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Development and characterization of new immortalized human breast cell lines

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Abstract

New human breast cell lines were developed from metastatic breast cancer tissues and normal breast tissues. Primary cultures were initiated from cellular outgrowths of explanted tissues or from mechanically isolated cells in two serum-free media. Cell cultures derived from both cancer and normal tissues were immortalized with pRSV-T plasmid to generate permanent breast cell lines that exhibited an epithelial morphology. Cell lines generated in this study were characterized with respect to morphology, growth rate, karyotype, presence of specific genes, and the expression of epithelial and breast markers. The cell lines expressed the epithelial cell markers, cytokeratins 8 and 18, and retained the capacity to produce human milk fat globulin. They also express the BRCA-1, erbB2, and EGF receptor genes and possess the H-*ras*, K-*ras*, and p53 genes. Preliminary data showed that one of the new cancer cell lines was highly sensitive to the cytotoxic action of taxol. It is envisioned that the new breast cell lines will be useful as targets for identification of therapeutic agents against breast cancer and as models for carcinogenesis studies.

Abbreviations: BRFF – Biological Research Faculty and Facility; CPDL – Cumulative Population Doubling Levels; EGFR – Epidermal Growth Factor Receptor; FBS – Fetal Bovine Serum; FNC – Fibronectin Collagen; HBS – HEPES Buffered Saline; HMFG – Human Milk Fat Globulin; PBS – Phosphate Buffered Saline; SFM – Serum Free Medium; SC – Subculture; SRB – Sulforhodamine B

Introduction

The availability of well-characterized human breast cell cultures capable of long-term growth will stimulate basic and applied cancer research. Establishment of permanent human breast cell lines has been difficult as evidenced by the relatively small number of such cell lines. To identify the molecular defects which accumulate during the initiation and progression of cancer, it is essential to study the basic biology, physiology, and biochemistry of normal breast cells. It is also important to develop cell lines derived from breast cancer patients of divergent ethnicity and from different stages of the disease to follow the carcinogenic process and to generate various targets for anticancer drug-screening. In this paper, we report the establishment of normal as well as cancer breast epithelial cell lines.

Several breast cancer cell lines have been established during the last 25 years (*inter alia,* Soule et al, 1973; Cailleau et al, 1974; Keydar et al, 1979; Band et al, 1990; Petersen et al, 1990; Kurebayashi et al, 1995; Ethier et al, 1996). All except one (Band et al, 1990) were developed from pleural effusions. A difficulty in establishing permanent cell lines from solid human breast tissue is the intrinsic inability of human cells to divide indefinitely (Hayflick, 1965). Since both normal and neoplastic cells usually have a limited culture life span, many investigators have transfected cell cultures from different organ sites with an 'immortalizing' gene (*inter alia* Kaighn et al, 1989; Stoner et al, 1991; Van Der Haegen and Shay, 1993) to create permanent cell lines. Another difficulty in developing permanent cell lines is the lack of optimal culture conditions (Wolman et al, 1985). The proliferation and differentiation of human breast epithelial cells are reg-

Table 1. Background of human breast tissue. The breast tissues listed in this table yielded cell strains or cell lines

BRFFTissueID	Donor age	Donor race	Tissue description
BRFF049	57	Caucasian	Metastatic
BRFF069[a,b]	55	Caucasian	Metastatic
BRFF071[a,b]	44	Caucasian	Metastatic
BRFF089	57	Caucasian	Metastatic
BRFF110	51	Caucasian	Metastatic
BRFF117	71	Caucasian	Metastatic
BRFF129	67	Caucasian	Metastatic
BRFF130	61	Caucasian	Metastatic
BRFF047	48	Caucasian	Primary cancer
BRFF096	64	Caucasian	Primary cancer
BRFF105	84	Caucasian	Primary cancer
BRFF109[c]	59	Black	Primary cancer
BRFF135	46	Caucasian	Primary cancer
BRFF029[c]	33	Caucasian	Normal tissue from cancer patient
BRFF036	52	Caucasian	Normal tissue from cancer patient
BRFF079	57	Caucasian	Normal tissue from cancer patient
BRFF087	84	Caucasian	Normal tissue from cancer patient
BRFF088	40	Caucasian	Normal tissue from cancer patient
BRFF099	53	Caucasian	Normal tissue from cancer patient
BRFF082[a]	27	Black	Normal tissue from mammoplasty
BRFF097[a,b]	39	Caucasian	Normal tissue from mammoplasty
BRFF098	34	Caucasian	Normal tissue from mammoplasty

[a] These tissues yielded epithelial cell lines after transfection with pRSV-T plasmid.
[b] The epithelial cell lines developed from these tissues are described in this paper.
[c] These tissues yielded fibroblastic cell lines.

ulated by multiple interacting factors. Moreover, there are significant differences between normal and neoplastic cell types in both their media requirements and their tolerance for cell dissociation procedures. Advances in the formulation of culture media (Hammond et al, 1984), tissue fractionation methods, and cell culture procedures (Dairkee et al, 1995) have facilitated development of human mammary epithelial cell lines. Despite these advances, the number of breast cell lines has remained relatively small. It is essential to generate additional breast cancer cell strains and cell lines from patients at different stages of breast cancer in order to understand the disease. To develop new epithelial cell strains, we mainly employed explant cultures, used gentle cell dissociation methods, minimized the use of serum, and applied coating mixtures to the plastic surface to support the attachment of the anchorage-dependant primary cells. In addition, we used experimental transfection of immortalizing genes to circumvent senescence of the cell strains thus yielding permanent epithelial cell lines.

Materials and methods

Human breast tissue

All tissues were received from Ohio State University, and included 19 primary breast carcinomas, 14 metastatic cancers (mostly to the lymph nodes), and 3 normal non-neoplastic samples from reductive mammoplasties. Information about those tissues which yielded cell strains or cell lines is listed in Table 1. Freshly excised tissues in cold transport medium were shipped from the collection center to BRFF for overnight delivery. In some cases, small fragments of cancer tissues were cryopreserved at the collection center and sent to BRFF in dry ice.

Culture reagents

Most reagents were obtained from the Sigma Chemical Company, St. Louis, Missouri. Cell culture media and FBS were from BRFF, Ijamsville, MD. Two serum-free media, BMZERO and BM2, were devel-

Table 2. Serum-free media developed for human breast cell lines. Two serum-free media were developed to establish and maintain human breast cell lines. These media are based on modifications of BRFF-HPC1 medium which was developed to grow human prostate cells (Iype and Kaighn, 1995). The growth factors and hormones in the SFM for breast cells are shown below

Growth factors and hormones	BMZERO	BM2
β-estradiol	Not present	10 nM
Prolactin	Not present	1 μg/ml
Dihydrotestosterone	Not present	0.1 nM
EGF	5 ng/ml	5 ng/ml
Insulin	5 μg/ml	5 μg/ml
Phosphoethanolamine	5.00 μM	5.00 μM
Hydrocortisone	0.28 μM	0.28 μM
Soy bean trypsin inhibitor	10 μg/ml	10 μg/ml
Bovine pituitary extract	40 μg/ml	40 μg/ml

oped by modifying BRFF-HPC1 medium used for growing human prostate cell lines (Iype and Kaighn, 1995). The growth factors and hormones in these two media are listed in Table 2.

Culture method

Primary cultures were initiated by two methods. For the explant method, crosses were made with a scalpel on plastic petri dishes (60 mm Falcon) and the dishes coated with FNC coating mixture (BRFF, Ijamsville, MD). To facilitate proper attachment to the growth surface, the tissues fragments (1–2 mm^3) were placed on the center of the crosses. Approximately 2 ml of cell culture medium was added so that the entire surface of the dish was covered without immersing the tissue fragments. These explants were incubated at 37 °C in a humidified incubator with a gas phase of 5% CO_2 in air. The cellular outgrowth which formed around the explanted tissue was used to establish cell lines.

The second method, a mechanical dissociation method, consisted of plating single-cell suspensions derived from minced tissue. The tissue was washed free of the transport medium, minced and pressed with the flat side of a scalpel to expel the cells. The cell suspension was sieved through a cell strainer (Falcon #2340) and centrifuged (100 $\times$ *g)* for 5 min. The resulting cell pellet was suspended in medium and incubated at 37 °C in a humidified incubator with a gas phase of 5% CO_2 in air. Monolayers from this primary culture were used to establish cell lines. For routine culture maintenance, the appropriate culture medium (see discussion) was changed 3 times per week. All the cell lines exhibited anchorage-dependent growth and required dissociation treatment before subculturing. Monolayers of confluent cultures were resuspended with PET (Lechner and LaVeck, 1985) dissociation medium (BRFF, Ijamsville, MD). The tryptic activity was then inhibited by addition of HBS containing 5% FBS. The cells were collected by centrifugation and resuspended in growth medium and plated on FNC-coated plastic dishes. The cell cultures were routinely checked under a phase contrast inverted microscope and photographed.

Transfection of breast cells with the SV40 T-antigen gene

Cell strains were transfected with the pRSV-T plasmid, which was custom-prepared (with permission from Dr. Bruce Howard) by Bioserve (College Park, MD). The pRSV-T plasmid is an SV40 ori- construct containing the SV40 early region genes and the Rous sarcoma virus long terminal repeat (Reddel, et al, 1988). Transfections with supercoiled pRSV-T DNA (Driscoll et al, 1995) were carried out using a lipofection method. For each transfection, 2.5–5 μg of plasmid DNA in 100 μl TE was mixed with 100 μl of 0.24 mg/ml lipofectamine (Gibco/BRL, Gaithersburg,MD) and incubated at room temperature for 45 min. The DNA-lipofectamine mixture was diluted to 2 ml with the culture medium and then added drop-wise to the culture dish containing either primary explant cultures with epithelial outgrowths, or cell strains. After 6–8 h of incubation at 37 °C, the medium was replaced with regular culture medium and the transfected cultures were maintained using the routine conditions described above.

Nomenclature of cell lines

The nomenclature of the cell lines developed is based on the BRFF Tissue ID number (e.g., the cell line BRF-69 is derived from BRFF Tissue No. 69). Cultures transfected with pRSV-T are identified with the character T after the BRF cell number. Cell lines established from the same tissue under different experimental conditions are identified with a 1 or 2 (e.g., the cell line, BRF71T1).

Growth in soft agarose

Nutrient agarose (0.75%) was prepared by mixing equal volumes of 1.5% Seaplaque agarose (FMC,

Rockland, ME) and a *2x* SFM, P4/8F (Kaighn et al, 1989) supplemented with 10% FBS. A base layer (4 ml) of 0.75% nutrient agarose was poured into 60 mm Petri dishes and allowed to solidify in the refrigerator. The top layer (1 ml) in each dish contained a final concentration of 0.375% agarose, 2.5% FBS, and 10^4 to 10^5 cells. After 10–12 days of incubation, the dishes were stained with 0.1% tetrazolium solution (MTT, Sigma) in PBS for 1 h. Stained three-dimensional colonies were counted under a stereomicroscope.

Karyotype analyses and DNA fingerprinting

These procedures were carried out by Dr. B. Hukku (Children's Hospital of Michigan, Detroit, MI). For trypsin Giemsa banding (GTG), karyotypes were prepared by a modified procedure (Seabright, 1972). A minimum of 7–10 karyotypes were prepared and arranged according to standard human karyotype. Well banded metaphases were karyotyped using the AKSII image analysis system. Genomic DNA samples isolated from the different cell lines and the original breast tissue were used for DNA fingerprinting (Yan et al, 1996).

Genomic DNA isolation and PCR amplification

Genomic DNA was isolated by proteinase K digestion of the cell pellets, phenol-chloroform extraction, and ethanol precipitation. PCR reactions of 50 μl were prepared: deoxynucleotides (200 μM), AmpliTaq DNA polymerase (0.03 unit/μl; Perkin-Elmer, Foster City, CA), PCR buffer (1x), 5′ and 3′ primers (0.4–0.6 μM each), and DNA (1 μg). PCR amplification for the SV40 early region T-antigen gene was performed using published primer sequences and PCR conditions (Driscoll et al, 1995). PCR amplification of codons 12/13 of the H-*ras* oncogene, codons 12/13 of the K-*ras* oncogene, and exon8 of the p53 tumor suppressor gene was performed using Clontech's primer sets (Clontech, Palo Alto, CA) and Clontech's suggested PCR conditions. Identification of the amplified products was performed by loading 20 μl of each PCR reaction mixture onto a NuSieve 3:1 agarose gel containing 0.5 μg/ml ethidium bromide and performing electrophoresis. For SV40 T-antigen PCR products, 2.5% agarose gels were used and for the H-*ras*, K-*ras*, and p53 PCR products, 4% agarose gels were used.

RNA isolation and reverse transcriptase-PCR (RT-PCR)

RNA samples from BRF-69T (at SC 7), BRF-71T1 (at SC10), BRF-71T2 (at SC8), and BRF-97T (at SC4) were isolated using TRIzol reagent (Gibco/BRL, Gaithersburg, MD) and the recommended Gibco/BRL protocol. For RT-PCR reactions, rTth polymerase and 5x EZ buffer (Perkin-Elmer, Foster City, CA) were utilized. RT-PCR of various genes was performed using published primer sequences (cytokeratin 8 and cytokeratin 18 (Traweek et al, 1993); EGFR (Patel et al, 1994); erbB2 (Lonn et al, 1995); BRCA-1 (Gowen et al, 1996)). Reverse transcriptase reactions of 50 μl were prepared: deoxynucleotides (300 μM), rTth polymerase (0.1 unit/μl), EZ buffer (1x), magnesium acetate (2.5 mM), 5′ and 3′ primers (100 ng), and RNA (1–3 μg). RT-PCR conditions for cytokeratin 8 and cytokeratin 18 were as follows: RT reaction of 1 cycle of 60 °C–30 min, 4 °C–5 min and PCR reaction of 94 °C–5 min; 30 cycles of 94 °C–1 min denaturation, 52 °C–1 min annealing, 72 °C–2 min extension; 72 °C–5 min. RT-PCR conditions for BRCA-1, erbB2, and EGFR were as follows: RT reaction of 1 cycle of 60 °C–30 min, 4 °C–5 min and PCR reaction of 94 °C–5 min; 40 cycles of 94 °C–2 min denaturation, 52 °C–2 min annealing, 72 °C–2 min extension; 72 °C–5 min. Identification of the amplified product was verified by loading 20 μl of the RT-PCR reaction mixture onto a 2.5% NuSieve 3:1 agarose gel containing 0.5 μg/ml ethidium bromide and performing electrophoresis.

Indirect immunofluorescent detection of human milk fat globulin (HMFG)

Cells were grown on micro slides (Cat No. 3032, Becton-Dickinson, Lincoln Park, NJ) which were coated with FNC. After 24 h, the slides were rinsed with PBS, fixed with cold acetone for 10 min, air dried and stored for the assay. The cells were rehydrated with PBS and then incubated with 1:100 dilution of the IgG fraction of a rabbit polyclonal antibody against HMFG (Cat. AHP216, Serotec Limited, Oxford, England) for 60 min at room temperature in a moist chamber. The slides were then rinsed 3 times with PBS and incubated with 1:100 dilution of fluorescein-labeled goat anti-rabbit IgG (Sigma) for 60 min. The slides were rinsed 5 times with PBS, mounted with a coverslip and examined under a Nikon fluorescence microscope. Epifluorescence (450-490 nm excitation

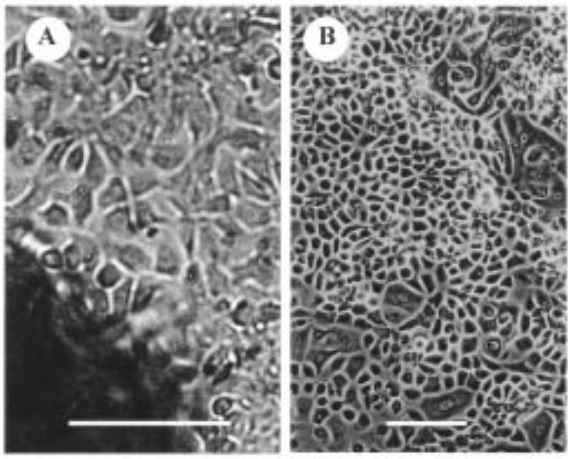

Figure 1. Explant cultures of human breast tissues. (A) Micrograph of an explant culture of BRFF-96 tissue plated in an FNC-coated dish. Cells grew from the tissue, the black area in the lower left section of the micrograph. (B) Micrograph of epithelial cells derived from an explant culture of BRFF-110 tissue. The scale bars represent 100 μm.

filter and 520 nm barrier filter) was used to observe and photograph the cells.

Effect of taxol on the BRF-71T2 cell line

A microassay in 96-well plates was employed using SRB staining of the cells as the endpoint (Skehan et al, 1990). After 7 days in culture with taxol, the cells were fixed directly with cold trichloroacetic acid (50%) for 60 min, rinsed 5 times with tap water, and stained with 0.4% SRB (w/v) for 15 min. The cells were then rinsed 5 times with 1% acetic acid. The plates were air-dried and the dye from dried cells was solubilized with 200 μl of 10 mM Tris base (pH 10.5) for 5 min on a gyrotory shaker. The optical density (OD) of each well was read on a Microplate Reader (MR 600; Dynatech, Chantilly, Virginia) at a wavelength of 550 nanometers.

Results

Establishment of cell lines

The first step in this study was to generate cell strains from tissues *via* an explant method or mechanical dissociation method. Metastatic tissues, primary breast cancer tissues, normal tissues from cancer patients, and normal tissues from reductive mammoplasty patients were processed (Table 1). Most of the explant

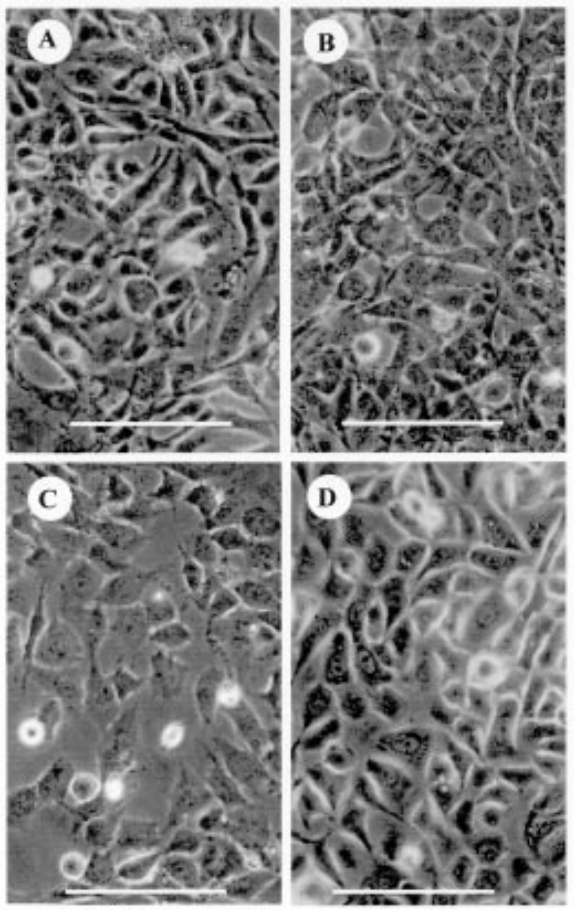

Figure 2. Micrographs of human breast cell lines. Three breast cancer epithelial cell lines, BRF-69T at SC4 (A), BRF-71T1 at SC35 (B), and BRF-71T2 at SC6 (C), and a normal breast epithelial cell line derived from reductive mammoplasty, BRF-97T at SC16 (D), are shown. The scale bars represent 100 μm.

cultures from the fresh tissues maintained on FNC-coated dishes in the appropriate SFM produced cellular outgrowths (Fig. 1). Mechanically dissociated cells also yielded cell strains in SFM on FNC-coated dishes. Primary cells isolated by enzymatic digestion using collagenase and trypsin did not yield subculturable monolayers and cryopreserved breast tissues were unsuitable for generating epithelial cell strains. Therefore, gentle treatment of the tissue was essential to develop cell strains.

The initial tissue samples and cell strains were used primarily to develop suitable SFMs that selected for the growth of epithelial cells. Fibroblastic outgrowths from explants were produced when low levels of FBS were added to the SFM, and therefore, SFM without added FBS was used to initiate epithelial cellular

outgrowths. The important components of the SFMs developed in this study are given in Table 2. The SFM that produced maximal growth of each epithelial cell strain was chosen for its continuous culture.

The second step was to generate *permanent cell lines* from the epithelial cell strains. As in other types of human cells, *all of the cell strains including those from cancer tissues* were found to undergo senescence in the absence of experimental immortalization. Many breast cancer cell strains and normal breast cell strains were cryopreserved (prior to senescence) for future experimentation. To make permanent (immortal) cell lines from cell strains, we chose to transfect the cells with the pRSV-T plasmid which contains the SV-40 early region T-antigen gene (Reddel et al, 1988). Not all transfected cell strains developed into cell lines. Some of the strains senesced after a few SC. We did cryopreserve some of the transfected cell strains prior to senescence. Several permanent cell lines were also generated in this study and some of the immortalized epithelial cell lines are described in detail below. It was relatively easy to produce normal breast cell lines from reductive mammoplasty tissue and normal tissues from cancer patients. We had difficulty developing permanent cell lines from primary breast cancer tissues, although we did develop and cryopreserve some primary cancer cell strains which had been transfected. (These strains may develop into primary breast cancer cell lines in the future). On the other hand, metastatic breast cancer tissues yielded many permanent cell lines after transfection with immortalizing genes.

BRF-69T was derived from a lymph node metastasis. After 35 days in culture, the explants along with the cellular outgrowth were transfected with pRSV-T. The cell line was carried for 11 subcultures. Fig. 2A shows a micrograph of BRF-69T at SC4. Two additional cell lines were developed from a single lymph node explant. BRF-71T1 and BRF-71T2 were derived from two separate transfections of the explant on days 12 and 19 and maintained in culture for 37 and 16 SCs, respectively. Micrographs of BRF-71T1 at SC35 and of BRF-71T2 at SC6 are given in Fig. 2B and C, respectively. BRF-97T was generated from cells isolated by mechanical dissociation of reductive mammoplasty tissue. After 25 days in the flask, the culture was transfected with pRSV-T, and carried for 25 subcultures. Fig. 2D shows a micrograph of BRF-97T at SC16. All cell lines were tested and found free of mycoplasma contamination by the ATCC.

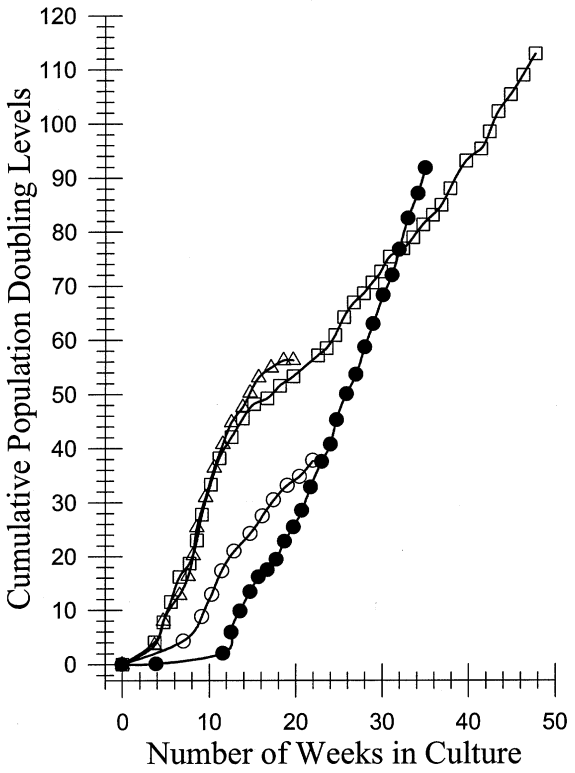

Figure 3. Cumulative Population Doubling Levels of human breast cell lines. BRF-69T (○), BRF-71T1 (□), BRF-71T2 (△), and BRF-97T (●). The number of cells obtained at each SC was used to calculate CPDL. Population doublings were determined by calculating the fold growth of the cell population (cell number yielded/cell number plated in previous SC). The log of the fold growth was then divided by log2 to give the number of population doublings during that period. Each point on the graph represents a subculture.

Characterization of cell lines

Cumulative Population Doubling Levels (CPDL) of the breast cell lines are given in Fig. 3. The cancer cell line, BRF-69T, underwent 38 population doublings in 22 weeks. The cancer cell lines derived from the same tissue, BRF-71T1 and BRF-71T2, had similar cell proliferation patterns as can be seen by the overlapping plots. BRF-71T2 underwent 56 population doublings in 20 weeks and was then cryopreserved. BRF-71T1 underwent 113 population doublings in 48 weeks. The normal cell line, BRF-97T, underwent an initial slow growth phase. There was an 8 week period before SC2 was possible. However, after this time the cells grew rapidly and the cell line underwent 92 population doublings in 35 weeks.

Selected cell lines were tested for their ability to grow in soft agarose. BRF-71T1 produced a small number of 3-dimensional colonies in soft agarose

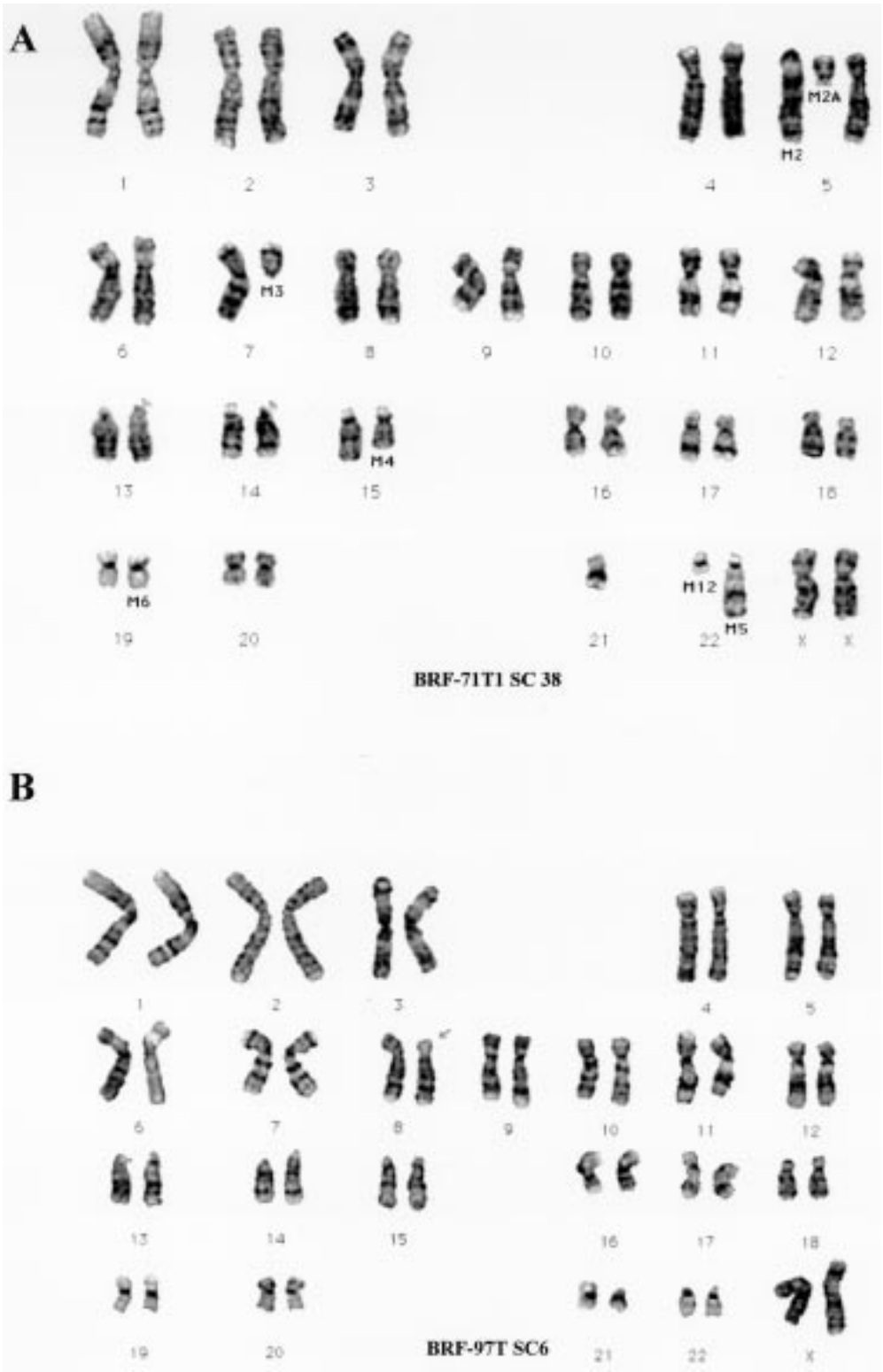

Figure 4. Representative karyotypes from BRF-71T1 at SC38 (A), and BRF-97T at SC6 (B). M indicates marker chromosomes.

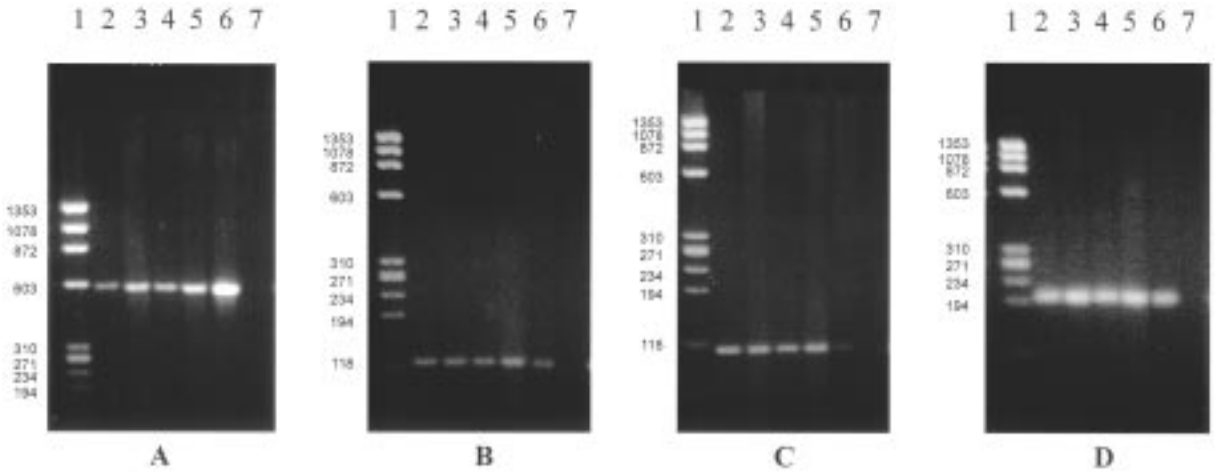

Figure 5. Analysis of PCR products generated from human breast cell lines. For each experiment, a negative control (sterile water) and a positive control (0.2 μg pRSVT plasmid for gel A or 0.5 μg human genomic DNA from Clontech for gels B-D) were included. The molecular weight marker is ϕx174 DNA digested with Hae III (Gibco/BRL). In gels A-D, lanes from left to right are: 1. Marker, 2. BRF-69T, 3. BRF-71T1, 4. BRF-71T2, 5. BRF-97T, 6. Positive Control, 7. Negative Control (A) PCR product for the SV40 T-antigen gene: expected size 580 bp. (B) PCR product for the 12/13th codon of the H-*ras* gene;expected size 123 bp. (C) PCR product for the the 12/13th codon of the K-*ras* gene; expected size 111 bp. (D) PCR product for exon 8 of the p53 gene; expected size 200 bp.

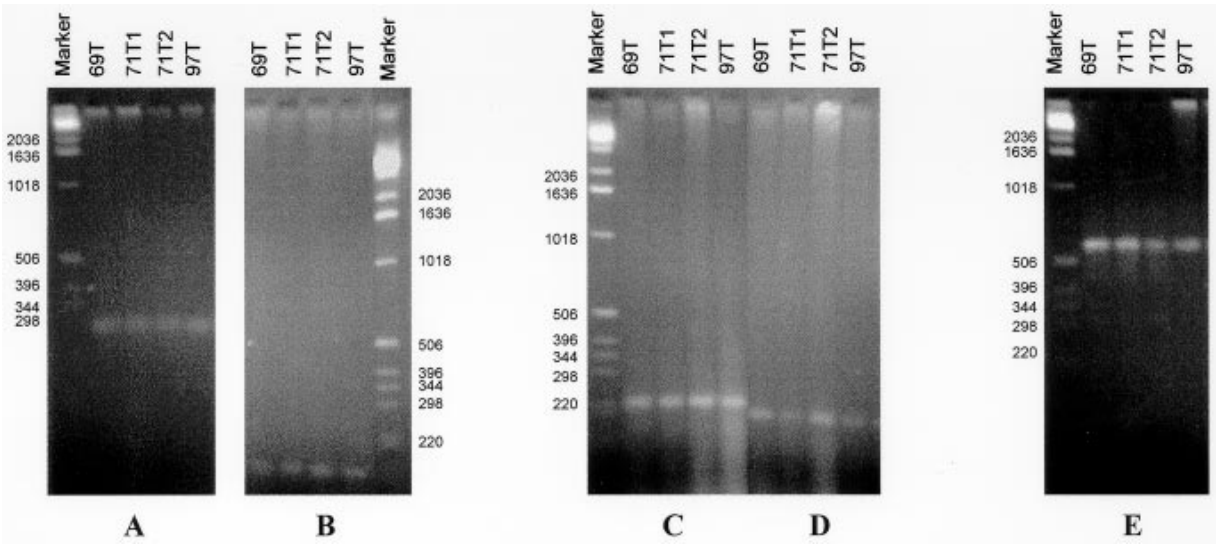

Figure 6. Analysis of RT-PCR products generated from human breast cell lines. In panels A-E, the molecular weight marker is the 1 kb DNA ladder (Gibco/BRL). The expected size of the RT-PCR products are: cytokeratin 8, 277 bp (A); cytokeratin 18, 135 bp (B); erbB2, 217 bp (C); EGFR, 202 bp (D); and BRCA-1 (exon 11), 581 bp (E).

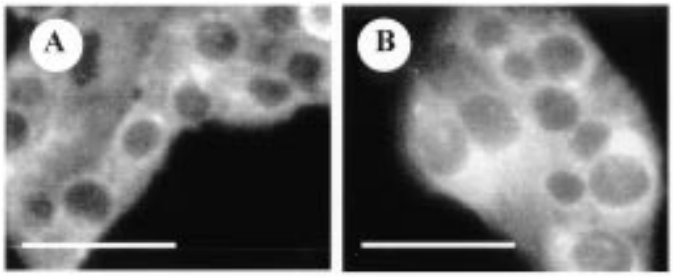

Figure 7. Indirect immunofluorescent detection of HMFG. (A) BRF-71T1 at SC39. (B) BRF-97T at SC9. The scale bars represent 50 μm.

when 1×10^4 cells were plated in the top layer; however, if the number of cells in the top layer was increased to 4×10^4 then a colony forming efficiency of approximately 2% was observed (data not shown). If the number of cells plated was further increased, the colonies formed were too numerous to count. The normal cell line, BRF-97T, at an early subculture (SC6) did not produce any colonies under the same experimental conditions whereas the same cell line at SC 19 did produce a few discernable colonies.

Karyotypic analysis of BRF-69T (at SC4), BRF-71T1 (at SC38), and BRF-97T (at SC6) showed that the Y chromosome (by QM staining) was not present. None of these cell lines were contaminated with other cell lines. Results of ploidy distribution studies from 100 metaphases showed that the cell lines had chromosome counts in the diploid range. BRF-69T and BRF-71T1 contained various marker chromosomes. A karyotype of BRF-71T1which shows some marker chromosomes is presented in Fig. 4A. No marker chromosomes were present in the BRF-97T cell line derived from reductive mammoplasty tissue (Fig. 4B).

The established cell lines were also examined by DNA fingerprinting (Yan et al, 1996). The number of repeats of each of six different fragment length polymorphism (FLP) loci was determined. The new cell lines have fingerprints identical to the tissue used to establish the cell line (data not shown). BRF-69T, the BRF-71T cell lines, and the BRF-97T cell line have *distinctly different* fingerprints. BRF-71T1 and BRF-71T2 share 5 out of 6 FLP indicating these cell lines have the same lineage.

Various techniques were used to further characterize the cell lines. DNA samples from all the transfected cell lines generated the PCR product of the expected size for the SV40 early region T-antigen gene (Fig. 5A) confirming the retention of the immortalizing gene in the cells. Moreover, all samples generated the specific PCR products for two proto-oncogenes, the H-*ras* and K-*ras* genes (Figs. 5B and C), as well as the p53 tumor suppressor gene (Fig. 5D). The RT-PCR results (Fig. 6) showed that the genes for cytokeratin 8, cytokeratin 18, erbB2, EGFR, and BRCA-1 are expressed in these cell lines. The immunofluorescence assay showed the presence of HMFG localized exclusively in the cytoplasm of both BRF-71T1 and BRF-97T (Fig. 7A and B).

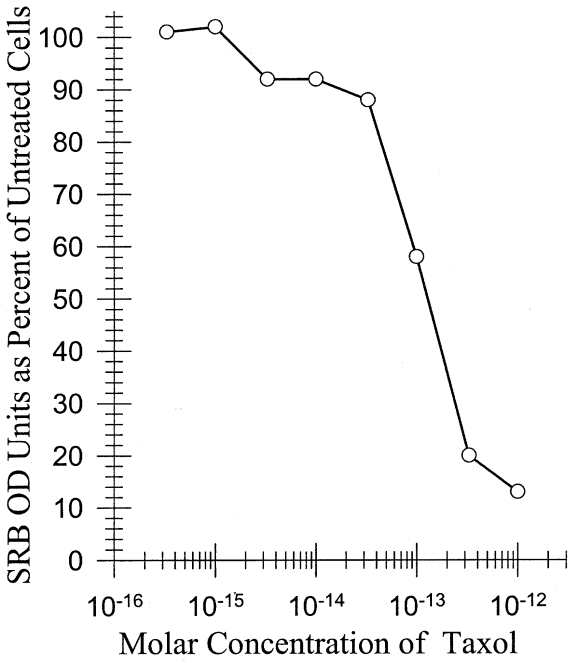

Figure 8. The effects of taxol on BRF-71T2.

Response of BRF-breast cancer cell lines to taxol

The dose response of the classical anti-cancer drugs, taxol (Kodali et al, 1994), used in the treatment of human breast cancer was determined using BRF-71T2 cells as the target cells. Taxol elicited significant cytotoxic effects in BRF-71T2 (Fig. 8).

Discussion

The primary goal of this study was to develop new human breast epithelial cell lines with extended culture potential. The establishment of such cell lines from different stages of breast cancer is a difficult process. We used metastatic tissues, primary cancer tissues, normal tissue from cancer patients, and normal tissue from reductive mammoplasty patients in this study (Table 1). The cell culture requirements for these samples were quite different. We found that normal cell lines and metastatic cancer cell lines were easier to develop than primary cancer cell lines. More research is required in order to understand the culture requirements for primary breast cancer cells. The success rate for developing cell strains from solid human tissues depends on many variables such as the condition of the original tissue, the media used, and the ability of cells

to attach to the growth surface. Using the experimental conditions described in Materials and methods, we could develop cell strains from almost all viable tissues received (as fresh tissue in the transport medium) within 1 to 2 days after removal of the tissue from the body. The success rate of developing permanent cell lines after transfection with immortalizing genes was unpredictable.

Based on information from previous studies by other investigators (Hammond et al, 1984; Stampfer and Bartley, 1988) and in our laboratory (Iype et al, 1993) several SFMs were prepared and tested using the explant cultures of the breast tissue. BRFF-BMZERO and BRFF-BM2 (Table 2), both generated excellent epithelial outgrowths with a predominantly epithelial morphology (Fig. 1) and were used for routine maintenance of the cell cultures. BRFF-BMZERO medium yielded better epithelial cellular outgrowths from explants of normal breast tissue whereas in most cases BRFF-BM2 medium produced better epithelial outgrowths from cancer tissue explants. For proper attachment of the cells to the substratum, the explants as well as the early subcultures required FNC-coated plastic dishes.

Many investigators have used transfection of cell lines with the SV40 T-antigen gene to circumvent cell senescence and to develop continuous cell lines from different normal human cell types (Kaighn et al, 1989; Pfeifer et al, 1989; Stoner et al, 1991). In our study, the normal breast cells as well as the cancer-derived breast cells were transfected with immortalizing genes because cultures that had not been experimentally immortalized *always failed* to develop long-term cell lines. It is important to note that cells from histologically identifiable early cancers need not be immortal when grown *in vitro* and therefore these cells may need to be experimentally immortalized. However, *autonomous* cancer cells such as those obtained from pleural effusions, are already immortal *in vivo* and therefore readily form permanent cell lines without further experimental immortalization. Indeed, most of the classical breast cancer cell lines (Soule et al, 1973; Cailleau et al, 1974; Keydar et al, 1979) as well as recent cell lines (Petersen et al, 1990; Kurebayashi et al, 1995; Ethier et al, 1996) have been derived from pleural fluids. The cancer cell lines developed in our study were initiated from solid lymph node metastasis.

There were subtle differences in the growth requirements of the cell lines developed. BRFF-BM2 containing the hormones β-estradiol, prolactin, and dihydrotestosterone was found to produce better cell growth in the three breast cancer lines. Indeed, breast cancer cells are known to have androgen receptors (Kuenen-Boumeester et al, 1996) and therefore, the inclusion of dihydrotestosterone in BM2 may have enhanced the growth of the cancer cell lines. However, the normal cell line, BRF-97T grew well in medium without any of these components (BMZERO).

Both the cancer and normal cell lines exhibited an epithelial morphology (Fig. 2), and expressed cytokeratin 8 and cytokeratin 18 (Fig. 6) which are epithelial cell markers (Jing et al, 1996). Specifically, luminal epithelial cells express cytokeratin 18 whereas basal epithelial cells express cytokeratin 14 (Taylor-Papadimitriou et al, 1989). All of the immortalized cell lines developed in this study are similar to the luminal epithelial cells since they express the cytokeratin 18 and cytokeratin 8 genes as tested by RT-PCR (Fig. 6).

Growth in soft agarose was observed in all cancer cell lines and in a late subculture of the normal cell line, BRF-97T, but not in the early culture of BRF-97T. Varying the percentage of agarose, the percentage of FBS, and the hormonal composition in soft agarose may generate more distinct differences in the anchorage-independent growth characteristics of the cancer and the normal cells. A preliminary experiment has shown that none of the cell lines formed soft agar colonies in the complete absence of FBS.

Karyotype analysis has shown that all cell lines are aneuploid female with most chromosome counts in the diploid range. At the early subcultures no marker chromosomes were seen in the normal cell line, BRF-97T, whereas the cancer cell lines contained a variety of marker chromosomes (Fig. 4). Different types of chromosomal abnormalities (Bièche et al, 1996) are reported in breast cell cultures derived from carcinomas. Further studies are required in order to assess whether the newly developed breast cell cultures possess these chromosomal abnormalities and to assess the karyotypic alterations, if any, as a function of the CPDL. DNA fingerprinting analysis of the cell lines showed that each cell line and the original tissue used to develop the cell line possessed identical DNA fingerprints. Therefore, there was no cross-contamination of the newly developed cell lines. Moreover, mycoplasma was not present in any of the cell lines.

The cell lines have also been characterized with respect to the presence or absence of important genes known to be associated with the carcinogenic process (the H-*ras* gene, K-*ras* gene, and p53 tumor suppressor gene). Alterations of proto-oncogenes and tumor

suppressor genes play a key role in cancer progression, and mutated oncogenes and suppressor genes are present in mammary tumors (Ozbun and Butel, 1995; Telang, 1996). All of the cell lines contain the H-*ras* and K-*ras* genes as well as the p53 gene (Fig. 5). (Determination of possible mutation in these genes was beyond the scope of this study). The cell lines were also characterized with respect to the expression of genes which may play a role in breast carcinogenesis – BRCA-1 (Castilla et al, 1994), erbB2 (Lonn et al, 1995), and EGFR (Klijn et al, 1992). All of the cell lines tested did express these markers (Fig. 6). We also examined the expression of human milk fat globulin in BRF-71T1 and BRF-97T and found that they retained this important breast-specific differentiated function (Fig. 7).

An experiment was performed to test the usefulness of the newly established breast cancer cell lines for detecting anti-cancer agents in an SRB assay (Skehan et al, 1990). Taxol, used extensively for breast cancer treatment, was found to elicit a highly significant cytotoxic effect on the BRF-71T2 cell line (Fig. 8). To obtain 50% killing of BRF-71T2 only about 1×10^{-13} M of taxol was required. In a study using the T47D breast cancer cell line (derived from pleural effusion) the 50% killing dose was reported to be 2×10^{-8} M (Kodali et al, 1994). The lower concentration of taxol required to produce an equitoxic effect on the BRF-71T2 cell line indicates that this cell line may be a more sensitive target cell for screening anticancer drugs. This preliminary experiment suggests that the BRF-71T2 cell line as well as others established in this study may help to facilitate the discovery of new therapeutic agents for breast cancer. If the toxicity results presented here can be validated in BRF-71T2 using a number of known antineoplastic drugs and appropriate controls, the BRF-71T2 cell line may turn out to be a good candidate for inclusion in the current NCI Cancer Cell Panel (Monks et al, 1991) used for cancer drug discovery.

We have succeeded in generating several immortalized breast cell lines from metastatic cancer and reductive mammoplasty. The new cancer cell lines may be useful as targets for the identification of therapeutic drugs against breast cancer. The new normal cell line may be useful as an *in vitro* model for studying the effects of hormones and carcinogenic agents. In addition, the experimental conditions described in this study were instrumental in the development of breast cell lines and these conditions may be useful to other investigators in the establishment of new normal and breast cancer epithelial cell lines.

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