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

JERRY W. SHAY,[1]* GAIL TOMLINSON,[2] MIECZYSLAW A. PIATYSZEK,[1] AND LAUREN S. GOLLAHON[1]

Department of Cell Biology and Neurosciences[1] and Harold C. Simmons Comprehensive Cancer Center,[2] University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9039

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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 overrepresented. 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 immortalization 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-

* Corresponding author. Mailing address: Department of Cell Biology and Neurosciences, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235-9039. Phone: (214) 648-3282. Fax: (214) 648-8694. Electronic mail address: Shay@UTSW.SWMED.EDU.

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 μg of insulin (Sigma, St. Louis, Mo.) per ml, 10 ng of epidermal growth factor (Collaborative Research, Bedford, Mass.) per ml, 0.5 μg of hydrocortisone (Sigma) per ml, 5 μg of transferrin per ml, and 25 μg 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 μg of insulin per ml and 0.5 μg 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-μm 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 μg 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 μg of G418 per ml in medium containing serum, while breast epithelial cells, which are more sensitive to G418, were selected on 50 to 100 μg 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 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.

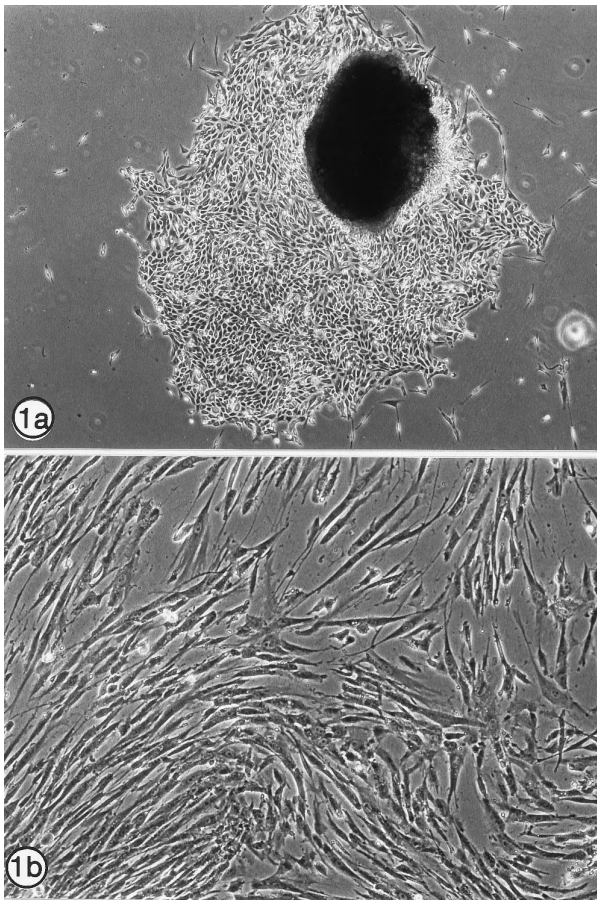

FIG. 1. The breast organoids obtained by overnight digestions with enzymes consist of epithelial, myoepithelial, stromal, and stem cells which are placed either in MCDB170 medium to select for the growth of mammary epithelial cells (a) or in Dulbecco modified Eagle medium-medium 199 with iron-supplemented 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).

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 μl 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 $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10% glycerol, 5 mM β-mercaptoethanol, 0.1 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride] (ICN Biomedical Inc., Aurora, Ohio), and leave them

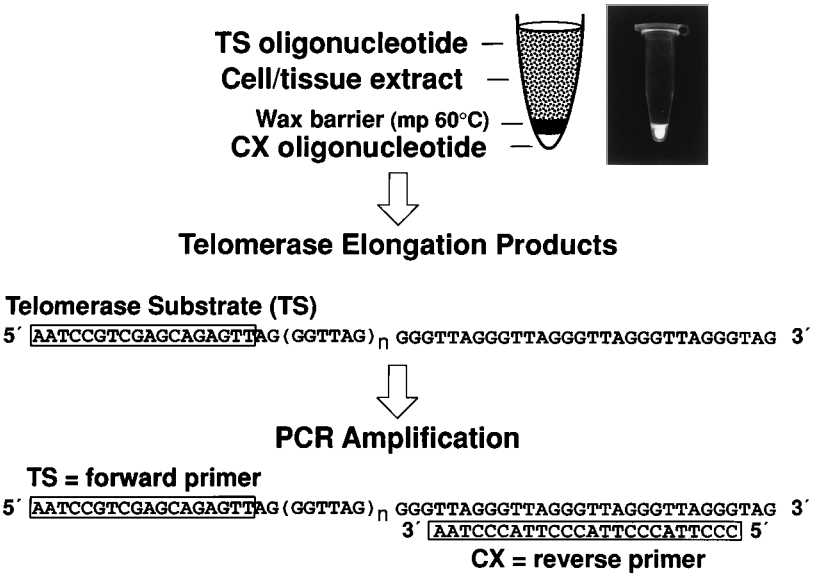

FIG. 2. Diagram of the PCR-based telomerase assay. PCR amplification of telomerase extension products is as detailed by Kim et al. (26). Telomerase synthesizes telomeric repeats [$(TTAGGG)_n$] onto the nontelomeric oligonucleotide (TS) which serves as a telomerase substrate. Such telomerase products are specifically amplified by PCR using the downstream primer CX [5′-$(CCCTTA)_3$CCCTAA-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 × *g* for 20 min at +4°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 × *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′-CCCTTAC CCTTACCCTTACCCTAA-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→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 methoxyspiroyl 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 (pLSXN) 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

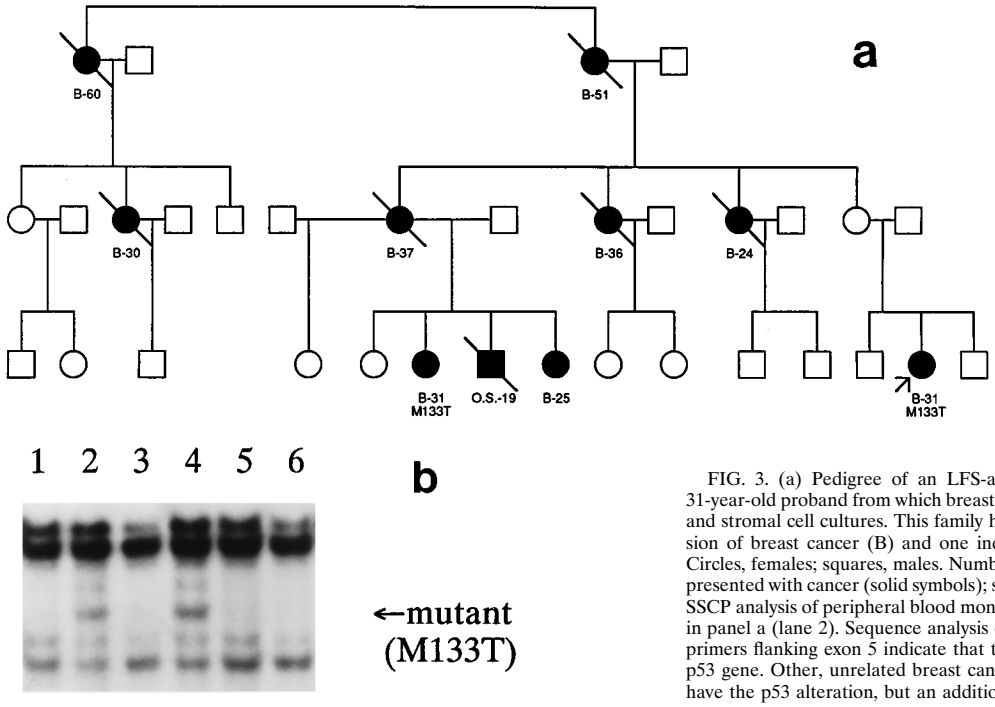

FIG. 3. (a) Pedigree of an LFS-affected family. The arrow indicates the 31-year-old proband from which breast tissue was obtained to establish epithelial and stromal cell cultures. This family has at least three generations of transmission of breast cancer (B) and one individual with osteogenic sarcoma (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).

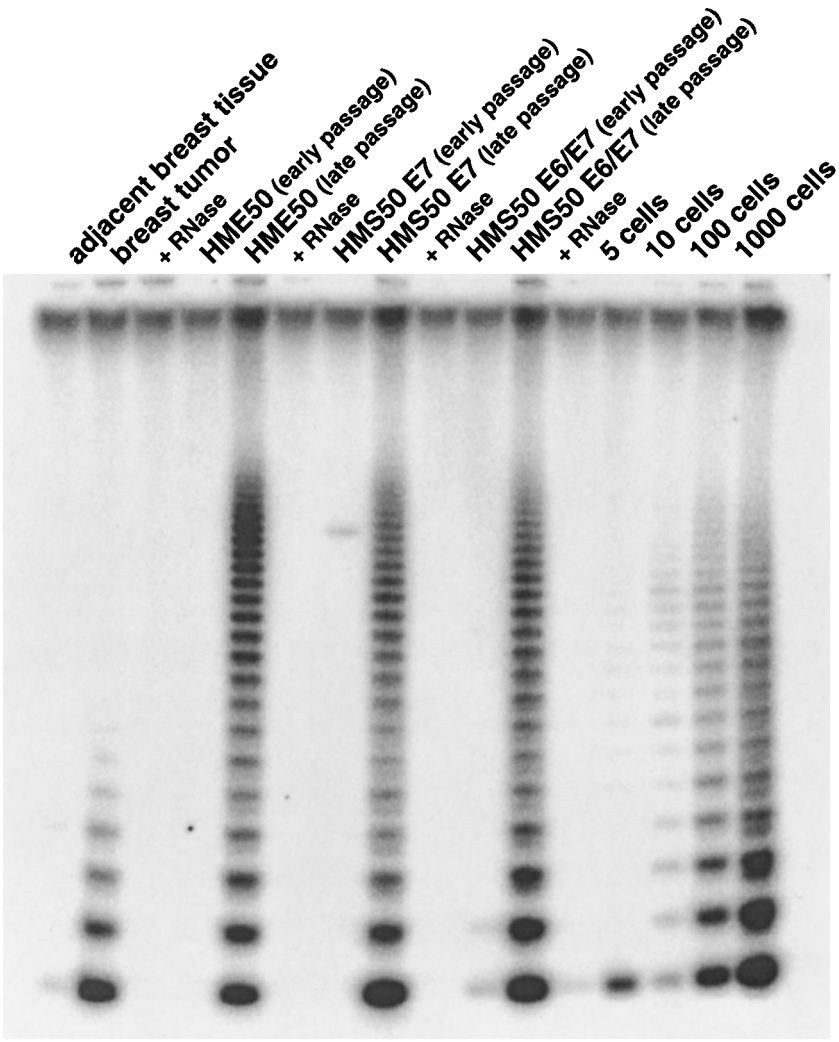

FIG. 4. An assay of telomerase activity using a PCR-based modification of the conventional assay indicates that the normal tissue obtained from this patient does not have detectable telomerase activity (lane 1) whereas the tumor tissue was telomerase positive (lane 2) and RNase sensitive (lane 3). Normal 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 mammoplasty 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 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.

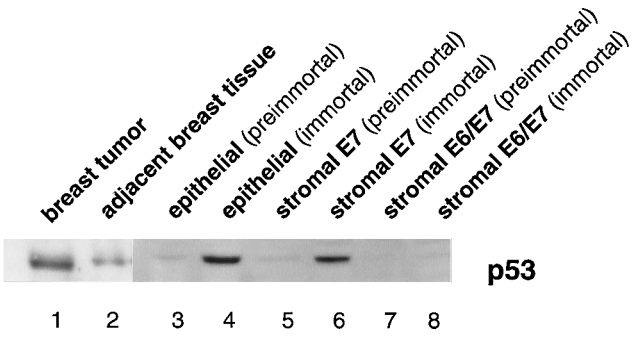

FIG. 5. Western blot analysis of p53 in protein extracts from the tissue and cells obtained from an LFS patient by using PAb1801 antibody, which recognizes both wild-type and mutant conformations of p53. Levels of p53 in the tumor are much higher than in the adjacent normal tissue from which epithelial 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.

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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