any, telomeric repeats remain at the end of the chromosomes, and may represent the critical shortening of telomeres (3). Only a rare event will allow cells to overcome M2, stabilize the telomeres, and become immortal (3). This rare event is most likely due to the reactivation of telomerase, the ribonucleoprotein responsible for the synthesis of the telomeric repeats at the ends of the chromosomes. Telomerase activity has been found only in germline tissues, immortal cells, and cancer cells, but not in somatic cells or tissues with the exception of stem cells (4,5).

Human papillomaviruses (HPV) are classified as small DNA tumor viruses and are implicated as the causative agents of papillomas or warts. A subset of HPVs has been further categorized into those that are associated with benign genital lesions (low risk) and with genital cancers (high risk). The high risk HPVs (HPV-16 and -18) are capable of extending the lifespan and immortalizing cervical epithelial cells, primary human keratinocytes and HME cells (6-10); in contrast, the low risk HPVs (HPV-6 and -11) are unable to extend the lifespan or immortalize any of these cells (7). The immortalizing function of the high risk HPVs was mapped to two early open reading frames, E6 and E7 (7,8). The HPV-16 E6 and E7 proteins are both required to immortalize primary human fibroblasts, keratinocytes, and cervical epithelial cells, presumably by associating with and/or degrading p53 and pRb, respectively (2,10,11). The E7 protein is able to bind to pRb, as well as the other pocket binding proteins, p107 and p130 (12). This association allows the release of the cellular transcription factor, E2F, resulting in the activation of cellular growth regulatory genes, such as *c-myc* and *B-myb* (13,14). However, HME cells require only the presence of the high risk E6 to immortalize, indicating that inactivating p53, and not pRb, is the only requirement for immortalization (6,10).

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Further evidence supporting a role for p53 as the only block necessary to overcome M1 in HMEs stems from results obtained with cells from patients with Li-Fraumeni syndrome (an inherited mutation in a single allele of p53), as these cells are able to overcome, and eventually reactivate telomerase and spontaneously immortalize in culture without the addition of any viral onco-proteins (15). Taken together, these results indicate that the requirement for pRb in the M1 mechanism of cellular senescence is not present in HME cells. This system provides an excellent model for the study of the *in vivo* interaction and mechanisms of E6 and p53 as they relate to the reactivation of telomerase and immortalization.

Both the high risk and low risk HPV E6 proteins are capable of forming a complex with p53, yet only the HPV-16 and -18 E6 proteins are able to associate with and degrade p53 through the ubiquitin pathway (16). This enhanced degradation is mediated by a cellular cofactor, E6-AP, which has been identified as a ubiquitin ligase (17). E6-AP associates with the E6-p53 complex and serves to allow degradation of p53 through the ubiquitin pathway. HPV-6 E6 associates with but does not degrade p53 (18,19), and mutational analysis of the HPV-16 E6 protein suggests that the regions required for binding and degradation of p53 are separable (18). Mutation of amino acid residues near the amino-terminus of HPV-16 E6 inactivates its ability to degrade p53, while retaining the ability to bind p53. Small deletions in the carboxy-terminus of E6 eliminate binding to p53. Crook *et al* found that a 16/6 chimera (made by fusing the amino-terminus of HPV-16 E6 to the carboxy-terminus of HPV-6 E6) is capable of binding and degrading p53 and that the 6/16 mutant (made by fusing the amino-terminus of HPV-6 E6 to the carboxy-terminus of HPV-16 E6) is only able to bind p53, indicating that the HPV-6 E6 was able to bind p53 but that its amino terminus lacked the ability to induce the degradation of p53 (18). These results suggest that the C-terminal half of the low risk E6 could functionally replace the C-terminus of the high risk protein but that the N-terminus of low risk E6 could not replace that of high risk. Since these experimental results were obtained using *in vitro* assays, it would be important to determine if these effects also occur *in vivo*. In addition, since HMEs immortalize when expressing high risk E6 alone, it would be important to know if the effect of E6 mutants on p53 degradation *in vitro* (cell-free) and *in vivo* correlates with their ability to reactivate telomerase and immortalize HME cells.

In this report, we describe the effect of specific HPV E6 mutants on p53 levels in primary human foreskin keratinocytes, two normal HME cell strains, and a cervical carcinoma-derived cell line containing mutant p53 and lacking any other HPV sequences (C33a). Defective retroviruses were constructed encoding the HPV-16 E6(Δ 9-13), HPV-16 E6(YYH), 6/16, and 16/6 mutations as developed by Crook and coworkers (18). After infection of the defective retrovirus clones and selection by drug-resistance, representative cell populations were analyzed for the presence of the integrated E6 mutations, p53 levels by Western analysis, extension of lifespan (HME only), and immortalization and/or reactivation of telomerase (HME only). p53 was not significantly degraded in the cell lines expressing the mutant E6 proteins when compared to those lines expressing wild-type HPV-16 E6, and only the wild-

type HPV-16 E6 reactivated telomerase and immortalized the HME cells. The results with the chimeric high/low risk E6 proteins appear to conflict with those generated from the *in vitro* studies, suggesting that the *in vitro* (cell-free) E6 mediated p53 degradation assays may not fully represent the activities of these mutant E6 proteins *in vivo*. In addition, the differences in residual p53 levels between human foreskin keratinocytes and mammary epithelial cells may provide insight into the differences in the susceptibility of those cell types to immortalize using high risk HPVs.

Materials and methods

Human cell lines and culture. Normal human mammary epithelial (HME) cells (HME 31 and HME 32) were obtained from primary cultures and maintained in serum-free medium (MEBM from Clonetics Corp., San Diego, CA) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, CA), 5 μ g/ml insulin (Sigma Chemical Co., St. Louis, MO), 10 ng/ml of epidermal growth factor (Collaborative Research, Bedford, MA), 0.5 μ g/ml hydrocortisone (Sigma), 5 μ g/ml transferrin (Sigma), 25 μ g/ml gentamycin (Sigma) with a change of medium every 2 to 3 days. Uninfected, normal HME cells, depending on the strain, grow for 25 to 50 population doublings (PDL). Human foreskin keratinocytes were maintained in complete keratinocyte growth medium (KGM from Clonetics). C33a cells, a cervical carcinoma line containing mutant p53 and no HPV sequences, were grown in 4:1 mixture of Dulbecco modified medium and Medium 199, containing 15% iron-supplemented calf serum (Hyclone Laboratories, Logan, UT) and 25 μ g/ml gentamycin.

Retroviral vectors and infection. HPV-16 E6 mutation clones, which were supplied by K. Vousden (Ludwig Institute for Cancer Research, London, UK), are E6(Δ 9-13), E6(YYH), 16/6 and 6/16 (Fig. 1) as previously described (18). Each of these mutations was cloned into the parental retroviral vector, pLXSN (a gift of Dr A.D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA), as was the wild-type HPV-16 E6. All of the E6 constructs are under the control of the Moloney murine leukemia virus promoter-enhancer sequences (a gift of Dr D. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA) and contain the neomycin resistance gene for selection. Recombinant viruses were generated and cell lines were infected as previously described (15). Keratinocytes and HME cells were selected on 50 to 100 μ g/ml of G418 for 10 to 14 days, while C33a cells were selected on 400 to 600 μ g/ml G418 for 10 to 14 days. All cells were maintained as populations, and HME cells were infected at 15 to 20 PDLs prior to senescence.

Gel electrophoresis and Western blotting. Proteins were extracted from cultured cells with 1X complete RIPA buffer [50 mM Tris, pH 7.4, 1 mM EDTA, 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS)] containing aprotinin (10 μ g/ml) and PMSF (200 μ M) on ice for 15 to 20 min. Lysates were drawn through a 27-gauge needle to shear DNA and centrifuged for 15 min. The supernatant was removed and protein concentration was quantitated using the BCA Protein Assay

kit as recommended by the manufacturer (Pierce Chemical Co., Rockford, IL). 40 μ g of total cellular protein was separated by 4-12% SDS-polyacrylamide gel electrophoresis using the mini-protein II apparatus (BioRad, Melville, NY) and transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA). The blot was incubated with a mouse anti-p53 antibody (PAb421) followed by incubation with the secondary anti-mouse IgG antibody conjugated to horseradish peroxidase. Detection was done using the enhanced chemiluminescence system as recommended (Amersham, Arlington Heights, IL).

Polymerase chain reaction (PCR). DNA from cultured cells was extracted by addition of lysis buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% SDS, 200 to 300 mM sodium chloride, 150 μ g/ml proteinase K) and incubated for 4 to 18 h at 55°C. DNA was extracted twice with an equal volume of phenol:chloroform mixture followed by one to two extractions with chloroform. DNA was precipitated with 2 volumes of ice-cold ethanol at -20°C for 2 to 4 h, followed by centrifugation to pellet the DNA. DNA pellets were washed with 70% ethanol, air dried for 60 min, and resuspended in TE containing 20 to 50 μ g/ml DNase-free RNase (Sigma). Purified genomic DNA was quantitated by spectrophotometry. Each PCR reaction consisted of 1X Taq reaction buffer, 2.5 mM MgCl₂, 200 μ M dNTPs, 10 pmoles of each primer, 2 units of Taq DNA polymerase (Gibco-BRL, Gaithersburg, MD), and 100 ng of purified DNA in a final volume of 100 μ l. The PCR program started with a 5 min incubation at 94°C, followed by 30 cycles of 1 min at 95°C, 2 min at 50°C, and 3 min at 72°C. The HPV-16 E6 5' and 3' primers, respectively, used for the PCR reactions were: 5'-TTTCAGGACCC ACAGGAGCG-3' and 5'-TTACAGCTGGGTTTCTCTA CGTG-3'. The HPV-6 E6 5' and 3' primers, respectively, used for PCR were: 5'-TTATGGAAAGTGCAAATGCCTCC-3' and 5'-GGGTAACATGTCTTCCATGCATG-3'. PCR amplified DNA was electrophoresed on 1% agarose gels and visualized using ethidium bromide.

Telomerase assay. Detection of telomerase activity in cultured cells is performed in 2 sequential steps: (i) telomerase-mediated extension of an oligonucleotide, which serves as the substrate for the telomerase enzyme, and (ii) PCR amplification of the resultant product with the forward (TS) and reverse (CX) primers, yielding an incremental 6 nucleotide single-stranded DNA ladder. The details for this telomeric repeat amplification protocol (TRAP assay) are discussed elsewhere (4,5). Briefly, for cultured cells, 100,000 cells were pelleted at low speed (6,000 $\times$ g for 6 min) in a sterile microcentrifuge tube, and aspirated cell pellets were quickly stored at -80°C until lysis was performed. Cells were lysed, left on ice for 20 min, and centrifuged at 14,000 $\times$ g for 20 min at 4°C. The supernatant was removed, flash-frozen in liquid nitrogen, and stored at -80°C. For the PCR reaction, an appropriate amount of extract (2 μ l, which corresponds to 1,000 cells) was combined with TS and CX primer, 50 μ M dNTPs, 0.5 μ M T4 gene 32 protein (US Biochemicals, Cleveland, Ohio), 2 μ Ci [α -³²P]dCTP, 5 attograms of a 160 base pair internal standard fragment derived from the human myogenin coding sequence (20), and 2 units of Taq DNA polymerase (Gibco) in a 50 μ l

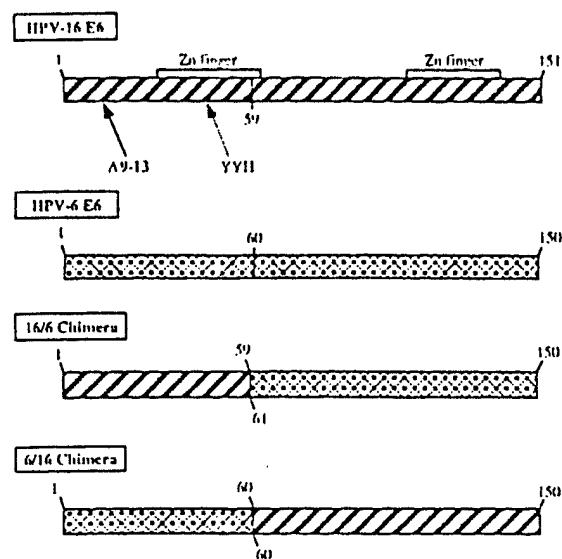


Figure 1. Schematic representation of the HPV-16 and HPV-6 E6 open reading frames and the location of the E6 mutations. The relative location of the HPV-16 E6(Δ 9-13) and E6(YYH) mutations is shown by the arrows. The 16/6 and 6/16 chimeras were made by fusing the amino-terminus of HPV-16 E6 to the carboxy-terminus of HPV-6 E6 and by fusing the amino-terminus of HPV-6 E6 to the carboxy-terminus of HPV-16 E6, respectively. These mutations were provided by Dr K. Vousden (Ludwig Institute for Cancer Research, London, UK) and were subsequently cloned into the retroviral vector, pLXSN. HME cells, keratinocytes and C33a cells were infected with the wild-type or mutant E6 constructs, or pLXSN.

reaction mixture. After incubation at room temperature for 30 min which is necessary for telomerase extension, the reactions were heated to 90°C to inactivate telomerase followed by PCR amplification as described (5). PCR products were electrophoresed on 10% polyacrylamide gels, and the gel was analyzed using the PhosphorImaging System from Molecular Dynamics.

Results

The use of viral oncoproteins to block the tumor suppression functions of p53 and pRb in cultured human cells has been widely established (2,7,9). It has been reported that the amino-terminus of HPV-16 is responsible for the degradation of p53 (18), and that the carboxy-termini of both HPV-16 and HPV-6 E6 proteins are responsible for the binding of p53, implying that these domains can functionally replace each another. To determine if the *in vitro* data reported by Crook *et al* (18) can be extrapolated to an *in vivo* system, we infected defective retrovirus clones containing wild-type E6, both chimeras, E6(Δ 9-13), and E6(YYH) (Fig. 1) into two normal HME cell lines (HME 31 and HME 32), human foreskin keratinocytes, and C33a cells (a cervical cancer cell line containing mutant p53 and no other papillomavirus sequences) (18). *In vitro* studies have suggested that the chimeric E6 proteins (Fig. 1) are both capable of binding p53, yet only the 16/6 chimera mediates the *in vitro* degradation of p53 (18). The 16/6 chimera was made by fusing, in frame, the N-terminal 59 amino acids of HPV-16 E6 to the C-terminal 90 amino acids of HPV-6 E6; likewise, the

Table I. p53 levels effected by HPV-16 E6 mutants.

	pLXSN	16 E6	p53 level per cell ^a		YYH	Δ9-13
			16/6	6/16		
HME 31 ^{b,c}	29.3	1.7	21.7	15.3	17.5	16.1
Keratinocytes	32.9	6.5	43.6	12.7	13.2	10.9
C33a	212.4	208.4	210.8	377.4	173.7	171.3

^aLevels of p53 were quantitated per cell from autoradiographs using densitometry from 3 to 5 independent experiments; ^bVariation between Western analysis of different cell lines was normalized to 20 μg of total protein from an uninfected C33a control cell line; ^cIn all cases except control, 40 μg of total protein were loaded per lane for Western analysis.

p53 Levels Effected by HPV E6 Mutants

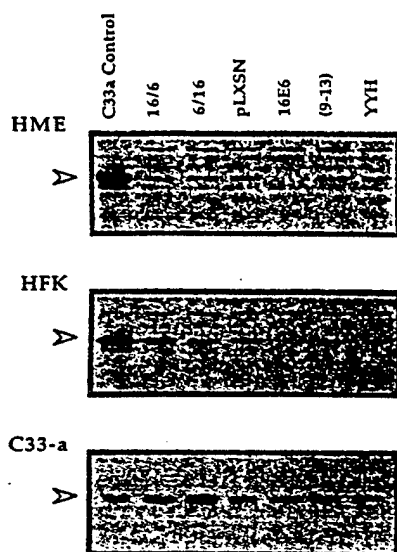


Figure 2. Western analysis of HPV E6-expressing for the presence of p53. 40 μg of protein extract from each cell type was separated on 10% polyacrylamide gels and subjected to Western blotting using PAb421, specific for human wild-type and mutant p53. Each experiment was repeated 3 to 5 times and quantitation was done using densitometry. 20 μg of C33a was electrophoresed on each gel and used to standardize the variation in intensity and quantitation. Arrows indicate the location of p53.

6/16 mutant was constructed by combining the N-terminal 60 amino acids of HPV-6 E6 with the C-terminal 90 amino acids of HPV-16 E6 (18). The E6(Δ9-13) mutant has a deletion of amino acids 9-13, and the E6(YYH) mutant has a 3 amino acid change at amino acid positions 45, 47, and 49, which changes the original amino acids to tyrosine (Y), tyrosine (Y), and histidine (H), respectively. Each of these mutants was tested for their ability to degrade p53 in the various cell types, and for immortalization as determined by reactivation of telomerase in the HMEs.

Degradation of p53 by E6 mutants *in vivo*. After infection, neomycin-resistant, E6 expressing cultures were maintained as populations. Harvested cells were subjected to denaturing electrophoresis and Western blotting using a p53-specific antibody (PAb421). Fig. 2 shows the Western analysis and Table I shows the quantitation results of p53 degradation as

mediated by the various E6 proteins in each of the three cell types used. For purposes of quantitation, 20 μg of a single stock of C33a protein extract was electrophoresed in addition to the extracts from the HPV E6 expressing cell lines for the sole purpose of normalization for variation in densitometric quantitation, in terms of intensity and exposure of each autoradiograph. We found that E6(Δ9-13) and E6(YYH) mutants had lost, to different degrees, the ability to degrade p53. HME cells and keratinocytes containing the chimeric 16/6 protein had p53 levels similar to those without E6 (pLXSN). In contrast, the same cells expressing the reciprocal construct (6/16) had about 50% of the amount of p53 present in cells when compared to control cells without E6 (pLXSN). The effect of HPV-16 E6 on mutant p53 present in C33a cells was essentially unchanged. Interestingly, the residual level of p53 in HPV-16 E6 containing primary foreskin keratinocytes was three times higher when compared to HME cells expressing wild-type HPV-16 E6. These results with the high/low risk chimeric E6 proteins differ from those generated from the *in vitro* studies (18), as no reduction of p53 with 16/6 mutant and a 50% reduction with the 6/16 mutant was observed *in vivo*. This suggests that cell-free p53 degradation assays with these chimeric E6 proteins may not correlate fully with the *in vivo* activities (18).

Detection of integrated HPV E6 constructs. After establishment of E6 expressing cell lines, keratinocytes, C33a, and HME cells were tested using the polymerase chain reaction (PCR) for the presence of integrated proviral plasmids, which would allow expression of the HPV E6 wild-type and mutant proteins. In addition, HME cells were maintained in culture until they either immortalized or became senescent and were also tested by PCR for the presence of the E6 open reading frame. Immediately after selection, the E6 wild-type and chimera constructs were confirmed to be in each individual cell line (data not shown). For the HME cells, DNA was isolated from each cell type at various PDLs, both pre-M1, the period between M1 and M2, and post-M2, and PCR was performed with each to confirm the maintenance of the E6 coding sequences. Using primers specific for the 5' or 3' sequences of HPV-16 E6 and HPV-6 E6 in various combinations, we found: (i) the wild-type E6 was amplified with HPV-16 5' and 3' primers and not with a combination of HPV-16 5' and HPV-6 3' or HPV-6 5' and HPV-16 3' primers; (ii) the 16/6 mutant was only amplifiable with HPV-16 5' and HPV-6 3' primers; and (iii) the 6/16 mutant

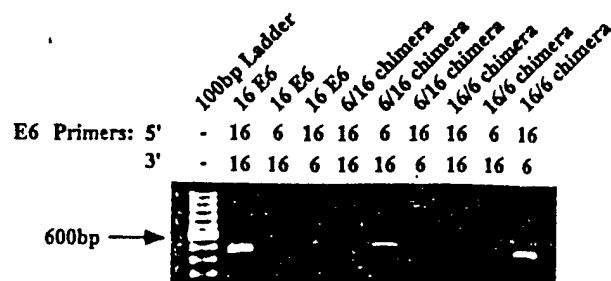


Figure 3. Detection of the wild-type and chimeric HPV E6 constructs in HME 31 cells. Genomic DNA was harvested from HME cells containing wild-type E6, the 16/6 chimera, and the 6/16 chimera at or near their senescence PDL and subjected to PCR using primer sets from HPV-16 E6 and HPV-6 E6. The numbers in the parentheses indicate the 5' and 3' primers used for PCR (i.e. 6 and 16 indicates 5' HPV-6 E6 primer and 3' HPV-16 E6 primer). Primers were designed to yield a 450 bp fragment, visualized on a 1% agarose gel containing ethidium bromide and a 100 bp ladder as a marker.

was only amplified using the HPV-6 5' and HPV-16 3' primers (Fig. 3). While this result was expected, it is further evidence that each of these E6 mutant constructs is stably maintained in the cell lines and that the p53 degradation and telomerase/immortalization data (discussed below) are the result of the activities corresponding to the E6 mutants *in vivo*.

Relationship of p53 degradation and telomerase activity/immortalization in HME cells. It has been previously shown that expression of HPV-16 E6 in HME cells results in the binding and degradation of p53, which allows cells to overcome the M1 mechanism of cellular senescence and eventually immortalize (6,10). We have recently shown that in tumor-derived and virally immortalized cell lines, telomerase is reactivated in greater than 95% of the cases and is the key step in immortalization (4,5). Using the HME cell system that requires only a block of p53, and not pRb, to immortalize, it is possible to assess the biological significance of these HPV E6 mutations as they relate to immortalization and the reactivation of telomerase. Two HME cell strains that contain a normal p53 gene were used: HME 31 cells, which senesce between PDL 45 and 50, and HME 32 cells, which senesce between PDL 25 and 30. The HPV E6 infected cell lines were propagated until they either senesced or until they immortalized.

HME 31 and 32 cells with and without the E6 wild-type and mutants E6 proteins were harvested for use in the PCR-based telomerase assay at varying PDLs. Fig. 4 shows the results of a standard telomerase assay. Human telomerase is an RNA-dependent, processive enzyme that, in this assay, synthesizes an RNase-sensitive (data not shown) 6 base pair ladder consisting of the telomeric repeat sequence TTAGGG (4,5). For the reaction to take place, cell extracts must contain the telomerase holoenzyme. The RNA component of telomerase serves as the primer for telomeric repeat synthesis. The prominent, constant band that migrates at approximately 160 base pairs is the internal TRAP assay standard (ITAS) that serves as a control for quantitation of relative telomerase activity to normalize for sample to sample variation (Fig. 4, all lanes) (20). The HME 31 HPV-16 E6 cell line appears to have increasing telomerase activity as PDL increases (Fig. 4,

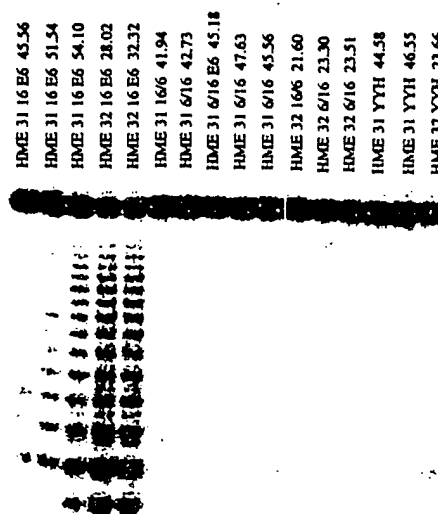


Figure 4. Telomerase activity in HME cells expressing wild-type or mutant E6. Using the PCR-based telomerase assay (4,5), each E6-expressing HME cell line was tested for telomerase activity at different PDLs post infection with each of the retroviral vectors. The 6 bp processive ladder seen is the result of the polymerization activity of the telomerase enzyme and the relatively constant band seen in each lane represents a 160 bp fragment used as an internal standard to quantitate relative amounts of telomerase activity, thus reducing sample to sample variation.

lanes 1-3). This can be explained by the heterogeneity of cell populations, in which only a rare cell immortalizes, and after several population doublings, the population becomes homogeneous for immortalized cells. At this point, cells that did not immortalize become senescent and the now homogeneous, immortalized cells have a relatively constant amount of telomerase activity (within a factor of 2 by quantitation using the ITAS).

We found that for both the HME 31 and HME 32 cell lines, only the expression of wild-type HPV-16 E6 protein (Fig. 4, lanes 1-5), and none of the mutants (Fig. 4, lanes 6-16), resulted in the reactivation of telomerase and immortalization, which is consistent with the p53 degradation results *in vivo* (Fig. 2) but is not consistent with the *in vitro* results obtained by Crook *et al* (18). Also, both the HME 31 cells and the HME 32 cells without infection of retroviral clones or with infection of pLXSN were negative for telomerase activity and eventually senesced (data not shown). Neither the chimeric proteins (16/6 and 6/16) nor the N-terminal HPV-16 mutations [E6(Δ9-13) and E6(YYH)] caused extension of lifespan, reactivation of telomerase, or immortalization of HME cells (summary shown in Table II). These results indicate that the p53 binding and degradation data obtained from the *in vitro* studies using this set of HPV E6 mutants does not correlate with the *in vivo* biologic activity of the mutants in terms of extension of lifespan in culture, telomerase activation or immortalization.

Discussion

High risk HPV E6 protein has been shown to associate with and enhance the degradation of human p53 (16,17). The low risk HPV E6 can associate with p53 to a lesser degree than

Table II. Summary of immortalization and telomerase activity.

Cells/Construct	Beginning PDL	Senescence PDL	Telomerase (+/-)	Immortal (+/-)	PCR confirmation
HME 31	30.1	45.7	-	-	-
HME 31-pLXSN	30.1	46.5	-	-	ND
HME 31-16 E6	30.1	N/A	+	+(PDL 120)	+
HME 31-16/6	30.1	44.4	-	-	+
HME 31-6/16	30.1	48.9	-	-	+
HME 31-YYH	30.1	48.9	-	-	ND
HME 31-Δ9-13	30.1	46.0	ND	-	ND
HME 32	16.4	26.3	-	-	-
HME 32-pLXSN	16.4	25.1	-	-	ND
HME 32-16 E6	16.4	N/A	+	+(PDL 95)	+
HME 32-16/6	16.4	26.7	-	-	+
HME 32-6/16	16.4	23.4	-	-	+
HME 32-YYH	16.4	26.1	-	-	ND
HME 32-Δ9-13	16.4	25.6	ND	-	ND

Abbreviations: PDL, population doublings; PCR, polymerase chain reaction; HME, human mammary epithelial cells; ND, not done; N/A, not applicable.

high risk, but does not mediate degradation (19). Presumably by association with and/or degradation of the tumor suppressors p53 and pRb, HPV-16 E6 and E7 are required for human diploid fibroblast cells to overcome the M1 and M2 blocks to cellular senescence, reactivate telomerase to stabilize telomere length, and eventually immortalize (2,21). We and others have shown that HME cells require only a functional HPV-16 E6 protein to eliminate the wild-type p53 function and eventually immortalize in culture, and is thus an ideal culture system in which to study the effect of HPV E6 mutants on p53 association and degradation as it relates to reactivation of telomerase and immortalization (6,10). It has also been previously shown that the low risk HPV-6 and bovine papillomavirus type 1 (BPV-1) E6 proteins can mediate the immortalization of human mammary epithelial cells (22). However, we have been unable to reactivate telomerase or immortalize HME cells using HPV-6 E6 (unpublished observations). This discrepancy may be explained by the differences in the cell strains used, in that using the HME cell strain 76N (used by Band and coworkers), the HPV-6 E6 protein may be capable of binding a separate and unique set of cellular proteins not found in the HME 31 or 32 cells (6,22). This set of cellular proteins may, in part, contribute to the immortalization event in the 76N cell strain. Yet, the BPV-1 E6 immortalization event is a surprising phenomenon, since it appears necessary to abrogate the normal functions of p53 in order to overcome the M1 mechanism and eventually reactivate telomerase and immortalize. As BPV-1 E6 does not interact with p53, it seems unlikely that expression of BPV-1 E6 in HME cells would allow these cells to bypass M1 and eventually immortalize (23).

In the present report, we describe the effect of specific HPV E6 mutants on the levels of p53 in primary human

foreskin keratinocytes, two normal HME cell strains, and a cervical carcinoma-derived cell line containing mutant p53 and lacking any other HPV sequences (C33a). We have found that wild-type HPV-16 E6 degrades, to varying degrees, p53 in HMEs and keratinocytes and that expression of high risk E6 in HMEs eventually results in extension of lifespan, cell immortalization, and telomerase reactivation. However, chimeras made by fusing the N- and C-terminal HPV-16 E6 with the reciprocal segment of HPV-6 E6 showed no significant decrease in p53 levels in HME cells or keratinocytes for either chimera. In addition, these mutants in HME cells exhibited no extension of lifespan in culture, immortalization or reactivation of telomerase. Similar results were obtained for the E6(Δ9-13) and E6(YYH) mutants. We also found no substantial degradation of p53 in the C33a cell line, which contains only mutant p53, a result that is consistent with the hypothesis that the E6-mediated degradation of p53 is specific for the wild-type p53 conformation.

Previous *in vitro* results with the 16/6 chimera have shown that all of the bound p53 was degraded, suggesting that the E6 domain for degradation was contained within the first 60 amino acids and the p53 binding region was in the carboxy-terminus of the protein (18). Yet, the present *in vivo* results show no substantial decrease in p53 levels in HME cells or keratinocytes containing the 16/6 mutant, indicating that the function of the 16/6 mutant *in vivo* differs from that seen *in vitro*. Crook and coworkers state that the 16/6 mutant only binds to approximately 32% of the p53 when compared to wild-type E6, yet it degrades p53 at wild-type levels. If the 16/6 mutant were able to degrade p53 at near wild-type levels *in vivo*, it seems likely that extension of lifespan in culture and/or reactivation of telomerase and immortalization of HME cells would have been observed. One explanation

for this discrepancy is that the *in vitro* degradation results using the 16/6 mutant do not correlate with the biologic activity found *in vivo* for this 16/6 mutant protein. Another possibility is that p53 is degraded by the 16/6 mutant and that the 16/6 mutant, via some as yet unidentified pathway, is able to induce a feedback stimulation of p53 protein synthesis, whereby p53 turnover remains constant. In addition, it is possible that p53, which interacts with numerous cellular proteins, is protected by its interaction with other proteins *in vivo*. Since the 16/6 mutant does not interact with p53 at or near wild-type HPV-16 E6 levels, the interactions with cellular proteins by p53 could serve to decrease the p53 degradation by the 16/6 mutant by essentially sequestering p53, not allowing the disruption of the p53:cellular protein interaction by the 16/6 mutant.

Using internal deletions of the E6 protein, Crook *et al* (18) were able to map the E6 domain for p53 association to amino acids 96 to 132, approximately 25% of the E6 protein. These data are difficult to interpret in that these deletions are likely to conformationally change the E6 protein, causing the reduction in p53 association. In these *in vitro* studies, they also reported no degradation of p53 by the 6/16 mutant, yet in our *in vivo* assay we found approximately a 50% reduction in the level of p53 for keratinocytes and HME cells. While experimental error cannot be ruled out as a cause of this decrease, it is possible that the domain for *in vivo* degradation of p53 is located in the carboxy-terminus of the HPV-16 E6 protein or that degradation of p53 is due to a specific conformation of E6, not found with some of the deletion mutants or the chimeras *in vivo*. The 6/16 mutant was shown *in vitro* to bind p53 at levels similar to HPV-16 E6 with no detectable degradation of p53 (18). The *in vivo* data shows that the 6/16 mutant is incapable of immortalizing HME cells. Additionally, we have tested the low risk E6 for the ability to immortalize HME cells and found that HPV-6 E6 does not mediate the extension of lifespan in culture, immortalization, or reactivation of telomerase in these cells (not shown). These results taken together indicate that binding of p53 alone by E6 is not sufficient for overcoming the M1 block to cellular senescence, further explaining the reason for the inability of low risk HPV E6 to reactivate telomerase or immortalize HME cells. It is plausible that the binding of p53 by the low risk, non-oncogenic form of the E6 protein could be necessary for benign papilloma formation and that the degradation of p53 induced by the high risk HPV E6 protein could be the cause of augmented growth, reactivation of telomerase, immortalization, and eventual progression to malignant tumor formation.

Our results indicate that the amount of residual p53 remaining in HME cells is significantly less than the amount observed in primary human foreskin keratinocytes. Using *in vitro* assays, Crook *et al* showed that all of the exogenous p53 is degraded upon the addition of wild-type HPV-16 E6 (18). However, detection of the degradation of p53 in HME and keratinocyte cell lines resulted in a substantial amount of residual p53 remaining in the cells. This discrepancy in the degradation of p53 *in vitro* and in HMEs and keratinocytes serves as a possible explanation as to the relative susceptibilities of these different cell lines to the onset of carcinogenesis. In HME cell strains, p53 is reduced to

virtually undetectable levels, which may be why only the addition of HPV-16 E6 is sufficient for HMEs to bypass the M1 block to cellular senescence and eventually immortalize. In the keratinocyte cell line, which needs the expression of HPV-16 E6 and E7 for overcoming M1 leading to immortalization, there remains a substantial amount of p53 in HPV-16 E6-expressing cells. It is important to determine if the residual p53 in both the HME and keratinocyte cell types is functional. If the remaining p53 has wild-type activity, its inactivation may eliminate the requirement for the second oncogene, HPV-16 E7.

Acknowledgements

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Spontaneous In Vitro Immortalization of Breast Epithelial Cells from a Patient with Li-Fraumeni Syndrome

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Individuals with germ line mutations in the p53 gene, such as Li-Fraumeni syndrome (LFS), have an increased occurrence of many types of cancer, including an unusually high incidence of breast cancer. This report documents that normal breast epithelial cells obtained from a patient with LFS (with a mutation at codon 133 of the p53 gene) spontaneously immortalized in cell culture while the breast stromal fibroblasts from this same patient did not. Spontaneous immortalization of human cells in vitro is an extremely rare event. This is the first documented case of the spontaneous immortalization of breast epithelial cells from a patient with LFS in culture. LFS patient breast stromal fibroblasts infected with a retroviral vector containing human papillomavirus type 16 E7 alone were able to immortalize, whereas stromal cells obtained from patients with wild-type p53, similarly infected with human papillomavirus type 16 E7, did not. The present results indicate a protective role of normal pRb-like functions in breast stromal fibroblasts but not in breast epithelial cells and reinforces an important role of wild-type p53 in the regulation of the normal growth and development of breast epithelial tissue.

Familial cancer syndromes with germ line mutations, such as the dominantly inherited p53 mutations present in Li-Fraumeni syndrome (LFS), have helped to illustrate the important role of tumor suppressor genes in the development of human cancers (3, 30, 31, 35, 36, 37, 52). The p53 gene is presently considered to be one of the most frequently mutated genes in human cancer (19, 22, 28, 29, 47, 56, 58), and the functional effects of mutations in evolutionarily conserved regions of the p53 phosphoprotein are currently a subject of intense study (24, 25, 28, 43, 46, 59-63). While a complete understanding of wild-type p53 function is not yet known, it is generally believed that perturbations of wild-type p53 function may lead to genomic instability and permit the expansion of the pool of proliferating cells, which leads to a cascade of additional mutations, increasing the probability of neoplastic transformation. The discovery of the importance of the tumor suppressor gene p53, and the identification of germ line mutations in p53 in LFS-affected families, has led to a growing awareness of the cancer risk to such families. Even though rare bone and soft tissue sarcomas are relatively common in families affected by LFS, other, more frequently occurring forms of cancer other than breast cancer (such as colorectal carcinoma) are not over-represented. Among women in families affected by LFS, breast tumors are the most prevalent cancer (afflicting at least 50%), with 28% of the breast cancers diagnosed before age 30 and 89% diagnosed before age 50 (21, 31, 37). A molecular explanation for the specifically increased incidence of breast cancer, particularly early-onset breast cancer, in families affected by LFS relative to other forms of cancer has not yet been elucidated (20, 41).

We and others have shown that spontaneous immortaliza-

tion of human cells in vitro (a cell culture term for unlimited proliferative capacity of cells) is an extremely rare event (23, 32, 50) requiring alteration or mutations in several genes which are normally involved in the regulation of cellular senescence (16, 42, 57). It has been suggested that cellular immortalization is a critical and perhaps rate-limiting step in the development of most human cancers (18, 50). It has previously been reported (1, 2, 45, 49) that the expression of viral oncoproteins such as large T antigen of simian virus 40 (SV40) and E6/E7 of high-risk strains of human papillomavirus (HPV) can cause human breast epithelial cells to immortalize at a much higher frequency than fibroblasts. In cell culture, while the stromal fibroblasts require abrogation of both p53 and retinoblastoma (pRb)-like functions to become immortalization competent, human breast epithelial cells appear to require abrogation only of p53 (1, 2, 45, 49). In either case, alteration of p53 in breast epithelial cells or p53 and a pRb-like function in breast stromal cells is only the first of two stages that need to be altered for cells to become immortal. While abrogation of this first stage (mortality stage 1 [M1]) generally results in extension of the in vitro life span, a second step, referred to as crisis or mortality stage 2 (M2), represents a condition in which most cells cease proliferation again.

In normal human somatic cells there is a gradual loss of the ends of chromosomes (telomeres), a process known as the telomere end replication problem (8, 14, 17). The loss of telomeric repeats in vitro and in vivo continues during the period between M1 and M2 (8, 48, 49). At M2 the telomeres reach a critically short length, resulting in destabilization of chromosomes and cessation of cell proliferation. It has been proposed that only if telomerase is reexpressed (an enzyme activity that adds DNA hexameric TTAGGG sequences to telomeres) does an immortalized cell line arise out of M2 (8, 9, 26, 46). Once a cell overcomes crisis (M2), telomerase appears to stabilize telomere length and permit indefinite cell division.

This study addresses the molecular basis for the increased frequency of immortalization-competent human breast epithe-

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lial cells, by testing the hypothesis that LFS patient breast epithelial cells containing a germ line p53 mutation would spontaneously immortalize at a relatively high frequency but fibroblasts from the same patient should only rarely immortalize (because of a pRb-like function preventing abrogation of M1). Previously, it was reported that skin fibroblasts derived from members of two separate LFS-affected families immortalized in cell culture at a very low frequency (4). However, efforts to reproduce these findings using the same primary fibroblasts were unsuccessful even though loss of the wild-type p53 allele occurred after long-term culture (33). In addition, Maclean et al. (34) originally were unable to observe spontaneous immortalization of fibroblasts obtained from other LFS-affected families, even though more recently they have succeeded (43a).

MATERIALS AND METHODS

Cells and culture. Primary tumor and adjacent normal tissue samples were obtained from a 31-year-old LFS patient undergoing surgery for breast cancer. The normal breast tissue was enzymatically digested by a combination of hyaluronidase and collagenase to separate breast epithelial and ductal tissue (organoids) from stromal cellular components (primarily adipocytes) (55). After dispersion, organoid clusters were cultured in serum-free medium (53) (MEBM from Clonetics Corp., San Diego, Calif.) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, Calif.), 5 μ g of insulin (Sigma, St. Louis, Mo.) per ml, 10 ng of epidermal growth factor (Collaborative Research, Bedford, Mass.) per ml, 0.5 μ g of hydrocortisone (Sigma) per ml, 5 μ g of transferrin per ml, and 25 μ g of gentamicin (Sigma) per ml to select for growth of epithelial cells (HME50; Fig. 1a). The medium was changed every 2 to 3 days. To select for the growth of stromal fibroblasts (HMS50; Fig. 1b), cells were grown in a 4:1 mixture of Dulbecco modified Eagle medium and medium 199 containing 15% iron-supplemented calf serum (Hyclone Laboratories, Logan, Utah) supplemented with 5 μ g of insulin per ml and 0.5 μ g of hydrocortisone (Sigma) per ml. Epithelial cells were continuously subcultured when near or at confluence, and the cumulative population-doubling level was recorded. Epithelial cells with a typical cobblestone morphology grew out of the organoids in MEBM and expressed cytokeratin 14 (a basal cell marker), cytokeratin 18 (a luminal cell marker), and involucrin (a marker associated with keratinizing squamous epithelium) (55). The epithelial cells growing in these conditions appeared to be from a stem cell population capable of differentiating into a number of different pathways. Breast epithelial cells obtained from milk appear to have more of a luminal cell phenotype (expressing cytokeratin 19), as is the case for the majority of breast tumors, with only a small subset showing some evidence of basal markers (54).

Retroviral vectors and transfection. Retroviral vectors consisted of the parent vector pLXSN (obtained from A. D. Miller) or pLXSN containing the genes for HPV type 16 (HPV16 E6, HPV16 E7, or both (designated HPV16 E6/E7) under the transcriptional regulation of the Moloney murine leukemia virus promoter-enhancer sequences (obtained from D. Galloway). These vectors also contain the gene conferring neomycin resistance under the transcriptional regulation of the SV40 promoters. Recombinant viruses were generated in the amphotrophic packaging line PA317 according to previously described procedures (38). Plasmid DNA was transfected into Psi-2 or PE501 cells by calcium phosphate precipitation. Viral supernatants derived from the Psi-2 cells were used to infect PA317 cells to generate clones containing unrearranged proviral copies of pLXSN, HPV16 E6, HPV16 E7, or HPV16 E6/E7. PA317 clones which had viral titers of approximately 3×10^4 to 5×10^4 PFU/ml (15) were selected on G418 (1 mg/ml). Medium containing released viruses produced from confluent dishes of each clone was filtered (0.4- μ m pore size) and used to infect human mammary epithelial cells and stromal cells as previously described (49). In brief, cells growing in 100-mm-diameter plates were approximately 30 to 50% confluent the day prior to infection. On the day of infection, the medium was removed and replaced with medium containing helper-free viral supernatant (preventing further spread of the vector after initial infection) in the presence of 2 to 4 μ g of Polybrene (Gibco/BRL, Gaithersburg, Md.) per ml. After 12 to 16 h the medium was replaced with fresh medium lacking viral supernatant. The next day the cells were split in a series of dilutions into several plates for isolation of clones and then selected on G418 for approximately 2 weeks (Gibco/BRL). Breast stromal cells were selected on 600 to 800 μ g of G418 per ml in medium containing serum, while breast epithelial cells, which are more sensitive to G418, were selected on 50 to 100 μ g of G418 per ml in serum-free medium. Infection frequencies of 10 to 25% were common, and donor age did not appear to alter this result as long as the cells were replication competent. These frequencies were determined by dividing the number of G418-resistant colonies by the number of colonies growing in the absence of selection.

Mutation analysis. Single-strand conformation polymorphism (SSCP) analysis (40) was used to screen for mutations of the p53 gene in cells and tissues from

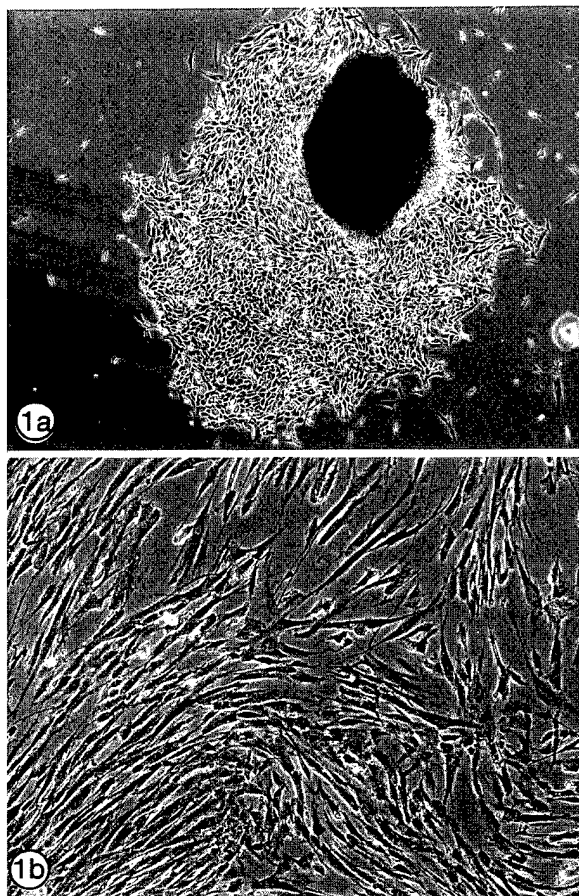


FIG. 1. The breast organoids obtained by overnight digestions with enzymes consist of epithelial, myoepithelial, stromal, and stem cells which are placed either in MCDB170 medium to select for the growth of mammary epithelial cells (a) or in Dulbecco modified Eagle medium-medium 199 with iron-supplemented bovine calf serum to select for the growth of stromal cells (b). Initially all types of cells grow in defined growth medium, but with continuous cell culture the epithelial cells predominate. The ductules of the human mammary gland are lined by a layer of luminal epithelial cells surrounded by a layer of basal or myoepithelial cells. The epithelial cells which grow out from the organoids have a cuboidal, cobblestone-like appearance (a) and are keratin positive (data not shown; see reference 55), whereas the stromal cells (b) are more fusiform, elongated, and vimentin positive (data not shown; see reference 55).

the proband and other patients. Exons 5 to 9 of p53 were PCR amplified with primers flanking coding regions (6). [α - ^{32} P]dCTP was incorporated into the PCR in order to obtain radiolabeled PCR products. Amplified products were treated with formamide, heated to 95°C, subjected to electrophoresis through 5% polyacrylamide gels, and visualized by direct autoradiography. A shift in electrophoretic mobility, suggestive of a change in conformation due to sequence variation, was confirmed by cloning the amplified fragments into M13 vectors and DNA sequencing.

Telomerase assays. The one-tube PCR-based telomerase assay is schematically presented in Fig. 2 and is based on the technique as originally described (26). The assay is performed in two steps: (i) telomerase-mediated extension of an oligonucleotide primer (TS), which serves as a substrate for telomerase, and (ii) PCR amplification of the resultant product (an incremental 6-nucleotide single-stranded DNA ladder) with the oligonucleotide primer pair TS (forward) and CX (reverse).

Details of the method are as follows. For cells in culture, pellet 100,000 cells ($3,000 \times g$ in a 1.5-ml microcentrifuge tube for 6 min [Eppendorf centrifuge]) in culture medium. Carefully remove the supernatant, and quickly store the pellet at -80°C. Washing the pellet is not necessary. Lyse the cells with 200 μ l of ice-cold lysis buffer consisting of 0.5% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM ethylene glycol-bis(8-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10% glycerol, 5 mM β -mercaptoethanol, 0.1 mM AEBF [4-(2-aminoethyl)-benzenesulfonyl fluoride] (ICN Biomedical Inc., Aurora, Ohio), and leave them

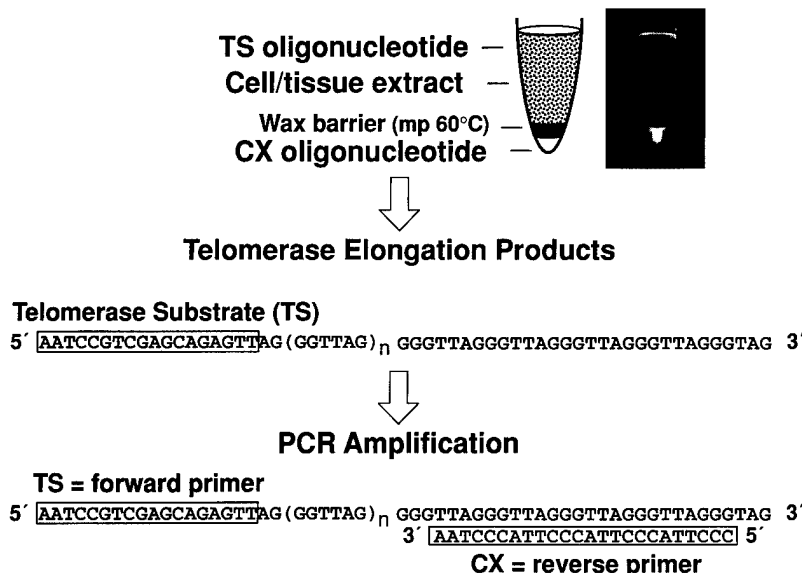


FIG. 2. Diagram of the PCR-based telomerase assay. PCR amplification of telomerase extension products is as detailed by Kim et al. (26). Telomerase synthesizes telomeric repeats [(TTAGGG)_n] onto the nontelomeric oligonucleotide (TS) which serves as a telomerase substrate. Such telomerase products are specifically amplified by PCR using the downstream primer CX [5'-(CCCTTA)₃CCCTAA-3'] and the upstream primer TS. As is illustrated in this figure, a single-tube assay is accomplished by initially separating the CX primer from the rest of the reaction mix by a wax barrier. The CX primer in the photograph in this figure was labelled at the 5' end with fluorescein to illustrate its sequestration below the wax barrier.

on ice for 30 min. Centrifuge the lysate at $16,000 \times g$ for 20 min at $+4^{\circ}\text{C}$. Collect 160 μl of supernatant into an Eppendorf tube, making sure that no traces of cell debris from pellet are withdrawn; flash-freeze the supernatant in liquid nitrogen; and then store it at -80°C . Generally 2 μl of each lysate is analyzed, which is equivalent to approximately 1,000 cells. Modifications of this procedure are required for analysis of primary tumor material. Each tissue sample of 50 to 100 mg of frozen (-80°C) tissue is first washed in ice-cold washing buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH [pH 7.5], 1.5 mM MgCl_2 , 10 mM KCl, 1 mM dithiothreitol) and then homogenized in 200 μl of ice-cold lysis buffer in Kontes tubes with matching disposable pestles (VWR, Vineland, N.J.) rotated at 450 rpm by a drill. After 25 min of incubation on ice, the lysate is centrifuged at $16,000 \times g$ for 20 min at 4°C , and the supernatant is rapidly frozen in liquid nitrogen and stored at -80°C . The concentration of protein is measured with the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, Ill.), and an aliquot of the extract containing 6 μg of protein is used for each telomerase assay.

An appropriate amount of extract is assayed in 50 μl of reaction mixture containing 50 μM each deoxynucleoside triphosphate, 344 nM TS primer (5'-AATCCGTCGAGCAGAGTT-3'), 0.5 μM T4 gene 32 protein (U.S. Biochemicals, Cleveland, Ohio), [α - ^{32}P]dCTP, [α - ^{32}P]TTP, and 2 U of *Taq* polymerase (Gibco/BRL) in a 0.5-ml tube which contains the CX primer (5'-CCCTTACCTTACCCTTACCCTAA-3') at the bottom sequestered by a wax barrier (Ampliwax; Perkin-Elmer, Foster City, Calif.). After 30 min of incubation at room temperature for telomerase-mediated extension of the TS primer, the reaction mixture is heated at 90°C for 90 s to inactivate telomerase and subjected to 31 PCR cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s (Fig. 2). As a control, 5 μl of extract is incubated with 1 μg of RNase (5Prime $\rightarrow$ 3Prime, Boulder, Colo.) for 20 min at 37°C prior to the telomerase assay. The PCR products are electrophoresed on a 10% acrylamide gel as previously described (26). Since human telomerase is processive, during the initial 30 min of incubation in the presence of the TS primer, various numbers of hexameric repeats are added to it and when subsequently amplified yield a 6-bp DNA incremental ladder. Extracts from tissues not containing telomerase do not extend the TS primer (26).

Gel electrophoresis and immunoblotting. Cell extracts were prepared according to published protocols (11) and analyzed for protein concentration (bicinchoninic acid protein assay; Pierce). Proteins were separated in one dimension by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 8% gels with 4% stacking gels using a minigel apparatus (Mini Protean II System; Bio-Rad, Richmond, Calif.). Immunoblotting, incubation, and developing procedures followed the protocol for chemiluminescence detection of proteins (5) as modified by Gillespie and Hudspeth (13). Briefly, after electrophoresis, gels were transferred to charged nylon (Nytran from Schleicher & Schuell, Keene, N.H.) or polyvinylidene difluoride membranes (Immobilon P from Millipore, Bedford, Mass.) and incubated with a primary antibody (anti-p53 clone PAb1801; Oncogene Science Inc., Manhasset, N.Y.) followed by a secondary antibody conjugated to alkaline phosphatase. The blot was then placed in an assay buffer

containing methoxyspirolyl phenyl phosphate for 5 min, blotted on filter paper, and exposed to X-ray film.

Immunoprecipitation procedures were modified from those of Zhang et al. (62, 63). Briefly, treated cells were washed with phosphate-buffered saline (PBS), incubated for 2 to 4 h in methionine- and cysteine-free medium, and then metabolically labelled with 200 μCi of [^{35}S]methionine per ml for 4 h at 37°C in a 5% CO_2 incubator. Cells were rinsed with PBS, placed for 1 h at 4°C in lysis buffer (150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 20 mM Tris-HCl [pH 8.0], 10 mM dithiothreitol, and 2.5 mM phenylmethylsulfonyl fluoride [Sigma]), and immunoprecipitated with anti-p53 antibodies, PAb240, PAb1620, and PAb1801 (Oncogene Science Inc.). Lysates were precleared with Protein-G Plus agarose and immunoprecipitated overnight at 4°C . Samples were then run on an SDS-10% acrylamide gel, dried, and exposed to Fuji X-ray film.

Metaphase spread analysis. Cultures were incubated with 0.01 μg of colcemid (Gibco/BRL) per ml for 4 h. After collection, cells were incubated for 1 h at 37°C in 0.067 M KCl and then fixed in 3:1 methanol-glacial acetic acid. Cell suspensions were dropped onto slides, and the resulting chromosome spreads were stained in 4% Giemsa stain (Sigma). Chromosomes were counted from 25 randomly chosen spreads per clone.

Fluctuation analysis. The frequency of escape from crisis (i.e., immortalization frequency of HME50 clones and HMS50 clones expressing HPV16 E7) was estimated by an approach based on what is essentially a fluctuation analysis as previously described (45, 48). Clones were expanded several population doublings before crisis into multiple series in several sizes of culture vessels at a constant cell density. Each series was subsequently maintained as a separate culture, so that at the end of the experiment the fraction of each series that gave rise to an immortal cell line could be determined. Using different sizes of vessels permitted setup of series which contained a different number of cells per dish while maintaining a constant culture environment (cells per square centimeter). Cultures were split at or just prior to confluence. Once cells reached crisis, they were split at least once every 3 weeks until virtually no surviving cells remained or the culture had immortalized. Stromal fibroblast clones were subcultivated at 6,667 cells per cm^2 , and mammary epithelial cells were subcultivated at 5,000 cells per cm^2 . When too few cells were obtained, all of the cells were put back into culture in a single dish. Mammary epithelial and stromal fibroblasts were considered immortal if they expressed telomerase or if vigorous growth occurred after crisis during two subcultivations in which 1,000 cells were seeded into a 50- cm^2 dish and allowed to proliferate for 3 weeks for each cycle.

Immortalization is expressed as the number of immortal lines per number of culture series. Frequency is expressed as the probability of obtaining an immortal cell line based on the total number of cells plated at each passage (not per cell division) and is calculated by dividing the total number of independent immortalization events by the total number of cells plated. For example, if one maintained nine series at a minimum population size of 10^6 cells per dish, for a total pool size of 9×10^6 , and three immortalization events were observed, this would yield a frequency of 3 divided by 9×10^6 , or 3.3×10^{-7} .

TABLE 1. Spontaneous immortalization of breast epithelial cells obtained from a patient with LFS containing a mutant p53 allele (HME50) but not in breast epithelial cells (HME31 and HME32) containing wild-type p53^a

Clone (n)	p53 alleles	No. of immortalized clones expressing:			
		pLXSN (vector only)	HPV16 E6	HPV16 E7	HPV16 E6/E7
HME50 (9)	+/-	4	ND ^b	ND	ND
HME31 (24)	+/+	0	4	0	7
HME32 (6)	+/+	0	1	0	2
HMS50 (6)	+/-	0	0	2	3
HMS31 (6)	+/+	0	0	0	2
HMS32 (6)	+/+	0	0	0	2

^a Five of the six HMS50 stromal fibroblasts senesced around population doubling 40 to 50, while one clone exhibited extended growth but then senesced at population doubling 68. This extended in vitro growth was not observed in HMS31 and HMS32 stromal fibroblasts. Immortalization occurred in LFS patient HMS50 cells expressing HPV16 E7 and containing mutant p53 but not in HPV16 E7-expressing HMS31 and HMS32 cells which contain wild-type p53.

^b ND, not done.

RESULTS

The frequency of in vitro spontaneous immortalization of an LFS patient's normal epithelial cells (HME50; Fig. 1a) was compared with that of breast stromal fibroblast cells (HMS50; Fig. 1b) derived from the same patient (Table 1). Both LFS-affected (HMS50) and normal (HMS31 and HMS32) stromal cells were infected shortly after isolation with the defective retrovirus (pLXSN) expressing HPV16 E6/E7 (as a positive control for immortalization), HPV16 E7 alone (15), or HPV16 E6 alone or the control vector pLXSN (38) lacking HPV16 inserted sequences (as a negative control) and cultured along with control (untransfected) populations of LFS patient stromal fibroblasts.

The results of these experiments confirmed our hypothesis

that breast epithelial cells from a patient with LFS can spontaneously immortalize (Table 1). While no spontaneous immortalization of the LFS patient control fibroblasts HMS50 (zero of six clones), HMS31 (zero of six clones), and HMS32 (zero of six clones) was observed, we did observe spontaneous immortalization of LFS patient breast epithelial cells (HME50) in cell culture (four of nine cultures) which followed a period of crisis (analyzed positively by the telomerase activity assay; Fig. 2). Breast epithelial cells containing wild-type p53 (HME31 and HME32) did not spontaneously immortalize (0 of 24 and 0 of 6 clones, respectively). Additionally, the immortalization of LFS patient HMS50 fibroblasts expressing HPV16 E7 alone was observed (two of six clones), but that of HPV16 E7-expressing normal breast stromal cells (HMS31, zero of six clones; HMS32, zero of six clones) was not. In these experiments, after infection of the retroviral vector and G418 selection, individual clones were isolated and maintained separately to determine if immortalization occurred. All immortalized clones were thus likely to be of independent origin. While most clones did not immortalize under these experimental conditions, the clones that did immortalize went through a period of crisis that varied in time for each of the individual clones (in some instances lasting several months). The probability of obtaining an immortalization event (in immortalization-competent clones) is generally proportional to the number of cells maintained at the time of crisis.

Figure 3a illustrates the LFS-affected family pedigree along with SSCP data (Fig. 3b) from the primary breast tumor demonstrating a p53 alteration. DNA sequencing (data not shown) confirmed that this alteration was a codon 133 mutation (Met to Thr [M133T]). This same p53 mutation was previously reported (27) in a large LFS-affected family pedigree also characterized by the frequent occurrence of very early-onset breast cancer. It has been reported that while some p53 mutations do not affect the wild-type p53 protein conformation, the p53 mutation M133T does (27, 52). SSCP analysis of DNA from

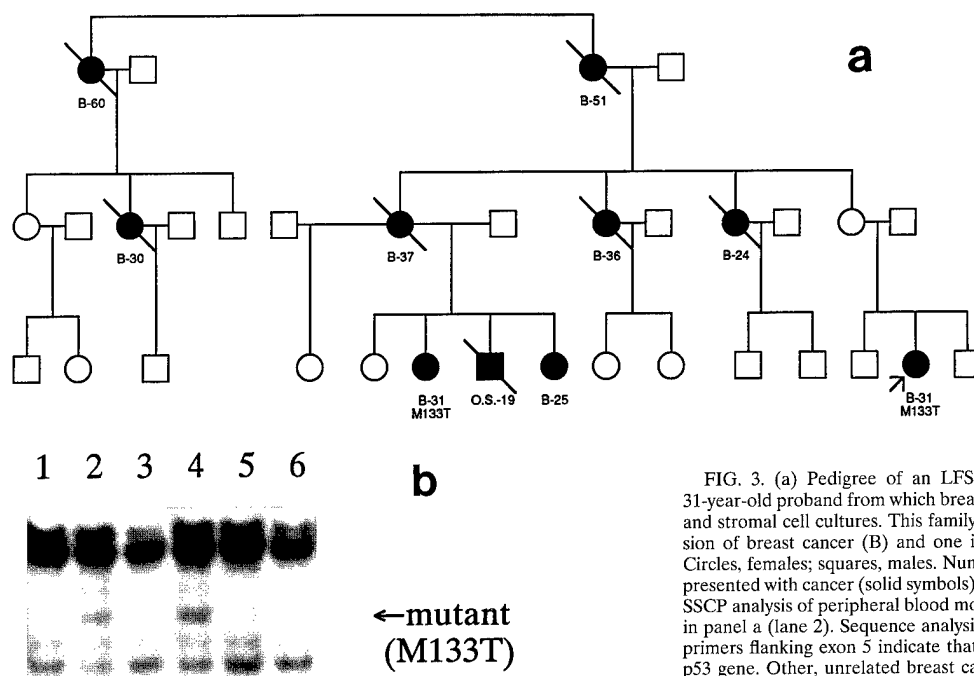


FIG. 3. (a) Pedigree of an LFS-affected family. The arrow indicates the 31-year-old proband from which breast tissue was obtained to establish epithelial and stromal cell cultures. This family has at least three generations of transmission of breast cancer (B) and one individual with osteogenic sarcoma (O.S.). Circles, females; squares, males. Numbers indicate the ages at which individuals presented with cancer (solid symbols); slashes indicate death from the cancer. (b) SSCP analysis of peripheral blood mononuclear cells obtained from the proband in panel a (lane 2). Sequence analysis of PCR-amplified fragments of p53 using primers flanking exon 5 indicate that there is an alteration at codon 133 of the p53 gene. Other, unrelated breast cancer patients (lanes 1, 3, 5, and 6) do not have the p53 alteration, but an additional member of this family does (lane 4).

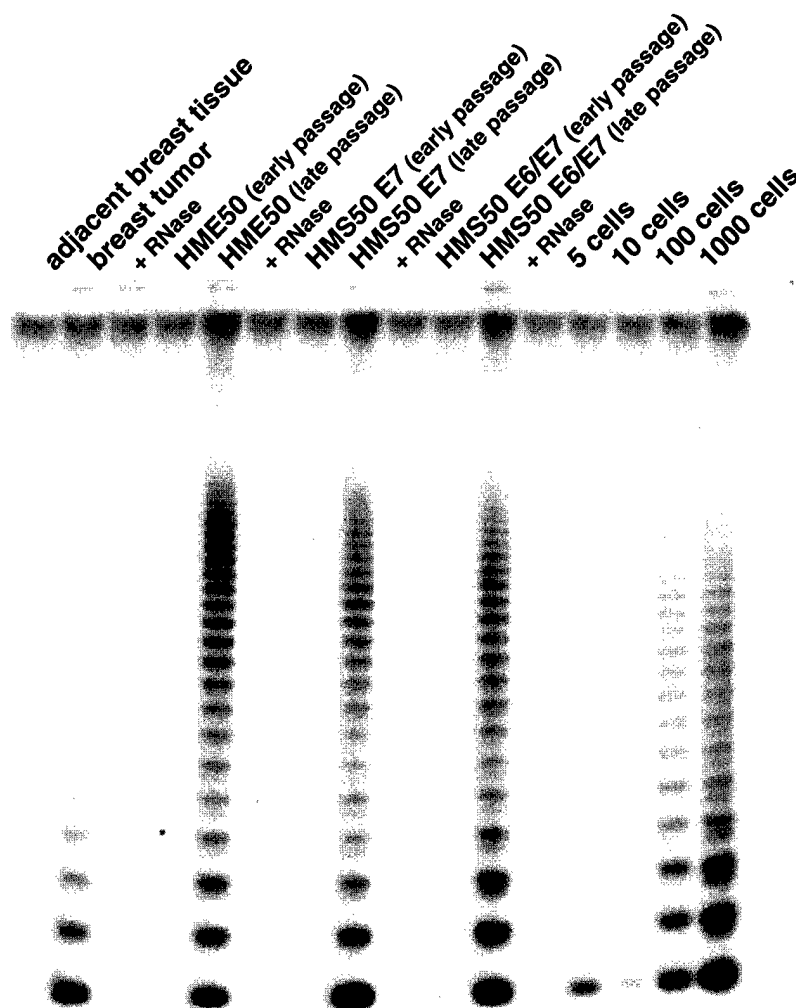


FIG. 4. An assay of telomerase activity using a PCR-based modification of the conventional assay indicates that the normal tissue obtained from this patient does not have detectable telomerase activity (lane 1) whereas the tumor tissue was telomerase positive (lane 2) and RNase sensitive (lane 3). Normal organoid primary explant cultures of both epithelial cells (HME50) and stromal cells expressing HPV16 E7 or E6/E7 (HMS50) were initially negative for telomerase activity (early-passage lanes). After growth in cell culture for several months and escape from crisis, telomerase activity was present (late-passage lanes) and was RNase sensitive (+RNase lanes). The four rightmost lanes are assays of cell equivalents from an established breast tumor cell line.

peripheral blood mononuclear cells (Fig. 3b, lane 4) from affected relatives in this family indicates that this p53 mutation is likely to underlie the high frequency of early-onset breast cancer in this family. Cancer incidence in this family was traced through three generations. Nine of eighteen women presented with breast cancer (the majority before age 40), and one male who presented with osteogenic sarcoma died at the age of 19.

The breast tumor and adjacent normal tissue were analyzed for telomerase activity (an indicator of immortalization) (8–10, 26, 51). Telomerase activity strongly correlates with immortalization events in both cell culture and primary tumors (9, 26). Using a PCR modification (Fig. 2 and reference 26) of the conventional telomerase activity assay (8, 9, 14, 39, 44), we observed that the primary tumor was telomerase positive whereas the normal breast tissue (consisting of both epithelial and stromal cells) was not (Fig. 4). The specificity of this activity is demonstrated by the presence of the hexanucleotide ladder and sensitivity of telomerase to RNase treatment of extracts prior to assay. The organoid explants (both epithelial

and stromal) derived from the normal breast tissue of this patient were telomerase negative (indicating that immortal cells are unlikely to preexist in this normal tissue).

Of nine breast epithelial organoid cultures initially isolated, four have continued to proliferate in culture. All nine cultures underwent a decrease in growth rate resembling crisis in virally transfected cells. The four cultures that escaped crisis continue to grow and express telomerase activity and an increased amount of p53 protein. The frequency of immortalization was approximately 5×10^{-7} for two of the organoid cultures and 1×10^{-6} for the other two organoid cultures. The frequency of escape from crisis was estimated by a fluctuation analysis approach described previously (45, 48).

Initially, all the stromal fibroblasts (i.e., control and pLXSN, HPV16 E6, HPV16 E7, and HPV16 E6/E7 infected) were telomerase negative. After 5 months in cell culture, most of the control stromal cell clones (five of six) slowed down in growth rate between population doublings 40 and 50, appeared to senesce, and remained telomerase negative (Table 1). One of

the stromal cell clones grew until population doubling 68, did not express telomerase, and did not immortalize. However, two of the six HPV16 E7-expressing and three of the six HPV16 E6/E7-expressing HMS50 stromal cell clones immortalized with a frequency of approximately 3×10^{-7} continue to grow vigorously (>100 population doublings) and most have acquired the ability to express telomerase activity (immortalized) that is RNase sensitive. Interestingly, one clone of HMS50 stromal cells expressing HPV16 E7 is presently beyond population doubling 130 and does not express telomerase. Similar results have been observed in some SV40 large T antigen immortalized human fibroblasts (26), but at present we do not have a molecular understanding of this phenomenon. The breast stromal fibroblasts obtained from patients undergoing mastoplasty for hypermastia (HMS32) and for prophylactic mastectomy (HMS31) neither spontaneously immortalized nor immortalized when expressing HPV16 E7 (Table 1). However, they did immortalize when expressing HPV16 E6/E7 (Table 1) or SV40 large T antigen (45, 55).

While it is difficult to accurately quantitate relative telomerase activity levels in each sample, we analyzed extracts from different numbers of cell equivalents of a telomerase-positive breast tumor cell line. The final three lanes of Fig. 4 illustrate that we can detect telomerase activity from as few as 10 to 100 cell equivalents (1 to 10% [by volume] of an extract of 10^3 cells). The primary breast tumor (second lane), though positive for telomerase, has a slightly less processive hexanucleotide ladder than the late-passage immortalized stromal and epithelial cell lines illustrated in Fig. 4. This could be explained by the fact that the primary breast tumor is a mixture of stromal cells (telomerase negative) and epithelial carcinoma cells (telomerase positive). In addition, during the early passages after an immortalization event has occurred in cell culture, there is often a weak telomerase signal which generally increases within several passages (data not shown). While it is possible that expression of telomerase activity increases with time, we believe that it is much more likely that in the early stages after immortalization there are still many mortal (telomerase-negative) cells mixed in the population with a few immortalized (telomerase-positive) cells.

Results of Western blot (immunoblot) analysis of protein extracts from representative epithelial and stromal cells probed with antibodies (recognizing both wild-type and mutant p53 [PAb1801]) are illustrated in Fig. 5. Protein extracts of breast tumor tissue from this LFS patient express more p53 than do those of the adjacent normal breast tissue. The organoid breast epithelial cultures (HME50) initially express low levels of p53 but appear to increase in total p53, a change which is presumed to be due to an increase in the abundance of mutant conformation of p53 as part of the immortalization process in cell culture (as previously reported [12, 33]). This indicates that while the HME50 breast epithelial cells in culture initially contain both mutant and wild-type p53 alleles, the allele containing the wild-type p53 appears to become inactivated (perhaps by mutation of the endogenous wild-type allele, by the loss of the wild-type p53 alleles, or by ectopic expression of a dominant-negative p53 allele). The breast stromal cells (HMS50) transfected with HPV16 E7 also initially express low levels of p53, but similarly to the breast epithelial cells, as part of the immortalization process in cell culture, the mutant p53 protein levels increase. Thus, loss of wild-type p53 function likely permits increased cell proliferation, ultimately resulting in immortalization. With time, the mortal cells stop dividing and/or die while the immortalized cells increase and dominate the population, leading to a stronger telomerase activity signal. We are not sure that there is a second p53 mutation in the

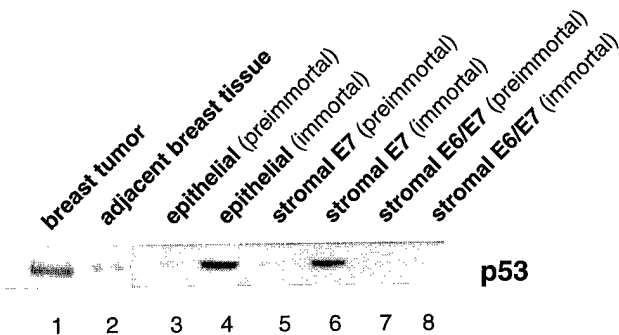


FIG. 5. Western blot analysis of p53 in protein extracts from the tissue and cells obtained from an LFS patient by using PAb1801 antibody, which recognizes both wild-type and mutant conformations of p53. Levels of p53 in the tumor are much higher than in the adjacent normal tissue from which epithelial and stromal cells were obtained. LFS patient epithelial and stromal cells in early passage also have low levels of expression of p53, while the epithelial cells that spontaneously immortalized and the stromal cells expressing HPV16 E7 that immortalized have increased expression of p53. The stromal cells that immortalized with HPV16 E6/E7 do not have increased expression of p53, since the E6 protein of HPV16 facilitates degradation of p53.

primary tumor, since by SSCP analysis the wild-type p53 signal is still present. This is likely due, in part, to the presence of connective tissue stromal cells mixed in with the primary tumor. In order to determine if wild-type p53 was expressed in the immortalized epithelial cells, we metabolically labelled HME50 cells pre- and postimmortalization with [^{35} S]methionine and then immunoprecipitated them using p53 antibodies that recognize wild-type p53 conformation (PAb1620), mutant p53 conformation (PAb240), or both the mutant and wild-type p53 conformations (PAb1801) (7, 61–63). While we could detect both a wild-type and mutant p53 conformation in early-passage HME50 cells (population doubling 22), only a stronger mutant conformation signal in late passage (population doubling >55) cells was detected (data not shown). This indicates that the mutant conformation of p53 increases as part of the immortalization process but does not exclude the possibility that a small amount of wild-type p53 remained undetectable by immunoprecipitation.

From our previous studies (45), we predicted that those clones which were able to remain near diploid were the most likely to immortalize. Chromosome analysis of metaphase spreads indicated that there was a higher fraction of breast epithelial clones remaining near diploid in three clones that spontaneously immortalized compared with three clones that did not spontaneously immortalize (Table 2). This was also true for the LFS patient breast stromal cells expressing HPV16 E7 and E6/E7 (Table 2).

DISCUSSION

This is the first report documenting spontaneous immortalization of human breast epithelial cells obtained from patients with LFS, although it has been reported that skin fibroblast cells from LFS patients can spontaneously immortalize (4). Even though we did not observe spontaneous immortalization of LFS patient stromal cells in the present study, we did observe immortalization of LFS patient stromal cells expressing HPV16 E7. These results indicate that wild-type p53 is important in regulating cellular senescence in breast epithelial cells and also suggest an important role for both p53 and a pRb-like function in the regulation of senescence of breast stromal cells.

Previously it has been reported that LFS patient skin fibroblast cells immortalize spontaneously at a very low frequency

TABLE 2. Chromosome analysis of human mammary epithelial (HME) and human mammary stromal (HMS) cells from an LFS patient

Clone name	Immortalized	Range of chromosomes/metaphase ^a	Median no. of chromosomes	% Diploid metaphases
HME50-5	Yes	44-123	47	60
HME50-8	Yes	38-101	46	68
HME50-9	Yes	41-107	49	55
HME50-3	No	42-99	59	42
HME50-6	No	32-155	75	20
HME50-7	No	37-174	78	36
HMS50-E7-5	Yes	41-113	47	72
HMS50-E7-pop	Yes	38-102	46	84
HMS50-E6/E7-2	Yes	40-133	46	68

^a Based on counts from 25 metaphase spreads.

(4), but this work has been difficult to confirm (33, 34). In a yet unpublished study (43a), stromal fibroblasts of an LFS patient appeared to senesce at 42 population doublings, but after several months of maintenance in the senescent state, cell proliferation which was associated with a loss of the wild-type p53 allele recommenced. One of these clones appeared to spontaneously immortalize even though after an additional 30 population doublings the rest of the clones again ceased proliferation (similar to crisis in SV40-transformed cells) and did not immortalize. These results and those in the present report suggest that wild-type p53 is important in maintenance of DNA stability and that loss of wild-type p53 function may be associated with a breakdown in cell growth control (loss of cellular homeostasis), causing increased proliferation ultimately resulting in immortalization, generally by the reactivation of telomerase activity. While our studies indicate that loss of p53 function may be sufficient to allow breast epithelial cells to immortalize, in the fibroblast lineages loss of p53 function alone may not be sufficient to obtain immortalization, and it is only after the additional loss of a pRb-like function that these cells become immortalization competent. Irrespective, loss of p53 function in breast epithelial cells or p53 and pRb-like function in stromal cells is only the first of two stages that must be overcome for cells to immortalize (46, 50). Thus, it is not surprising that fibroblasts obtained from LFS patients are difficult to immortalize and that even with the complete loss of wild-type p53 immortalization was not observed (33).

The implications of these findings are potentially important, not only because they concern LFS patients' risks of developing cancer but also because they indicate the important role of normal p53 in protecting human breast epithelial cells from immortalizing and progressing to malignant carcinoma. These studies may also provide one reason for the high frequency of breast cancer in LFS-affected families. While alterations in p53 appear to be a central factor for the development of breast cancer in LFS patients, tissue-specific changes (perhaps related to differentiation) are also likely to be important, since LFS-affected families do not have a high incidence of sporadic colorectal cancer although such cancers are associated with a high prevalence of p53 mutations (22). Finally, the spontaneously immortalized breast epithelial cell lines obtained in the present study may be useful in the elucidation of additional critical steps in the development of breast cancer.

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