

Isolation and Characterization of a Spontaneously Immortalized Human Breast Epithelial Cell Line, MCF-10¹

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ABSTRACT

Two sublines of a breast epithelial cell culture, MCF-10, derived from human fibrocystic mammary tissue exhibit immortality after extended cultivation in low calcium concentrations (0.03–0.06 mM) and floating transfers in low calcium (MCF-10F), or by trypsin-Versene passages in the customary (normal) calcium levels, 1.05 mM (MCF-10A). Both sublines have been maintained as separate entities after 2.3 years (849 days) *in vitro* and at present have been in culture for longer than 4 years. MCF-10 has the characteristics of normal breast epithelium by the following criteria: (a) lack of tumorigenicity in nude mice; (b) three-dimensional growth in collagen; (c) growth in culture that is controlled by hormones and growth factors; (d) lack of anchorage-independent growth; and (e) dome formation in confluent cultures. Cytogenetic analysis prior to immortalization showed normal diploid cells; although later passages showed minimal rearrangement and near-diploidy, the immortal cells were not karyotypically normal. The emergence of an immortal culture in normal calcium media was not an inherent characteristic of the original tissue from which MCF-10 was derived since reactivated cryopreserved cells from cultures grown for 0.3 and 1.2 years in low calcium were incapable of sustained growth in normal calcium.

INTRODUCTION

A reliable source of normal HMEC² is required to delineate sequential development of differentiated or malignant states. Recently devised culture techniques and media have permitted the long term cultivation of normal HMEC (1–3). Nevertheless, the establishment of immortal human mammary epithelial cell lines of nonmalignant origin has been rare; two such lines were derived from patients with tumors (4, 5), two others contain SV40 genetic information [one of which was transformed with SV40 (6–8)], and two lines were established after exposure to benzo(a)pyrene (9). It has been reported recently (10) that normal mammary epithelium was cultured for 10–20 passages in an improved medium. There are no reports of immortalization occurring in cultures of untreated normal human breast epithelium. Senescence usually occurs at passages 18–20 of serially transferred cells (11).

It has been demonstrated that decreasing the Ca²⁺ concentration in culture media will increase the *in vitro* longevity of normal epithelial cells, particularly with epidermal (12), bronchial (13), and esophageal (14) cells. We have shown previously the increased viability of normal human mammary epithelial

cells in low Ca²⁺ (<0.04 mM) medium (15). The epithelial and mammary characteristics of these long-term cultures (>1000 days) in low Ca²⁺ was established by the antiserum reactions with keratin and epithelial membrane antigens (16).

The present study reports the establishment of an immortal cell line that arose spontaneously, without viral or chemical intervention, from mortal human diploid mammary epithelial cells of extended life span (>1.2 years). The diploid mortal cells (MCF-10M) senesce when transferred serially in 1.05 mM Ca²⁺, whereas the immortal line, designated MCF-10A (attached cells) and MCF-10F (floating cells) has proliferated for more than 4 years in medium either with the customary Ca²⁺ concentration (1.05 mM) or in low Ca²⁺ medium (0.04 mM).

MATERIALS AND METHODS

Tissue. Breast tissue was obtained from a s.c. mastectomy performed on a 36-year-old parous premenopausal woman with no family history of breast malignancy. The breast histopathological diagnosis was extensive fibrocystic disease, consisting of increased mammary fibrous stroma containing numerous dilated mammary ducts, benign apocrine metaplasia, and small focal areas of intraductal hyperplasia with no evidence of atypia. The patient is free of disease. Procedures for cultivation of breast epithelial cells have been published previously (3, 15). Briefly, s.c. mammary tissue (identified as MCF-10M) was digested with collagenase and hyaluronidase as described elsewhere (1). Organoids obtained from the digestion mixture were washed by repeated unit gravity sedimentation in medium with 5% equine serum and plated at 1×10^5 cells/cm² in T₇₅ flasks (Corning Glass Works, Park Ridge, IL).

Media. Medium containing the customary Ca²⁺ concentration (1.05 mM) was prepared by mixing equal amounts of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (GIBCO, Grand Island, NY) containing L-glutamine and 4.5 g glucose/liter. Sterilization of this medium (designated DMEM-H) by filtration through 0.22 μm Millipore filters was followed by the addition of 5% equine serum (GIBCO).

LCM consisted of DMEM-H prepared *de novo* without CaCl₂, utilizing components purchased from Sigma Chemical Company, St. Louis, MO. This medium was sterilized by filtration and supplemented with 5% equine serum which had been treated with Chelex 100 (Bio-Rad Laboratories, Richmond, CA) to remove divalent ions. Calcium was added from a 28.5 mM sterile solution of CaCl₂ to a final concentration of 0.03, 0.04, or 0.06 mM depending on the experiment.

SFM consisted of the mixture of Dulbecco's and Ham's F-12 supplemented with fetuin (1 mg/ml) and transferrin (5 μg/ml; Sigma) with various levels of Ca²⁺.

The three media were supplemented with antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml; and amphotericin, 0.25 μg/ml), insulin (10 μg/ml), cortisol (1.4×10^{-6} M; Sigma), cholera enterotoxin (100 ng/ml; ICN Biomedicals, Cleveland, OH), and epidermal growth factor (20 ng/ml; Collaborative Research, Bedford, MA).

CM was prepared by centrifuging the culture containing floating cells (1–3 days after medium change) for 5 min at 200 g (23°C). This supernatant was decanted and used as CM in mixtures with fresh media containing identical constituents.

Cell Culture. Mortal MCF-10 cells were transferred by plating pel-

Received 11/27/89; accepted 6/12/90.

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¹These investigations were supported in part by NIH Grants CA28999, CA36399, and CA38921 and institutional grants to the Michigan Cancer Foundation from the United Foundation of Greater Detroit and from Abbott Laboratories. Cell characterization studies were carried out under National Cancer Institute Contract N01-CP-21017 to Children's Hospital of Michigan.

²The abbreviations used are: HMEC, human mammary epithelial cells; DMEM-H, Dulbecco's modified Eagle's medium-Ham's F-12 medium containing L-glutamine and 4.5 g glucose/liter; LCM, low Ca²⁺ medium; SFM, serum-free medium; CM, conditioned medium; FFC, free-floating cells; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; EGF, epidermal growth factor; AIG, anchorage-independent growth; CE, cholera enterotoxin.

leted FFC in fresh medium and CM (1:1). FFC of MCF-10F were plated by suspending pelleted cells in fresh medium and CM (2:1), or in some cases CM was omitted. MCF-10A cells were suspended for transfer after treatment with Versene (0.025%) and trypsin (0.05%) in a Ca²⁺-Mg²⁺-free balanced salt solution and then plated in fresh medium. The total number of days (d) *in vitro* was recorded by adding this number to the name of the subline, MCF-10M, F or A. Total and viable cell counts were determined by hemocytometer following trypan blue staining.

Chromosome and Isoenzyme Profile. The isoenzyme profile was performed on MCF-10A(109d) (17, 18). Chromosome analyses with trypsin-Giemsa and quinacrine banding were performed with modifications of published techniques (19). The following cultures were evaluated: MCF-10A(109d) (reactivated from the freezer), 1229d, 1359d, and 1527d; MCF-10F(920d) (reactivated from freezer), 1422d and 1524d; MCF-10M 350d and 459d (reactivated from freezer) and maintained in LCM and a lymphocyte culture from the patient who supplied the breast tissue from which the MCF-10 cell lines were derived.

DNA Isolation and Mapping. Genomic DNA was prepared from MCF-10 cells at the specified days of *in vitro* culture, from the patient's lymphocytes, from lymphocytes of other individuals, from breast tissues of other patients, or from the human breast cancer cell lines MCF-7, BT-20, or SKBR-3 (American Type Culture Collection), using an RNase and proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation protocol (20).

For restriction mapping, 5 µg of cellular DNA were digested to completion with *EcoRI*, *BamHI*, or *HindIII*, fractionated through a 0.7% agarose gel, and transferred to Biotrace RP (Gelman Sciences) as described (20). For mapping minisatellite sequences, 0.5 µg of cellular DNA was digested with *HaeIII* and fractionated through a 1% agarose gel. Phage λ-*HindIII*-digested DNA was used as a molecular size marker. Conditions for hybridization with 1 ng probe/ml for stringent washing and for autoradiography were as described (20). Hybridization probes, radiolabeled by the random priming method (21), were restriction fragments purified by size fractionation through a 1.2% low melting agarose gel. The 309-base pair *HaeIII* fragment of phage M13mp8 was used as a probe for detection of human moderately repetitive hypervariable minisatellites (22). Other hybridization probes were the 1.6-kilobase *EcoRI* insert of the human highly polymorphic epithelial-urinary mucin (*PUM*) complementary DNA clone pMUC10 (23), a 3-kilobase *HindIII-KpnI* fragment from the human *c-erbB-2* complementary DNA clone CER-204 (24), the 0.9-kilobase *SstI* or 1-kilobase *BamHI-KpnI* inserts of the human *int-2* genomic clones SS6 and BK4, respectively (25), a 2.9-kilobase *EcoRI-HindIII* fragment from the human *c-erbA-1* 3.9-kilobase genomic *EcoRI* clone HEA-1 (26), the 2.8-kilobase *HindIII* insert of the human *int-1* genomic clone AL-1 (27), and the 0.4-kilobase *MspI* fragment of the *Ha-ras-1* pKY-1 clone (28).

For dot blot hybridization analysis, five dilutions of each genomic DNA were blotted in duplicate in 0.4 N NaOH onto a Zetaprobe nylon membrane as described by the supplier (Bio-Rad) to give 10, 3.16, 1.0, 0.316, or 0.10 µg/dot. SV40 virion DNA was blotted in duplicate at 100, 31.6, 10, 3.16, or 1.0 pg/dot (plus salmon sperm carrier DNA). The samples were hybridized in 50% formamide/4× standard saline-citrate at 42°C using a ³²P-labeled probe prepared from cloned SV40 DNA, and an autoradiograph was prepared following stringent washing. Hybridization was then repeated using a ³²P-labeled genomic probe to demonstrate that equivalent amounts of DNA were present for each sample. Autoradiograms were analyzed using a Molecular Dynamics model 300A scanning densitometer.

Analysis of *Ha-ras-1* Genes for Activating Mutations. *Ha-ras-1* was amplified from genomic DNAs using the PCR (29) with Taq polymerase (New England Biolabs). The oligonucleotide primers used for amplification were based on the human *c-Ha-ras-1* sequence (Ref. 30; where +1 is the first base of codon 1); HR/1+, bases -2 through +26 (at the 5' end of exon 1); and HR/d-, bases +534 through +563 (at the 3' end of exon 2). The 565-base pair PCR product contained both the codon 12 and codon 61 regions, in which *ras*-activating mutations from clinical and experimental neoplasms are clustered. *MspI* RFLP

analysis was used to detect base substitutions at codon 12. All single-base *ras*-activating substitutions there eliminate an *MspI* site (CCGG) and give a novel 392-base pair *MspI* fragment instead of the normal 355-base pair fragment under these conditions. *MspI*-cut DNAs were analyzed by electrophoresis through 1.8% agarose gels containing 0.5 µg ethidium bromide per ml. For sequencing, PCR-amplified DNA fragments of interest were purified by electrophoresis through 2% NuSieve (FMC Corporation) gels and sequenced with a Sequenase kit (U. S. Biochemicals) by the dideoxy method of Sanger *et al.* (31) using Nonidet P-40 and Tween 20 (32).

Immunoperoxidase Procedure. Attached cells cultured in normal Ca²⁺ were removed from T₂₅ flasks with Versene-trypsin, whereas the free-floating cells were pelleted from the culture medium. After cells were washed in phosphate-buffered saline, 2 × 10⁵ cells were cytospun onto lysine-treated slides. These cells were fixed with methanol:acetone (1:1) at -20°C for 10 min, washed in phosphate-buffered saline, and exposed to mouse monoclonal antibody (OncoGene Science, Manhasset, NY) directed against SV40 large and small T-antigen (33). Experimental and positive-control cells were treated overnight with test and control mouse IgG at a concentration of 1.25 µg protein/ml. Following overnight incubation, washing and staining were followed as directed by antibody kits (Vector Laboratories, Burlingame, CA).

Methocel Cultures. Anchorage-independent growth was determined by the method of Stoker *et al.* (34) with volumetric variations to accommodate the adaptation to 24-multiwell plate (35) and with the final concentration of Methocel being maintained at 1.17%. The Methocel and agar solutions contained the same components as DMEM-H. All additions of nutrients or neutral red (1:10,000) were made in 0.4-ml volumes. Duplicate counts were made for each cell density plated after the addition of neutral red to determine the plating and cloning efficiencies. The number of cells that stained red 1 day postplating was counted and used in calculating the plating efficiency. Colony-forming efficiencies were determined by dividing the number of colonies greater than 50 µm (counted and sized using a calibrated ocular grid) by the number of viable cells counted 1 day postplating. A mouse mammary cancer cell line, 66.1 (36), was plated directly from the freezer with viable cells determined by trypan blue exclusion test and used as control.

Collagen Cultures. Collagen gels were prepared from either rat tail collagen fibers as originally described (37) or type 1 collagen solution (Collagen Corp., Palo Alto, CA). A 79.6% collagen solution was prepared and supplemented with 10% 0.1 M NaOH, 2% 0.7 M NaHCO₃, 8% F-12 at 12.5×, and 0.4% antibiotics, as reported by Yang *et al.* (38). A base layer of 300 µl was added per well in a 24-multiwell plate and allowed to gel at room temperature. For localized growth, cells were added in 1 µl of collagen which was allowed to solidify before layering on top an additional 300 µl of collagen. Dispersed single cells were plated in 300 µl of collagen and permitted to gel. Cultures were fed 3 times/week with DMEM-H containing equine serum at 5.0 or 20.0%.

Tumorigenic Assay. The tumorigenic assay was performed in three different groups of mice. Group A: Five female Harlan-Sprague-Dawley athymic mice were inoculated s.c. in the axilla with 17.4 × 10⁶ MCF-10A cells (895d) in a 10% brain extract which promotes vascularization at the site (39). Inoculation s.c. of MCF-7 human breast cancer cells with brain extract produces tumors from a 10-fold lower inoculation than is required for cells inoculated alone.³ Group B: Twenty-two athymic males were castrated at 6 weeks of age and treated with implanted (s.c.) pellets containing 10 mg 17β-estradiol prior to the injection of MCF-10A cells into the axillary fat pad (1 × 10⁶ cells, 1014d, were injected into 14 animals, or 1 × 10⁷ cells, 1141d, were injected into 8 animals). Group C: Seven 6-week-old athymic female mice received i.v. 1 × 10⁶ MCF-10A cells at 1141d. For Group A, mice were observed at weekly intervals for bolus and tumor formation. One mouse was sacrificed after 7 days and the bolus was fixed in Bouin's solution. The remaining mice were sacrificed after 8 months and the inoculation site was removed, fixed, and processed for histological examinations. For groups B and C, the animals were examined and palpated at weekly intervals and sacrificed after 6 months. The animals

³ T. M. Maloney, unpublished observation.

underwent complete autopsy, and injection sites (group B) and lungs, spleen, and liver (group C) were fixed in 10% neutral buffered formalin and processed for histological examination.

RESULTS

Primary cultures of human breast epithelial cells remain confluent when switched to LCM with the concurrent liberation of viable FFC. These FFC yield seed stock for secondary passages (3, 40). Serial transfer of FFC has been demonstrated by maintaining the primary and subsequent passages in LCM for over 1000 days. However, the number of FFC decreased over 100-fold/feeding in primary cultures (3, 16). Nonserial passages were obtained by combination of FFC from primary cultures and from serially transferred FFC passages in LCM. For example the 2nd–10th passages would represent the plating of FFC combined from the primary and the 2nd to the 9th passages. Therefore, a low passage number does not necessarily indicate a short *in vitro* life.

Derivation of MCF-10 Sublines (Fig. 1). After 6 days in DMEM-H and 10 days in LCM (0.06 mM Ca²⁺), FFC of the primary passage of human breast epithelial cells were transferred into low calcium (0.06 mM Ca²⁺) SFM (Fig. 1). The primary cultures were maintained in LCM and FFC served as seed stock for transfer in LCM or SFM. Cells of the primary to the 6th passage (1–6) in LCM were cryopreserved after 342 (50 doublings) and 429 days *in vitro* (3). Attached cells and FFC maintained in SFM were serially transferred and frozen after 108 and 341 days *in vitro*. After an initial burst of growth in SFM, the proliferative rate decreased relative to cells cultured in LCM (30 doublings at 341 days). These primary cultures and cells grown in LCM and SFM (designated MCF-10M) displayed mortality by undergoing senescence when cultured in DMEM-H (containing 1.05 mM Ca²⁺).

The yield of FFC from cultures in SFM depended on the degree of confluence and to some extent on the level of calcium in the medium (Fig. 2). The numbers of FFC were minimal before confluence and by 47 days after plating a constant level of cells was maintained in the medium above the attached confluent layer of MCF-10M cells. These FFC were capable of serial transfer in media with each of the 3 Ca²⁺ concentrations (0.0, 0.03, and 0.06 mM Ca²⁺). However, passage was more efficient in a medium with 0.03 mM Ca²⁺ and it was possible to carry out only 1 additional passage in Ca²⁺-free medium. After

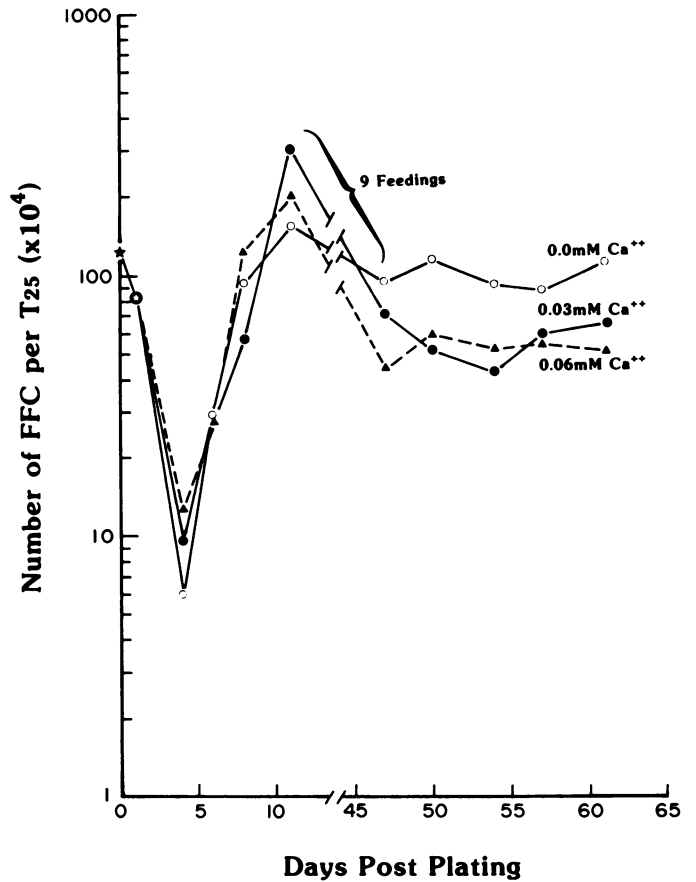


Fig. 2. After 29 days *in vitro*, free-floating cells in the 3rd passage of human breast cells as a function of calcium concentrations in SFM. FFC from 0.0, 0.03, and 0.06 mM Ca²⁺ in SFM medium of the 2nd passage were pooled and plated at 1.25 × 10⁶ cells/T₂₅ flask in 0.06 mM Ca²⁺. Day 1 posttransfer, 8.25 × 10⁵ cells were attached and the Ca²⁺ level was changed in duplicate flasks to 0.0, 0.03, and 0.06 mM. Confluency was 20, 90, and 100% on days 1, 4, and 6, respectively. FFC were counted from duplicate flasks: 0.0 mM Ca²⁺; 0.03 mM Ca²⁺; and 0.06 mM Ca²⁺.

661 days in SFM (total, 677 days), the second passage did not maintain confluence. Essentially, cells were maintained in SFM from 341 to 677 days *in vitro* with minimal increase in cell number (>30 to <35 doublings). Thereafter, subsequent passages were carried out in serum-containing medium with 0.04 mM Ca²⁺ (Fig. 1).

A gradual increase in FFC occurred in the 2nd–7th passages after serum-containing medium was added (LCM). The FFC of the 2nd–7th passages were pooled to initiate the 8th passage at 754 days. Transfer of FFC continued up to passage 12 (840 days) when the first of the immortal cells were cryopreserved. Since the 12th passage all subsequent passages have been listed serially in this subline. These cells then were cultured in a medium containing 5% Chelexed serum and 0.04 mM Ca²⁺. Substitution of this LCM with DMEM-H did not induce senescence. This line of immortal cells was designated MCF-10F (Fig. 1, *stippled area*).

MCF-10F. FFC and attached cells were counted after 1051 days *in vitro* in 11 separate passages (27th through 37th; the days in passage ranged from 7 to 76). FFC varied from 12 to 41% of the total (free floating and attached) cells in a T₂₅ flask. Cell densities ranged from 6 to 15 × 10⁶ cells/cm² and FFC were more numerous in younger cultures.

FFC and attached cells of MCF-10F removed with trypsin and EDTA were readily cryopreserved and reactivated in LCM. These cultures have been serially transferred as FFC and con-

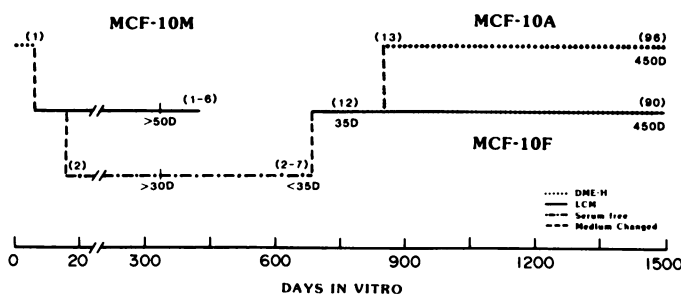


Fig. 1. Origin of MCF-10. Breast tissue and mortal cells (MCF-10M) with finite life span, clear area. DMEM-H complete medium in 1.05 mM Ca²⁺ (.....). LCM, complete medium in 0.04 or 0.06 mM Ca²⁺ (—). Serum-free medium in 0.0, 0.03, 0.04, or 0.06 mM Ca²⁺ (- - - -). Abscissa, total days *in vitro*. MCF-10M became immortal MCF-10F (*stippled area*) during the time period indicated by *solid line* between 2–7 and 12 passages. MCF-10A (*striped area*) was derived from MCF-10F when transferred serially with trypsin-EDTA in DMEM-H. Numbers in parentheses, passages when initiating or terminating cultures in the different media. Approximate doublings (D) are depicted at various stages in the evolution of these cell lines.

tinued to yield numerous FFC, unlike the other cultures which have been repeatedly transferred with trypsin and EDTA.

Fig. 3 illustrates essentially the same growth pattern for MCF-10F over 7 consecutive passages, whether the cells were maintained in continuous culture or had been reactivated after 254 days in liquid N₂. Ninety-five % confluence was attained on day 3 and essentially no FFC were apparent until after confluence was obtained. Greater than 3 × 10⁶ cells were free floating for the next 17 days during 5 nutrient changes. On the 24th day posttransfer, 17 ± 4 and 19 ± 5% of the cells in different T₂₅ flasks were free floating and 96% of these cells were viable, whether they had been reactivated from the frozen state or continuously cultured.

MCF-10A. After 849 days *in vitro* a second subline was initiated by plating FFC into DMEM-H at the 13th passage (Fig. 1). This subline of adherent cells, designated MCF-10A, has been transferred serially since the 13th passage with trypsin and EDTA (Fig. 1, striped area). The 13th passage was divided, with one culture being maintained in DMEM-H and the other in LCM. Growth rates of the two sublimes were determined after 897 days *in vitro* (Table 1). The growth rate of DMEM-H and LCM cultures decreased when the cell density reached 2.2 × 10⁵ and 1.8 × 10⁵ cells/cm², respectively. Complete confluence was attained by day 15 in LCM, whereas only 95% confluence was reached in DMEM-H. After day 9, DMEM-H cultures exhibited domes in localized confluent areas. Only 3–4% of the total cell number was free floating by day 15 when MCF-10A was cultured in LCM. This was approximately one-fifth of the FFC derived from MCF-10F cultured in LCM (Fig.

Table 1 Cell number, doublings, and doubling times of two passages of MCF-10A in LCM and DMEM-H

Day in passage	Passage 19 ^a			Passage 20 ^b		
	Cells × 10 ³	Doublings	Doubling time (h)	Cells × 10 ³	Doublings	Doubling time (h)
0	60.0 ^c			20.0 ^d		
1	8.6 ± 2.9 ^e			2.2 ± 0.9		
3	14.2 ± 14	0.65	73.8	5.0 ± 2.2	1.14	42.1
6	106 ± 6	2.87	25.1	18.9 ± 6	1.89	38.1
9	438 ± 121	2.03	35.5	86 ± 25	2.14	33.6
12 ^f	739 ± 59	0.69	104.3	349 ± 54	2.02	35.6
15	741 ± 116 ^g	0.003	24,000.0	525 ± 18	0.5	144.0
		6.243 ^h			7.69 ^h	

^a The 19th passage was transferred by trypsin-Versene since the 13th passage with all passages except the 13th cultured with LCM.

^b The 20th passage was cultured with DMEM-H since the 13th passage.

^c Corning 24-well plates were plated at 6.0 × 10⁴ cells/well in LCM.

^d Corning 24-well plates were plated with 2.0 × 10⁴ cells/well in DMEM-H.

^e Counts based on trypsin-Versene counts of 4 wells/point ± SD.

^f Nutrients were replenished on days 1, 3, 6, 9, and 12.

^g From 3 to 4% of cells were free floating.

^h Total doublings.

3). Doubling times in the two media were essentially similar when compared for 6 days of maximum growth, 30.4 versus 34.6 h for LCM and DMEM-H cultures, respectively. At this point the subline in LCM was no longer maintained. The subline in DMEM-H, which displayed resistance to Ca²⁺-induced senescence, has continued in culture (MCF-10A). MCF-10A has averaged over 5 doublings per weekly transfer since the 20th passage; therefore, there were approximately 450 doublings at the 96th passage.

Finite Life Span Substrains (MCF-10M). Cells maintained in 5% Chelexed serum in primary culture and the 2nd–6th passages were frozen after 429 days *in vitro* (Fig. 1). In addition, cells of the identical lineage from which MCF-10 was derived, growing in serum-free media, were cryopreserved after 108 days *in vitro* (3rd–8th passages; Fig. 1). These two “finite life span” sublimes were reactivated from liquid nitrogen and cultured in LCM with 5% serum to obtain cells for cytochemistry,⁴ oncogene analysis, and immortality determination and aliquots were refrozen after an additional 282 days *in vitro*. Unlike MCF-10F or A, both sublimes succumbed to calcium-induced senescence in DMEM-H, as indicated by fewer than 22 doublings, increased doubling times with succeeding passages (≥460 h), numerous senescent cells and the inability to produce monolayers. These experiments were terminated after 492 and 774 days *in vitro*.

Calcium-induced senescence also has been observed with other cultures of FFC from reduction mammaplasties, s.c. mastectomies, or tumor tissues when they were exposed to the 1.05 mM Ca²⁺ of DMEM-H after having been cultured for longer than 1 year in LCM (data not shown).

Growth Requirements. After 3.0, 3.5, and 3.9 years *in vitro*, the 50th, 71st, and 90th passages of MCF-10A demonstrated essentially no growth unless the medium, which contained 5% equine serum, was supplemented with growth factors and hormones, (Fig. 4). The growth requirements were identical to those previously reported for short-term human mammary secondary cultures [cortisol, EGF, insulin, and cholera enterotoxin (15, 38, 41)]. The 71st and 90th passages were assayed as continuously transferred cultures, whereas the 50th passage of MCF-10A(1014d) was tested after being reactivated from a

⁴ Cytochemical data, to be published separately, confirm the epithelial nature of MCF-10, which was proven by dome formation in confluent monolayer cultures maintained in medium with 1.05 mM Ca²⁺. (Tait, L., Soule, H. D., and Russo, J. Ultrastructural and Immunocytochemical Characterization of an Immortalized Human Breast Epithelial Cell Line, MCF-10. *Cancer Res.*, 50: 6087–6094, 1990.)

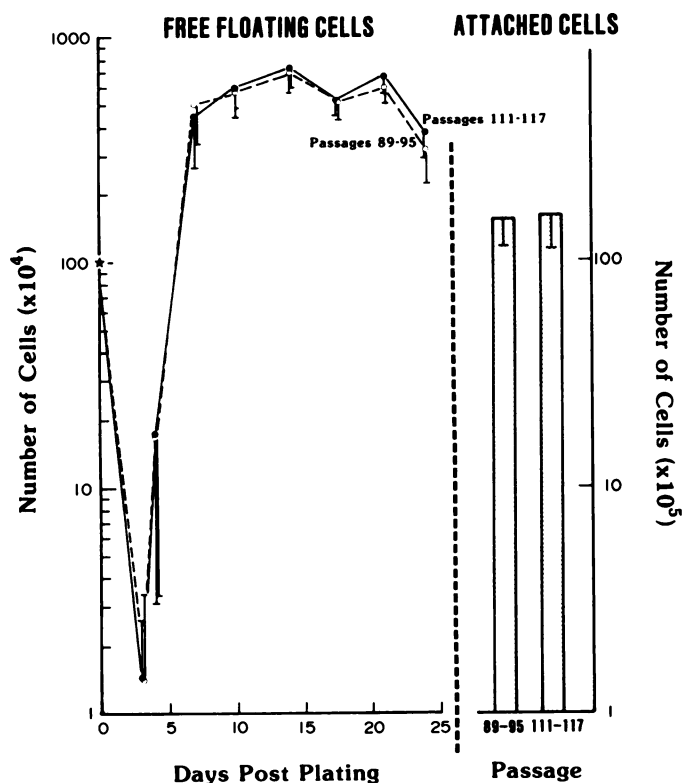


Fig. 3. Free-floating and attached MCF-10F cells as a function of passage, before and after cryopreservation. One T₂₅ flask was plated with 10⁶ viable FFC from a 7-day culture of cells reactivated at passage 85 (1445d) and FFC from passage 110 for 7 consecutive passages. Viable cells were counted (trypan blue exclusion) each time the medium was changed on days 3, 4, 7, 10, 14, 17, 21, and 24. On day 24, the attached cells were counted after the addition of trypsin-EDTA. Bars, SD.

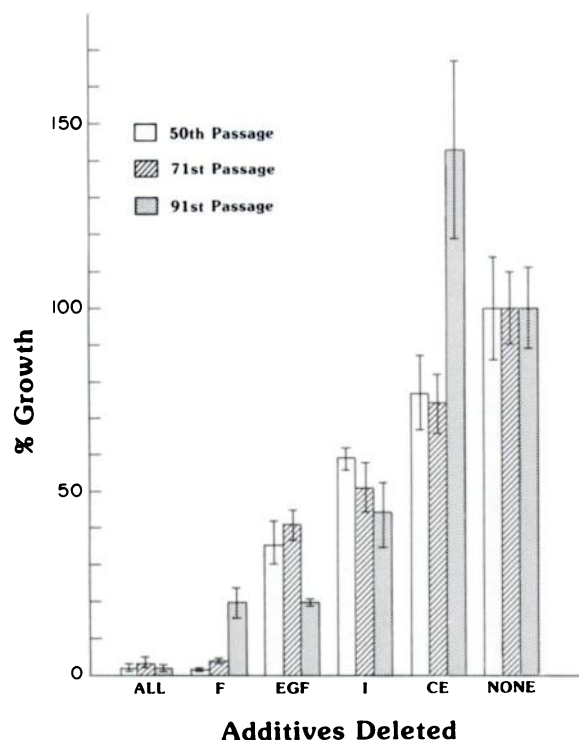


Fig. 4. Effect of additives on growth of 3 different passages of MCF-10A. Passage 71 (1266d) was plated at 1.17×10^5 cells/ T_{25} flask and the 50th (1014d) and 91st (1406d) passages at 6.25×10^4 cells. One day after passage, cells of 3 T_{25} flasks were counted to calculate plating efficiency and to determine the number of doublings. The plating efficiency was 69, 66, and 86% for the 50th, 71st, and 91st passages, respectively. Experimental additives (cortisol, F; insulin, I), and nutrient replacements were carried out on day 1 and at 3-day intervals thereafter with cell counts being determined on days 13, 9, and 8 for the 50, 71, and 91 passages, respectively. To permit a direct comparison of cell growth in the 3 different passages, the cell counts were converted to percent growth with the counts in complete medium assessed as 100%. The counts for succeeding passages in complete medium were $2.9 \times 10^6 \pm 0.4$ (SD), $3.3 \times 10^6 \pm 0.3$, and $6.3 \times 10^6 \pm 0.7$.

cryopreserved state (Fig. 1). Both the 50th and 91st passages were plated on the same day and received medium from the same stock solution. Cholera enterotoxin was the least stimulatory of the additives to the 50th and 71st passage, and it was inhibitory to the 91st passage. Dome formation, characteristic of epithelial cells in culture, was noted in all 3 passages when cultured in a medium containing all added growth factors or in medium lacking cholera enterotoxin.

Cell counts were performed once a monolayer was attained in the complete medium. An obvious result of the continuous passage of MCF-10A was the decrease in doubling times and the shorter incubation periods (8–13 days posttransfer) required to attain a monolayer in complete medium. These changes were accompanied by an increase in cell number per cm^2 . The doubling times varied from 20.2 h for the 91st passage lacking CE to infinity for the 50th passage without additional growth factors. The cell densities for these two cultures varied from 2 to 362×10^3 cells/ cm^2 .

After 4 weeks in DMEM-H, MCF-10F(1573d) demonstrated a 19.3-fold increase in cell number when complete medium and cortisol-depleted medium (both with DMEM-H) were compared (67.7 and 3.5×10^5 cells, respectively). In order to demonstrate that the ability to respond to cortisol was a constitutive characteristic of these cells and not a characteristic that had developed during the 4-week culture period, FFC of the reactivated 94th passage of MCF-10F were plated directly into DMEM-H and LCM. The doubling times were essentially

the same (greater than 40 h) in both media when all supplements were depleted, but MCF-10F possessed a faster growth rate in the complete medium with low Ca^{2+} concentrations as indicated by a 6-h reduction in doubling time, 22 versus 28 h.

MCF-10 Cytogenetics. The chromosome pattern of lymphocytes obtained from the patient who provided the tissue from which MCF-10 was derived was that of a normal diploid female. The cultured cells before immortalization (MCF-10M, reactivated from cryopreserved cells after 341 and 429 days *in vitro*) were normal diploid after 350 and 459 days *in vitro* and identical to those of the patient from whom the original tissue was obtained.

In the period between 754 and 849 days, before separation of the MCF-10A and MCF-10F sublines but after "transformation to an immortal state," the cultured cells acquired a balanced reciprocal translocation, $t(3;9)(3p13;9p22)^5$ and were otherwise normal. It is not known when, in the intervening period, this rearrangement occurred or whether it was correlated in time with acquisition of immortality. Other nonclonal changes were also seen. After separation, at the 93rd passage of MCF-10F(1524d), approximately 85% of the cells remained diploid (46 chromosomes), retained the same reciprocal translocation $t(3;9)$, and had also acquired an unbalanced rearrangement (1p+) (Fig. 5).

The other subline, MCF-10A, was examined at the 34th, 43rd, and later passages (from 1009 to 1527 days in culture). The stemline karyotypes were uniformly monosomic for chromosomes 3 and 9, although other sporadic losses were seen. All but one of the remaining chromosomes were normal and paired. Derivatives were identified for the missing chromosomes: M1 was identical to the der(3) resulting from the $t(3;9)$ described above and M2 was interpreted as a derivative arising from the reciprocal 9p+ that had undergone further rearrangement and translocation. The new translocation at the 9p22 breakpoint was interpreted as originating from partial duplication of 9q and probably a small portion of 5q. In addition, a 3rd marker (M3), an unbalanced rearrangement of chromosome 6, was interpreted as $t(6;19)(p25;q12)$ (Fig. 6); it was not present at 1046 days and was first observed in harvests at 1229 days in culture. The cells showed some fluctuation in chromosome number; a subline with 48 chromosomes was identical to the stemline except for acquisition of extra copies of No. 8 and No. 16; 5% were tetraploid with duplication of markers.

Genotypic Characterization of MCF-10 Cells. To further define the human origin and lineage of the MCF-10M, MCF-10F, and MCF-10A cells, genomic DNA was analyzed by hybridization with probes for highly polymorphic sequences (22, 23, 43, 44). In *EcoRI* digests of MCF-10 cells (Fig. 7A, Lanes 5–8) identical 10- and 6.35-kilobase fragments were detected by hybridization with a probe, which is specific for hybridization to human DNA, to the hypervariable single copy gene *PUM*, the highly polymorphic epithelial-urinary mucin gene (23, 44). Distinct size fragments were detected in MCF-7, BT-20, and SKBR-3 DNA, human breast cell lines that are carried in our laboratories, and an unrelated individual's lymphocyte DNA (Fig. 7A, Lanes 1–4).

The relationship of MCF-10 cells to the donor was demonstrated by hybridization of identical size *HaeIII* fragments (Fig. 7B, Lanes 1–4) with a M13 probe (22) that detects multiple hypervariable minisatellites (43). In contrast, DNA from MCF-

⁵ Cytogenetic nomenclature is based on ISCN 1985 (42).

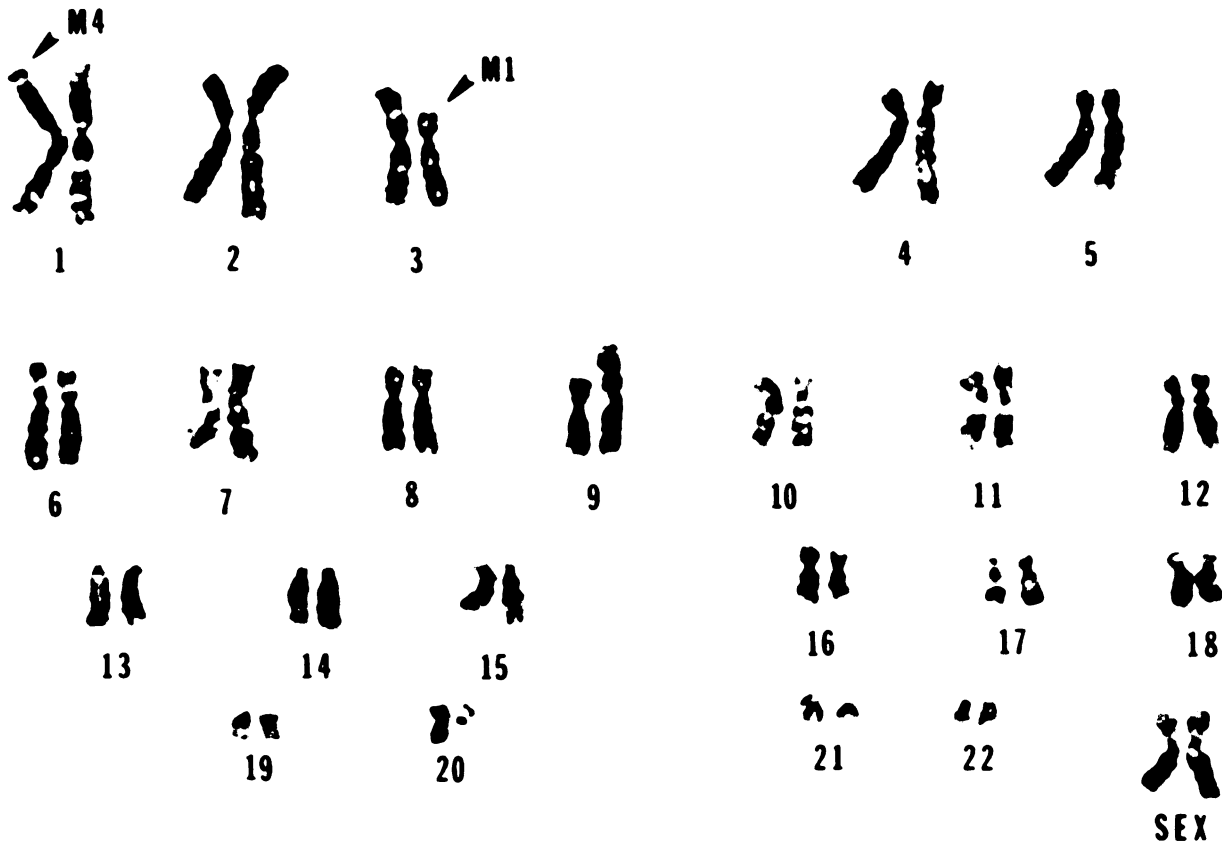


Fig. 5. Karyotype of MCF-10F(1524d) prepared from cells of passage 93. The pattern shown is 46,XX,1p+,t(3;9)(p13;p22). The markers are numbered in order of their time of appearance.

7 and short-term cultures of another individual's reduction mammoplasty cells demonstrated distinct, different restriction fragments (Fig. 7B, Lanes 5 and 6, respectively). These results demonstrate the human origin and, by these criteria, the genotypic identity of MCF-10M, MCF-10F, and MCF-10A cells.

Some primary breast tumors and cell lines contain alterations in protooncogenes on chromosome 17, *c-erbB-2/HER-2/neu* and *erbA-1*, or on chromosome 11, *int-2* (45, 46), that contribute to the development of the neoplastic phenotype. In contrast to the amplification of the *c-erbB-2* gene observed in SKBR-3 cells, neither mortal nor immortal MCF-10 cells nor MCF-7 cells demonstrated amplification (Fig. 8A). Similarly, MCF-10M, MCF-10A, and MCF-10F cells, which are heterozygous for a *int-2* *Bam*HI restriction fragment length polymorphism (data not shown), as well as SKBR-3 and MCF-7 cells, lacked amplification of the *int-2* locus (Fig. 8B). Amplification and rearrangement of the *c-erbA-1* and the *int-1* loci in MCF-10F cells were not detected (data not shown).

Likewise, no rearrangements of *c-Ha-ras-1* genes were detected in Southern blot analysis of *Bam*HI-digested MCF-10A genomic DNA (Fig. 9A). Comparison with serial dilutions of human placental DNA and normal human breast epithelial cells (S154) indicates that normal 2S levels of *Ha-ras-1* DNA were present in MCF-10A. Whereas the placenta and S154 were polymorphic for *Ha-ras* alleles, MCF-10 (and EJ/T24) did not show *Bam*HI RFLPs. Hybridization with a *Ha-ras* VNTR probe (47) of Southern blots of *Msp*I-digested MCF-10A, placental and other human breast DNAs indicated that MCF-10A is homozygous for the common *a1* VNTR allele (data not shown).

Ha-ras-1 genes were analyzed for activating mutations at

codon 12 by *Msp*I RFLP analysis. The exon 1 through exon 2 region of MCF-10A, EJ/T24, and human placental genomic DNAs were PCR amplified, digested with *Msp*I and analyzed by gel electrophoresis. MCF-10A and placenta showed the normal 355-base pair *Msp*I fragment (terminating in codon 12). In contrast, the EJ/T24 DNA sample, which is homozygous for a GCC→GTC substitution in codon 12 (and therefore has lost that *Msp*I site) has a longer 392-base pair *Msp*I fragment instead. This *Msp*I RFLP is diagnostic for all codon 12 activating base substitutions, and was not apparent in MCF-10A. The other region in which oncogenic mutations are clustered in *ras* genes is centered at codon 61 in exon 2. Exon 2 of *Ha-ras-1* was amplified from MCF-10A DNA and sequenced as shown in Fig. 9C. No base substitutions were apparent in this region, including three codons known to activate *ras* genes: 59, 61, and 63.

Analysis for the Presence of SV40. Unlike Chang's fR and Gaffney's HBL-100 cell lines (7, 8), MCF-10A and F did not contain SV40 T-antigen as determined by immunoperoxidase. The commercially available monoclonal antibody (PAb 419) stained essentially all nuclei of WI-26 VA SV40-transformed cells (48) with a very high intensity, whereas MCF-10A and F were negative. SV40 gene sequences were not detectable in either MCF-10A or MCF-10F DNAs (973d and 990d, respectively) using dot blot hybridization analysis. The limit of detection was approximately 0.3 pg SV40 DNA/dot, or 0.05 copy of SV40/haploid cell. In contrast, WI-26 cell DNA (included as a positive control) was found to have approximately 0.5 pg SV40 DNA/ μ g cell DNA, or 1 copy of SV40/WI-26 cell.

Growth in Collagen Gel. Single cells, clumps of cells, and duct-like structures were observed when MCF-10A, MCF-10F,

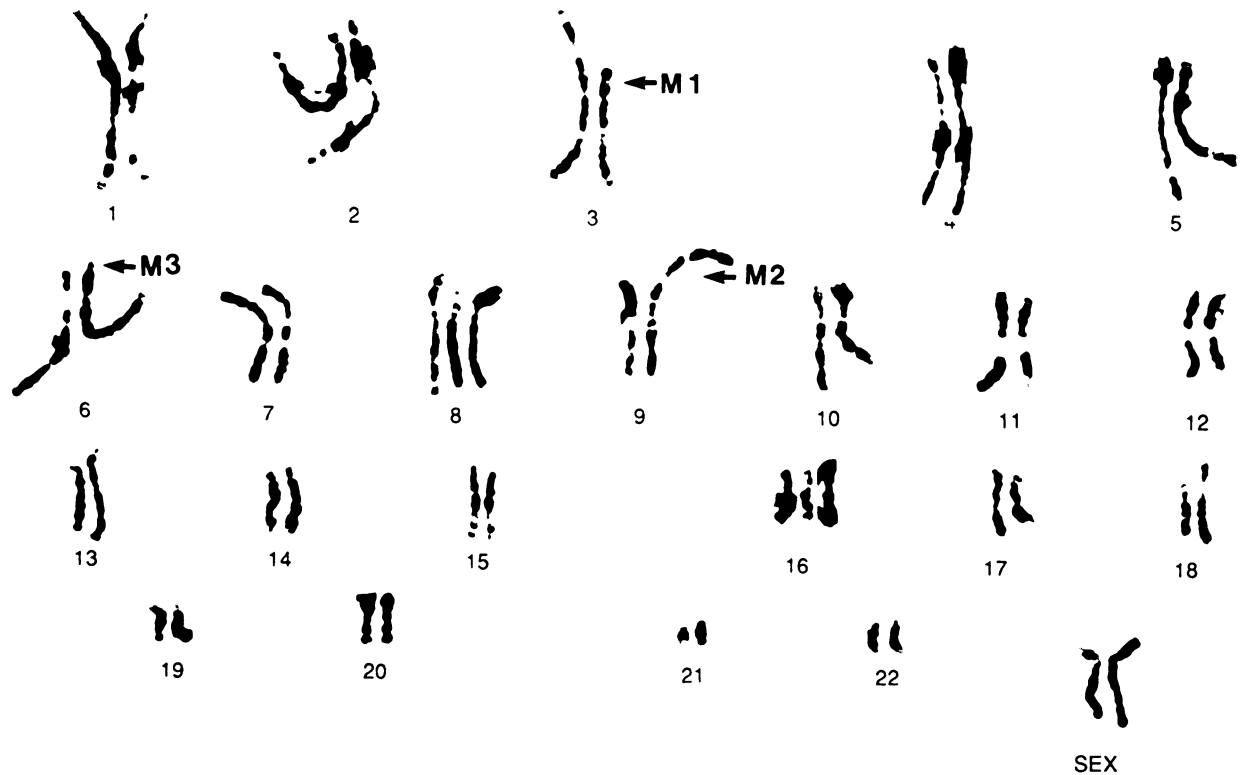


Fig. 6. Karyotype of MCF-10A(1359d) prepared from cells of passage 84. The pattern is interpreted as 48,XX,3p-,6p+, +8,9p+,+16. It had evolved as follows: By the 846th day, a balanced translocation t(3;9)(p13;p22) was present (shown above). The der(3) (M1) persisted but the der(9) was further modified before the 1009th day. The additional changes were interpreted as sequential duplication and addition of 9q(q21-q33) and 5q(q31-qter) to the original 9p22 breakpoint with loss of the translocated 3p segment (M2). The other structural aberration, the 6p+ (M3), was first observed after 1009 days in culture and was interpreted as duplication and translocation of a segment from 19q(q12-qter). Overlapping chromosomes distort the banding patterns of several chromosomes.

or the mortal sublines (which had been frozen after 108 or 429 days *in vitro*) were grown in collagen gels with 5 or 20% equine serum. A more pronounced duct-like growth was observed in 5% serum, as compared to the rapid and disorganized overgrowth characteristic of cultures grown in collagen gels with 20% equine serum. MCF-10F(1536d) produced ductal growth in collagen gels (1 or 300 μ l) in the absence of EGF, CE, or insulin. Duct-like structures, however, were absent from cultures that were not supplemented with cortisol. After 38 days in collagen gel, cultures displayed varied growth patterns (Fig. 10): (a) clump of cells with minimal duct formation and single cells radiating from the clump (Fig. 10A, complete medium); (b) haphazardly arranged single cells (Fig. 10B, medium without CE); (c) horizontally spread cells with duct-like structures and single cells radiating from the colony (Fig. 10C, medium without insulin); and (d) horizontally spread cells with numerous well organized duct-like structures containing essentially no single cells (Fig. 10D, medium without EGF). The compact colonies without single cells were most numerous in cultures lacking EGF.

Anchorage-independent Growth. MCF-10 was assayed for AIG in Methocel (Table 2). The plating efficiencies were determined 1 day postplating and varied from 25 to 70% of the cells plated. Viable cells in wells plated at 2×10^5 could not be enumerated due to an extremely dense cell concentration. Although no colonies were recorded which reached 50 μ m in diameter, occasional small clumps of cells were observed. Preliminary experiments indicated that the maximal AIG efficiency of the positive control culture [a mouse mammary tumor cell line, 66.1 (36)] was obtained when plated at $1-10 \times 10^3$ cells.

However, in the present study, AIG was obtained with up to 4×10^4 cells, the highest cell number tested.

Tumorigenic Assay. After injecting 17.4×10^6 MCF-10A cells into nude mice, the average volume of the bolus at the site of inoculation was 168 and 70.4 mm³ at 1 and 2 weeks postinoculation, respectively. No sign of enlargement was observed after 21 days. Histological sections of the nodule from a mouse sacrificed after 1 week exhibited a central area of necrosis surrounded by hyperplastic connective tissue infiltrated with mononuclear cells. Clumps of surviving nondividing MCF-10 cells were noted among the mouse fat cells and vascularization was prominent. No surviving MCF-10 cells were detected in the 4 remaining mice that were sacrificed after 8 months. Castrated estrogen-supplemented athymic male mice implanted in the axillary fat pad and female athymic mice given i.v. injections of MCF-10A did not develop tumors at the site of inoculation or in distant sites 6 months after inoculation.

DISCUSSION

The present study demonstrates that a primary culture derived from normal human mammary epithelial cells can be maintained in culture for longer than 1 year and up to 6 free-floating passages can be obtained. This longevity has been accomplished by decreasing the level of Ca²⁺ in 5% serum fortified medium from the customary 1.05 to 0.06 mM or below. Human mammary epithelial cells possessed greater *in vitro* longevity in low Ca²⁺ medium, possibly due to the inhibition of differentiation (3), but eventually these passages senesced as reported by others (49, 50), while the primary culture remained

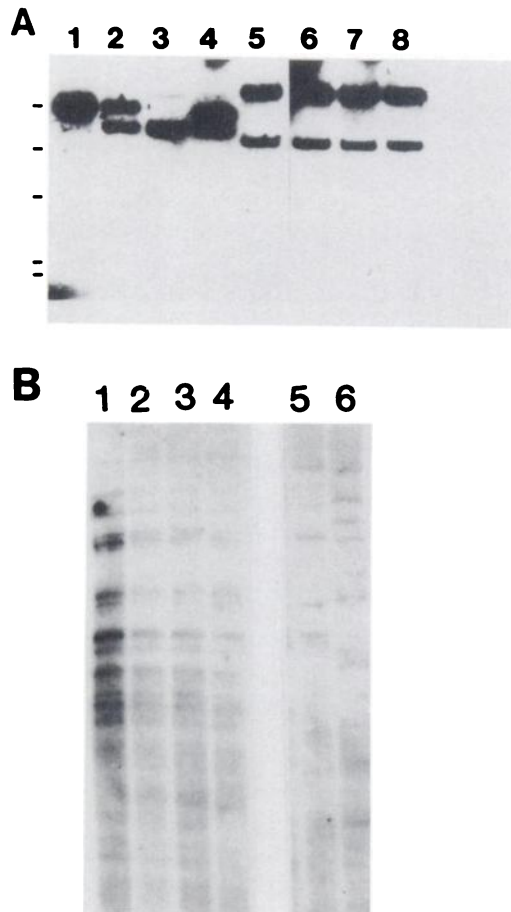


Fig. 7. Characterization of MCF-10 cells by hypervariable DNA sequences. In *A*, *EcoRI* digests hybridized with the human specific *PUM* locus probe were from human lymphocytes (Lane 1), MCF-7 (Lane 2), BT-20 (Lane 3), SKBR-3 (Lane 4), MCF-10F at 867 days (Lane 5), MCF-10A at 935 days (Lane 6), MCF-10A at 1445 days (Lane 7) and MCF-10M at 445 days (Lane 8). The migration of λ -*HindIII* size standards of 9.4, 6.6, 4.4, 2.3, and 2.0 kilobases top to bottom, is specified on the left margin. Autoradiography was for 3 days. In *B*, *HaeIII* digests hybridized with the M13-mp8 fragment that detects hypervariable minisatellite DNA were from the lymphocytes of the MCF-10M donor (Lane 1), MCF-10M at 458 days (Lane 2), MCF-10A at 944 days (Lane 3), MCF-10A at 1285 days (Lane 4), MCF-7 (Lane 5), and short-term culture of reduction mammoplasty cells of another individual (Lane 6).

viable (16). The fact that only primary cultures repeatedly can initiate new cultures with FFC suggests several possible explanations: (a) primary cultures may contain a finite number of stem cells; (b) required growth factors may be produced only in the primary culture by the attached cells; (c) the initial cell density in the transferred cells may be too low to maintain adequate growth factor production; or (d) the putative growth factor-producing cells may be adversely affected by passage in LCM.

The use of low levels of Ca^{2+} (<0.06 mM) also permitted long-term culture of MCF-10M in SFM supplemented with growth factors. A Ca^{2+} concentration in SFM of 0.03 mM yielded the optimum number of viable FFC for passage. However, these passages eventually exhibited senescence. Addition of 5% Chelexed equine serum extended the life of the MCF-10M cultures and within 86 days these cells became immortal (8th passage, 754–840 days *in vitro*). Serial passage in DMEM-H (1.05 mM Ca^{2+}) established the immortality of these cells. MCF-10F originated from the FFC produced by these immortal cells. Continual passage of the attached cells (grown in 1.05 mM Ca^{2+}) with trypsin-EDTA resulted in a cell line, MCF-10A,

which did not produce FFC, even when exposed to LCM, indicating that the method of transfer can provide a selective advantage for a different phenotype. Typically, neoplastic or nonmalignant cultures which become immortal are capable of withstanding repeated passages after trypsin-EDTA treatments (4–7, 9, 51–53). The relationship of the mortal MCF-10M and the immortalized MCF-10F and MCF-10A cells is substantiated by the genotypic identity of their DNA determined by analyses of hypervariable minisatellites and of the highly polymorphic human epithelial mucin gene, *PUM*.

MCF-10 may have become immortal following the apparently spontaneous reciprocal translocation between chromosomes 3 and 9 in the period from 754 to 840 days *in vitro*. The maintenance of diploidy and the balanced $t(3;9)$ in MCF-10F cells after 93 passages and 1524 days *in vitro* indicates a relative stability of this cell line; MCF-10A was relatively less stable with addition of markers and numerous chromosome changes over the same time period. The identity of the breakpoint on 9p suggests that the $t(3;9)$ antedated formation of the more complex chromosome 9 rearrangement observed in MCF-10A that remained as a constant marker (M2) in later passages. The absence of the $1p+$ unbalanced rearrangement from MCF-10A suggests that it was acquired after the two lines diverged, as was the $6p+$ in MCF-10A. In fact, the repeated treatment of

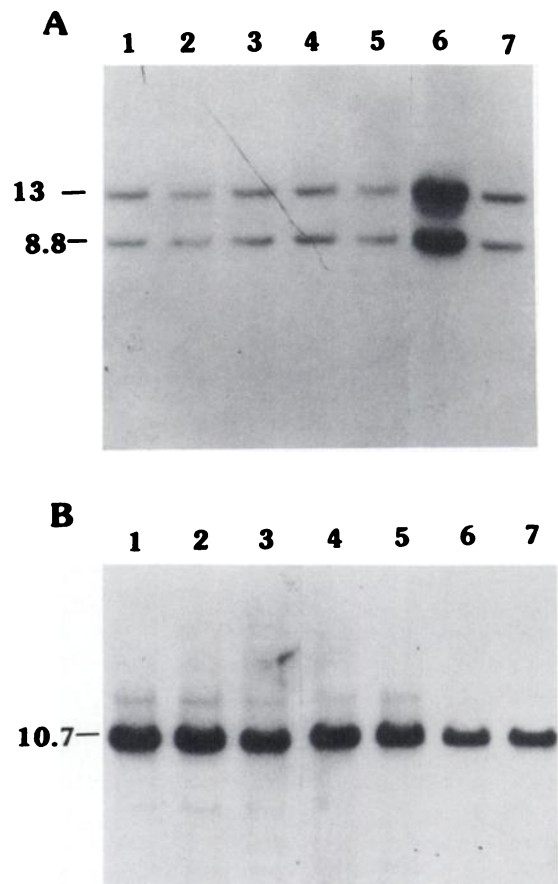


Fig. 8. Analysis of the *c-erbB-2* and the *int-2* genes in MCF-10 cells. Five μg of DNA were digested with *HindIII* (A) or *EcoRI* (B) and analyzed by the Southern blotting technique. DNA was from MCF-10A at 935d, MCF-10A at 1702d, MCF-10M at 395d, MCF-10F at 867d, MCF-10F at 1852d, SKBR-3 or MCF-7 cells (Lanes 1 to 7, respectively). The filter in A was hybridized with a 3-kilobase (kb) *HindIII*-*KpnI* fragment containing *c-erbB-2* complementary DNA sequences. Genomic *int-2* sequences from the SS-6 and BK-4 clones, 0.9-kilobase *SsrI* and 1-kilobase *KpnI*-*BamHI* fragments, respectively, were hybridized with the filter in B. The size in kilobases of hybridizing *c-erbB-2* and of *int-2* genomic fragments were deduced from λ -*HindIII* molecular size standards.

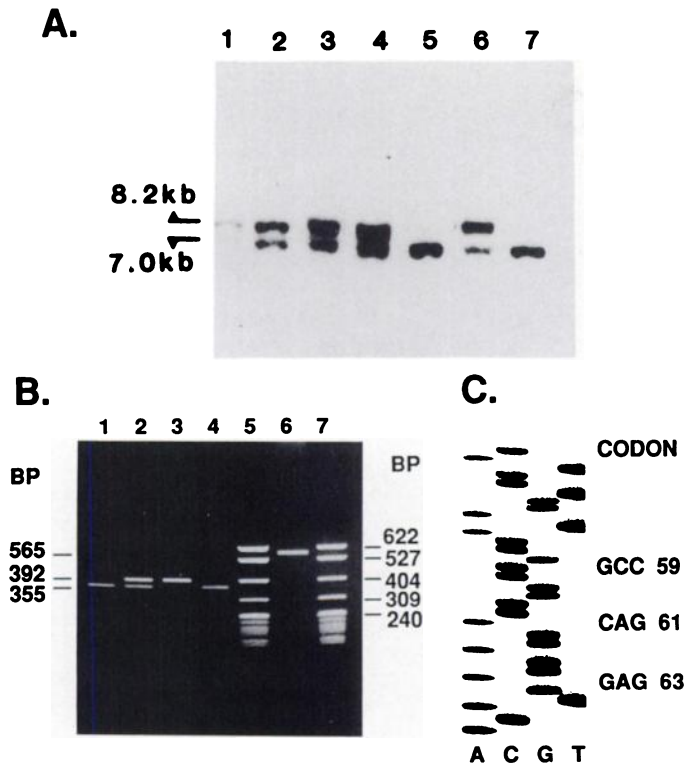


Fig. 9. Analysis of *Ha-ras-1* genes for activating mutations or other alterations. In *A*, genomic DNAs from the indicated samples were restricted with *Bam*HI and analyzed by Southern blot hybridization with a human *Ha-ras* probe (pT24c-3). *Left ordinate*, approximate *Ha-ras* fragment sizes (estimated from DNA size markers). The sources of the DNAs and amounts analyzed were: *Lane 1*, human placenta, 5 μ g; *Lane 2*, placenta, 10 μ g; *Lane 3*, placenta, 15 μ g; *Lane 4*, placenta, 20 μ g; *Lane 5*, EJ/T24, 10 μ g; *Lane 6*, S154 (normal human breast epithelial cells), 10 μ g; *Lane 7*, MCF-10A, 10 μ g. In *B*, the exon 1 through 2 region of *Ha-ras-1* genes from the indicated samples was PCR amplified with primers HR/a+ and HR/d-, giving approximately the expected 565-base pair (bp) product size. These amplified DNAs were restricted with *Msp*I, electrophoresed through agarose/ethidium bromide gels, and photographed. *Left ordinate*, estimated fragment sizes. *Right ordinate*, DNA size marker fragment sizes. The samples in *Lanes 1* through 4 were cut with *Msp*I. *Lane 1*, MCF-10A; *Lane 2*, MCF-10-A plus EJ/T24; *Lane 3*, EJ/T24 alone; *Lane 4*, human placenta; *Lane 5*, DNA size markers (*Msp*I-cut pBR322 plasmid DNA; New England Biolabs); *Lane 6*, MCF-10A, as in *Lane 1* but not cut with *Msp*I; *Lane 7*, pBR322 plasmid DNA, cut with *Msp*I in parallel with the samples in *Lanes 1* through 4. [Note that the other *Ha-ras* *Msp*I fragments (34, 45, 56, and 75 base pairs) are too small to be seen under these conditions.] In *C*, *Ha-ras-1* DNA, enzymatically amplified from MCF-10A as in *B*, was sequenced by the Sanger method. *Abscissa*, A, C, G, and T lanes. *Right ordinate*, normal codons 59, 61, and 63. MCF-10A had the normal sequence throughout this region.

MCF-10A with trypsin-EDTA may have had a deleterious effect. Immortalization appears to be a late stage in the progression of breast cancer cells (54). However, it remains to be demonstrated that phenotypically normal near-diploid immortal human breast epithelial cells can indeed be transformed to the neoplastic state spontaneously, as has been reported repeatedly for rodent cells (55).

The absence of anchorage-independent growth would suggest that MCF-10 is not a culture of malignant cells. However, many examples of growth of normal cells in supplemented Methocel or agar, and the subsequent inability of cells isolated from Methocel to produce tumors, suggest that AIG is not a dependable criterion (35, 56, 57). The inability of MCF-10A estrogen receptor-negative cells to produce progressively growing tumors in athymic mice, using different protocols with and without hormonal supplementation, is the most substantial criterion to indicate that this cell line is not malignant. Using the same protocol, receptor-negative breast tumor cell lines

(MDA-MB-231 and BT-20) form tumors without hormonal support, whereas the estrogen receptor-positive MCF-7 cell line requires estrogen supplements to promote growth of the cellular implant in athymic mice (58, 59). In contrast to some human breast tumors and cell lines (46, 60, 61), mortal and immortalized MCF-10 cells lacked amplification, rearrangement, or mutational activation of cellular protooncogenes on chromosome 17, *c-erbB-2/HER-2/neu* and *c-erbA-1*, or chromosome 11, *int-2* and *c-Ha-ras-1*. Also, using RFLP analysis and DNA sequencing we detected no activating missense mutations in the common activation sites of *Ha-ras* oncogenes. Furthermore, at least 99% of *Ha-ras* alleles in MCF-10A were found to be normal in the codon 12/13 and 59/61/63 regions as assessed by allele-specific oligonucleotide plaque hybridization of *Ha-ras* libraries constructed from PCR-amplified *Ha-ras* genes cloned into bacteriophage M13.⁶ *H-ras* was of particular interest because (a) of reports that this oncogene could both block calcium-induced senescence in keratinocytes and initiate epidermal neoplasia (62, 63); (b) *v-Ha-ras* is a potent initiator of mammary neoplasia in transgenic mice (64); and (c) *Ha-ras* is activated in nearly all mammary tumors induced by nitrosomethylurea in rats (65, 66). However, it does not appear that alterations in any of these (proto)oncogenes were involved in the immortalization or loss of calcium-induced senescence in MCF-10. Furthermore, activation of *ras* genes is rare in human breast cancer (61, 67).

Maximal growth of MCF-10A and F monolayer cultures required essentially the same supplements reported for human mammary epithelial primary cultures in collagen (15, 68, 69). However, MCF-10A and F plated in the dispersed state in collagen required cortisol for growth, even with 5% serum. Cortisol produced a diversified growth pattern for MCF-10 that comprised a mixture of ductal forms, spheroidal masses, or mixed outgrowths, as reported for primary cultures in collagen (68). Although the optimal growth medium for MCF-10A and F cultures was essentially identical to that required for short- or long-term primary cultures (38, 41), three variations were noted with increasing time in culture: (a) cholera enterotoxin inhibited growth; (b) the doubling time decreased from 48 to 20 h; and (c) the cell number increased 30-fold per T_{25} flask. These alterations could be expected after prolonged *in vitro* growth (6, 70). Therefore, it is suggested that growth rates, saturation densities, growth requirements, growth patterns in collagen, and karyotypes of MCF-10 should be obtained from long-term cultures or reactivated cryopreserved cells before comparative studies are carried out with other normal or malignant mammary epithelial cells.

Two human, immortal, breast epithelial cultures (184A1 and 184B5) were developed previously from normal HMEC by Stampfer and Bartley (9) after exposure to the procarcinogen benzo(a)pyrene. These two lines "contained several unrelated chromosome deletions, translocations and replicate chromosomes," although the cells had been diploid prior to carcinogen exposure. The fR lines of Chang *et al.* (7) were transformed by SV40 and passed through a crisis stage before attaining immortality. Both precrisis and postcrisis cell lines were hypotetraploid. Other reported lines, established from nonmalignant breast epithelium, HBL 100 and HMT 3522, differ from MCF-10 in that both were aneuploid when first examined cytogenetically. HBL 100, which originated from milk, was karyotyped at the 5th and 50th passages, but the overall time in culture

⁶ R. Jones, unpublished results.

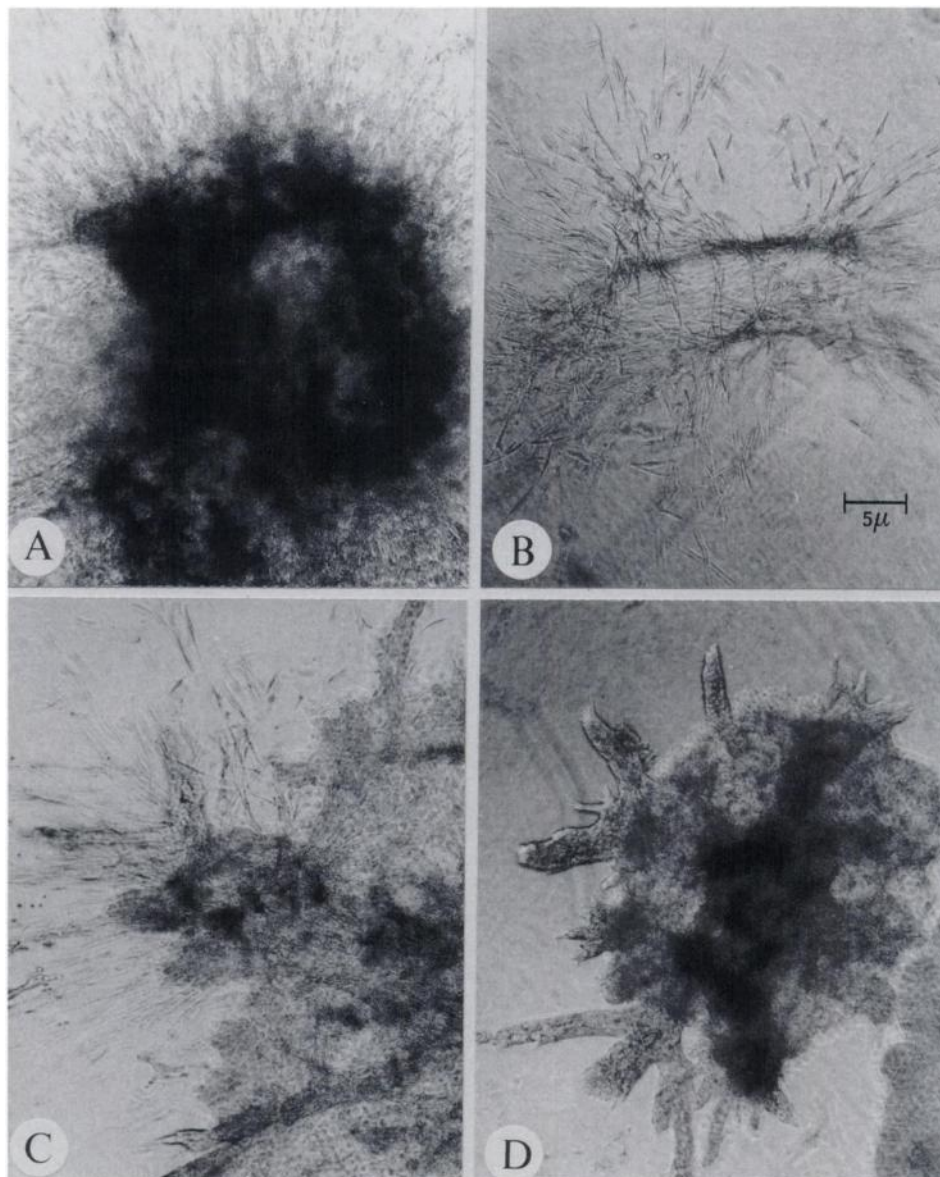


Fig. 10. Growth in collagen. Cells were passed into 24-well plates at 5×10^3 cells/300 μ l of medium containing rat tail collagen. Cultures were incubated for 38 days with triweekly feedings of complete medium (A) and in medium with the following growth factors deleted: (B) cholera enterotoxin, (C) insulin, or (D) epidermal growth factor.

was not specified. Chromosome counts ranged from 58 to 72, with a modal number of 63 at the later passage. Because these cells were not stained with banding procedures, many markers may have gone unrecognized, but a long acrocentric marker chromosome was present uniformly. The derivation of HMT 3522 is unclear: although stated to originate from breast tissue with fibrocystic disease, the origin was defined as "a benign breast tumor" and the description is consistent with a fibroadenoma, a benign, but neoplastic, lesion (5). HMT 3522 was examined at passage 16 after 10 months in culture (after reactivation from frozen cultures). Extensive aberrations were observed with a stemline pattern interpreted as $45,XX,-6,t(6p;8q),13p+,t(5;14)(q22;q34),t(6;17)(q11;q25)$. Emergence of a modified stemline and numerous sidelines in later passages with occasional appearance of double minutes was reported [an unusual observation for cells of nonmalignant derivation (71)]. There was no overlap between the initial chromosomal recombination sites of MCF-10 and HMT 3522; the definitive changes in HBL 100 have still to be reported. A third, Hs 578 Bst, was originally diploid, but probably derived from

myoepithelium, and did not evolve to aneuploidy in later passages prior to senescence.⁷

An isozyme analysis performed on MCF-10A after 1009 days in culture substantiated the human origin of the culture. The enzymes detected were: glucose-6-phosphate dehydrogenase, type B; phosphoglucomutase 1, type 1-2; phosphoglucomutase 3, type 1; esterase D, type 1; adenylate kinase, type 1; glyoxalase 1, type 1-2; and lactic dehydrogenase (human). Similar to other cultures of nonmalignant HMEC, the mortal MCF-10M and immortal MCF-10A and F cultures lacked estrogen receptors when assayed by either a whole cell or immunocytochemical assay (data not shown).

Thus, MCF-10 is the first spontaneous line of cultured breast epithelium for which there is direct evidence that the normal diploid chromosome pattern of the original explanted tissues was retained for over a year of growth in culture. The correspondence of aneuploidy with evolution to a cell line in all five of the cases cited above indicates that aneuploidy may contrib-

⁷ H. Smith, personal communication.

Table 2 Anchorage-independent growth in Methocel

Cultures	Days <i>in vitro</i>	Cells plated ^a / well × 10 ³	Plating ^{b,c} efficiency (%)	Colony-forming ^d efficiency (%)		
				Day 7	Days 15–16	Days 18–21
MCF-10A	1398	25–200	40–58	NT ^e	0	0
MCF-10A	1080	25–200	43–70	NT	0	0
MCF-10F	1398	25–200	40–48	NT	0	0
66.1		1–10	13–36	NT	10–64	10–64
MCF-10A	1427	10–50	25–31	0	0	0
66.1		1–10	18–33	19–34	46–48	25–52

^a Cells plated in 0.4 ml Methocel (1.17%/2-cm² well. New Methocel added on day 1 postplating and on days 7 and 14.

^b Neutral red added at 1:10,000 in Methocel on day 1 postplating and 1 day before colony formation efficiency was determined. Plating efficiencies and colony-forming efficiency were determined in duplicate wells for control culture 66.1 plated at 1 and 10 × 10³ cells/well. Plating efficiencies for MCF-10 were calculated 1 day after passage by dividing the number of neutral red-stained cells per duplicate well by the number of viable cells plated. For colony-forming efficiencies of MCF-10, duplicate wells of 2 cm² were observed.

^c Neutral red-staining cells counted in 5 random fields/well = 0.049 cm².

^d Neutral red-staining colonies greater than 50 μm in diameter counted in 5 random fields, 0.049 cm², and divided by viable cells determined when counting plating efficiencies.

^e NT, not tested.

ute to immortalization *in vitro*. Lack of evidence for preferential sites of chromosome rearrangement suggests that genetic pathways to acquisition of immortality are nonuniform.

A new immortal nonneoplastic cell line of human mammary epithelium is described herein. These cells have been maintained in low Ca²⁺ medium as the mortal phenotype (MCF-10M) and as an attached immortal cell line (MCF-10A) as well as the immortal FFC (MCF-10F). MCF-10 has several advantages over other breast cell lines currently available, in that (a) the cells were not treated with a procarcinogen, (b) the cells do not harbor SV40 genetic information, and (c) the karyotype is near-diploid with minimal rearrangement. The potential value of these cells for the study of mechanisms which promote immortality and transformation in human breast epithelium is manifest.

ACKNOWLEDGMENTS

The authors wish to express their appreciation for the expert technical assistance of Nanci Wappler.

REFERENCES

- Stampfer, M. R., Hallows, R. C., and Hackett, A. J. Growth of normal human mammary cells in culture. *In Vitro (Rockville)*, **16**: 415–425, 1980.
- Hammond, S. L., Ham, R. G., and Stampfer, M. R. Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. *Proc. Natl. Acad. Sci. USA*, **81**: 5435–5439, 1984.
- Soule, H. D., and McGrath, C. A simplified method for passage and long-term growth of human mammary cells. *In Vitro Cell. Dev. Biol.*, **22**: 6–12, 1986.
- Hackett, A. J., Smith, H. S., Springer, E. L., Owens, R. B., Nelson Rees, N. A., Riggs, J. L., and Gardner, M. B. Two syngeneic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs 578T) and the diploid myoepithelial (Hs 578Bst) cell lines. *J. Natl. Cancer Inst.*, **58**: 1795–1806, 1977.
- Briand, P., Petersen, O. W., and van Deurs, B. A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. *In Vitro Cell. Dev. Biol.*, **23**: 181–188, 1987.
- Gaffney, E. V. A cell line (HBL-100) established from breast milk. *Cell Tissue Res.*, **227**: 563–568, 1982.
- Chang, S. E., Keen, J., Lane, E. B., and Taylor-Papadimitriou, J. Establishment and characterization of SV40-transformed human breast epithelial cell lines. *Cancer Res.*, **42**: 2040–2053, 1982.
- Caron de Fromental, C., Nardeux, P. C., Soressi, T., Lavialle, C., Estrade, S., Carloni, G., Chandrasekaran, K., and Carsingena, R. Epithelial HBL-100 cell line derived from milk of an apparently healthy woman harbors SV40 genetic information. *Exp. Cell Res.*, **16**: 83–94, 1985.
- Stampfer, M. R., and Bartley, J. C. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. *Prcc. Natl. Acad. Sci. USA*, **82**: 2394–2398, 1984.
- Band, V., and Sager, R. Distinctive traits of normal and tumor-derived human mammary epithelial cell expressed in a medium that supports long-term growth of both cell types. *Proc. Natl. Acad. Sci. USA*, **86**: 1249–1253, 1989.
- Band, V., Zajcowski, D., Kulesa, V., and Sager, R. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. *Proc. Natl. Acad. Sci. USA*, **87**: 463–467, 1990.
- Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell*, **19**: 245–254, 1980.
- Lechner, J., Haugen, A., Autrup, H., McClendon, I., Trump, B., and Harris, C. Clonal growth of epithelial cells from normal human bronchus. *Cancer Res.*, **2294**–2304, 1981.
- Stoner, G., and Babcock, M. Influence of growth factors on proliferation of normal and chemically transformed rat esophageal epithelial cells. *Proc. Am. Assoc. Cancer Res.*, **23**: 42, 1982.
- McGrath, C. M., and Soule, H. D. Calcium regulation of normal human mammary epithelial cell growth in culture. *In Vitro Cell Dev. Biol.*, **20**: 652–662, 1984.
- Soule, H. D., Maloney, T., and Brenz, R. Characterization of long-term growth of normal human mammary epithelial (NHME) cells. *Proc. Am. Assoc. Cancer Res.*, **28**: 30, 1987.
- Ottenbreit, M. J., Halton, D. M., and Peterson, W. D. Jr. Rapid isoenzyme analysis of cell cultures by agarose electrophoresis. II. Intraspecies identification. *J. Tissue Culture Methods*, **6**: 107–110, 1982.
- Seabright, M. A rapid banding technique for human chromosomes. *Lancet*, **2**: 971–972, 1971.
- Wolman, S. R., Smith, H. S., Stampfer, M., and Hackett, A. J. Growth of diploid cells from breast cancers. *Cancer Genet. Cytogenet.*, **16**: 49–64, 1985.
- Pauley, R. J., Parks, W. P., and Popko, B. J. Expression and demethylation of germinally-transmitted BALB/c mouse mammary tumor virus DNA in Abelson MuLV B-lymphoid cell lines. *Virus Res.*, **1**: 381–400, 1984.
- Feinberg, A. P., and Vogelstein, B. A technique for radiolabelling DNA restriction fragments to a high specific activity. *Anal. Biochem.*, **132**: 6–13, 1983.
- Vassart, G., Georges, M., Monsieur, R., Brocas, H., Lequarre, A. S., and Christophe, D. A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA. *Science (Washington DC)*, **235**: 683–684, 1987.
- Gendler, S. J., Burchell, J. M., Duhig, T., Lampert, D., White, R., Parker, M., and Taylor-Papadimitriou, J. Cloning of partial cDNA encoding differentiation and tumor-associated mucin glycoproteins expressed by human mammary epithelium. *Proc. Natl. Acad. Sci. USA*, **84**: 6060–6064, 1987.
- Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T., and Toyoshima, K. Similarity of protein encoded by the human *c-erbB-2* gene to epidermal growth factor receptor. *Nature (Lond.)*, **319**: 230–234, 1986.
- Casey, G., Smith, R., McGillivray, D., Peters, G., and Dickson, C. Characterization and chromosome assignment of the human homolog of *int-2*, a potential proto-oncogene. *Mol. Cell. Biol.*, **6**: 502–510, 1986.
- Jansson, M., Philipson, L., and Vennstrom, B. Isolation and characterization of multiple human genes homologous to the oncogenes of avian erythroblastosis virus. *EMBO J.*, **2**: 561–565, 1983.
- van't Veer, L. J., van Kessel, A. G., van Heerikhuizen, H., van Ooyen, A., and Nusse, R. Molecular cloning and chromosomal assignment of the human homolog of *int-1*, a mouse gene implicated in mammary tumorigenesis. *Mol. Cell. Biol.*, **4**: 2532–2534, 1984.
- Fujita, J., Srivastava, S. K., Kraus, M. H., Rhins, J. S., and Aaronson, S. A. Frequency of molecular alterations affecting *ras* proto-oncogenes in human urinary tract tumors. *Proc. Natl. Acad. Sci. USA*, **82**: 3849–3853, 1985.
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Washington DC)*, **239**: 487–491, 1988.
- Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H., and Goeddel, D. V. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature (Lond.)*, **302**: 33–37, 1983.
- Sanger, F., Nicklen, S., and Coulson, A. R. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**: 5463–5467, 1977.
- Bachmann, B., Luke, W., and Hunsmann, G. Improvement of PCR amplified DNA sequencing with the aid of detergents. *Nucleic Acids Res.*, **18**: 1309, 1990.
- Harlow, E., Crawford, L. V., Prim, D. C., and Williamson, N. M. Monoclonal antibodies specific for simian virus 40 tumor antigens. *J. Virol.*, **39**: 861–869, 1981.
- Stoker, M., O'Neill, G., Benyman, S., and Waxman, V. Anchorage growth regulation in normal and virus-transformed cells. *Int. J. Cancer*, **3**: 683–693, 1968.
- Soule, H. D., Maloney, T., and McGrath, C. M. Phenotypic variance among cells isolated from spontaneous mouse mammary tumors in primary suspension culture. *Cancer Res.*, **41**: 1154–1167, 1981.
- Dexter, D. L., Kowalski, H. M., Blazor, B. A., Fligiell, Z., Vogel, R., and Heppner, G. H. Heterogeneity of tumor cells from a single mouse mammary tumor. *Cancer Res.*, **38**: 3174–3181, 1978.

37. Michalopoulos, G., and Pitot, H. C. Primary culture of parenchymal liver cells on collagen membranes. *Exp. Cell Res.*, *94*: 70-78, 1975.
38. Yang, J., Richards, J., Bowman, P., Guzman, R., Enami, J., McCormick, K., Hamamoto, S., Pitelka, D., and Nandi, S. Sustained growth and three-dimensional organization of primary mammary tumor epithelial cells embedded in collagen gels. *Proc. Natl. Acad. Sci. USA*, *76*: 3401-3405, 1979.
39. Peters, L. J., and Hewitt, H. B. The influence of fibrin formation on the transplantability of murine tumor cells: implications for the mechanisms of the R'evesz effect. *Br. J. Cancer*, *29*: 279-291, 1974.
40. Dupressoir, T., Vanacker, J. M., Cornelis, J. J., Duponchel, N., and Romnelaere, J. Inhibition by parvovirus H-1 of the formation of tumors in nude mice and colonies *in vitro* by transformed human mammary epithelial cells. *Cancer Res.*, *49*: 3203-3208, 1989.
41. Yang, J., Balakrishnan, A., Hamamoto, S., Elias, J. J., Rosenau, W., Beattie, C. W., Das Gupta, T. K., Wellings, S. R., and Nandi, S. Human breast epithelial cells in serum-free collagen gel primary culture: growth, morphological, and immunocytochemical analysis. *J. Cell. Physiol.*, *133*: 228-234, 1987.
42. An International System for Human Cytogenetic Nomenclature. Basel: S. Karger AG, 1985.
43. Jeffreys, A. J., Wilson, V., and Thein, S. L. Hypervariable minisatellite regions in human DNA. *Nature (Lond.)*, *314*: 67-73, 1985.
44. Swallow, D. M., Gendler, S., Griffiths, B., Corney, G., Taylor-Papadimitriou, J., and Bramwell, M. E. The human tumour-associated epithelial mucins are coded by an expressed hypervariable gene locus *PUM*. *Nature (Lond.)*, *328*: 82-84, 1987.
45. van de Vijver, M., van de Bersselaar, R., Devilee, P., Cornelisse, C., Peterse, J., and Nusse, R. Amplification of the *neu (c-erbB-2)* oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked *c-erbA* oncogene. *Mol. Cell. Biol.*, *7*: 2019-2023, 1987.
46. Lidereau, R., Callahan, R., Dickson, C., Peters, G., Escot, C., and Ali, I. U. Amplification of the *int-2* gene in primary human breast tumors. *Oncogene Res.*, *2*: 285-291, 1988.
47. Krantiris, T. G., DiMartino, N. A., Colb, M., and Parkinson, D. R. Unique allelic restriction fragments of the *Ha-ras* locus in leukocyte and tumour DNAs of cancer patients. *Nature (Lond.)*, *313*: 369-374, 1985.
48. Jensen, F., Koprowski, H., Pagano, J. S., Ponten, J., and Ravdin, R. G. Autologous and homologous implantation of human cells transformed by simian virus 40. *J. Natl. Cancer Inst.*, *32*: 917-937, 1964.
49. Hayflick, L., and Moorhead, P. S. The cultivation of human diploid cell strains. *Exp. Cell Res.*, *25*: 585-621, 1961.
50. Ponten, T. P., and Westermark, B. Cell generation and aging of nontransformed glia cells from adult humans. In: S. Fedoroff and L. Hertz (eds.), *Advances in Cellular Neurobiology*, Vol. 1, pp. 209-227. New York: Academic Press, 1980.
51. Baden, H. P., Kubilus, J., Kvedar, J. C., Steinberg, M. L., and Wolman, S. R. Isolation and characterization of a spontaneously arising long-lived line of human keratinocytes (MM1). *In Vitro Cell. Dev. Biol.*, *23*: 205-213, 1987.
52. Woodworth, C. D., Bowden, P. E., Doniger, J., Pirijsi, L., Barnes, W., Lancaster, W. D., and DiPaolo, J. A. Characterization of normal human cervical cells immortalized *in vitro* by papillomavirus types 16 and 18 DNA. *Cancer Res.*, *48*: 4620-4628, 1988.
53. Rudland, P. S., Hallows, R. C., Cox, S. A., Ormerod, E. J., and Wasburton, M. J. Loss of production of myoepithelial cells and basement membrane proteins but retention of response to certain growth factors and hormones by a new malignant human breast cancer cell strain. *Cancer Res.*, *45*: 3864-3877, 1985.
54. Smith, H. S., Wolman, S. R., Dairkee, S. H., Hancock, M. C., Lippman, M., Leff, A., and Hackett, A. J. Immortalization in culture: occurrence at a late stage in the progression of breast cancer. *J. Natl. Cancer Inst.*, *78*: 611-615, 1977.
55. Sanford, K. K., Handiman, S. L., and Jones, G. M. Morphology and serum dependence of cloned cell lines undergoing spontaneous malignant transformation in culture. *Cancer Res.*, *37*: 821-830, 1977.
56. Sporn, M. B., and Roberts, A. B. Autocrine growth factors and cancer. *Nature (Lond.)*, *313*: 745-747, 1985.
57. Lang, W. E., Tokes, Z. A., Benedict, W. F., and Sargente, N. Anchorage independent growth and plasminogen activator production by bovine endothelial cells. *J. Cell. Biol.*, *84*: 281-293, 1980.
58. Soule, H. D., and McGrath, C. M. Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. *Cancer Lett.*, *10*: 177-189, 1980.
59. Shafie, S. M., and Giartham, F. H. Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic mice. *J. Natl. Cancer Inst.*, *67*: 51-56, 1981.
60. Kraus, M. H., Yuasa, Y., and Aaronson, S. A. A position 12-activated *H-ras* oncogene in all HS578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. *Proc. Natl. Acad. Sci. USA*, *81*: 5384-5388, 1984.
61. Sukumar, S., Carney, W. P., and Barbacid, M. Independent molecular pathways in initiation and loss of hormone responsiveness of breast carcinomas. *Science (Washington DC)*, *240*: 524-526, 1988.
62. Yuspa, S. H., Kilkenny, A. E., Stanley, J., and Lichti, U. Harvey and Kirsten sarcoma viruses block keratinocytes in an early, phorbol ester responsive stage of terminal differentiation. *Nature (Lond.)*, *314*: 459-467, 1985.
63. Brown, K., Quintanilla, M., Ramsden, M., Kerr, I. B., and Blamain, A. *v-ras* genes from Harvey and Balb murine sarcoma viruses can act as initiators of two-stage mouse skin carcinogenesis. *Cell*, *46*: 447-456, 1986.
64. Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., and Leder, P. Coexpression of MMTV/*v-Ha-ras* and MMTV/*c-myc* genes in transgenic mice: synergistic action of oncogenes *in vivo*. *Cell*, *49*: 465-475, 1987.
65. Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zanca, D., and Barbacid, M. Direct mutagenesis of *Ha-ras-1* oncogenes by *N*-nitroso-*N*-methylurea. *Nature (Lond.)*, *315*: 382-385, 1985.
66. Barbacid, M. *ras* genes. *Annu. Rev. Biochem.*, *56*: 779-827, 1987.
67. Rochlitz, C. F., Scott, G. K., Dodson, J. M., Liu, E., Dollbaum, C., Smith, H. S., and Benz, C. C. Incidence of activating *ras* oncogene mutations associated with primary and metastatic human breast cancer. *Cancer Res.*, *49*: 357-360, 1989.
68. Yang, J., Elias, J. J., Petrolis, L., Wellings, S. R., and Nandi, S. Effects of hormones and growth factors on human mammary epithelial cells in collagen gel cultures. *Cancer Res.*, *41*: 1021-1027, 1981.
69. Balakrishnan, A., Cramer, S., Bandyopadhyay, G. K., Imagawa, W., Yang, J., Elias, J., Beattie, C. W., Das Gupta, T. K., and Nandi, S. Differential proliferative response to linoleate in cultures of epithelial cells from normal human breast and fibroadenomas. *Cancer Res.*, *49*: 857-862, 1989.
70. Rudland, P. S. Stem cells and the development of mammary cancers in experimental rats and in humans. *Cancer Metastasis Rev.*, *6*: 55-83, 1987.
71. Cowell, J. K. Double minutes and homogeneously staining regions: gene amplification in mammalian cells. *Annu. Rev. Genet.*, *16*: 21-59, 1982.