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Suppression of tumor-forming ability and related traits in MCF-7 human breast cancer cells by fusion with immortal mammary epithelial cells

(tumor suppression/somatic cell hybrids/keratins/thrombospondin/fibronectin)

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ABSTRACT **Somatic cell hybrids between MCF-7 human breast cancer cells and normal immortalized human mammary epithelial cells have been obtained by polyethylene glycol-mediated cell fusion. The hybrid cells are suppressed in their ability to form tumors in nude mice, as well as in traits specific to the tumorigenic MCF-7 parent: growth factor independence, tumor necrosis factor sensitivity, and pS2 gene expression. In addition, they display other characteristics of the "normal" parent, including increased expression relative to the MCF-7 cells of the genes for the extracellular matrix component fibronectin, the intermediate filament keratin 5, and the angiogenesis inhibitor thrombospondin. The levels of keratins 8 and 18 also resemble those of the nontumorigenic parent. These results provide evidence for the existence of tumor suppressor gene products in immortal mammary epithelial cells. We propose a characteristic "suppressed" tumor cell phenotype, which encompasses altered cytoarchitecture, angiogenesis capabilities, and growth factor requirements.**

Evidence for the multistage nature of the carcinogenic process has been rapidly forthcoming in recent years. The genetic changes which foster the development of cancer can be separated into two classes. One includes mutations which affect cellular genes involved in proliferation so that their gene products are altered or inappropriately expressed (the classical oncogene concept). The other class targets the dominant-acting gene products of normal cells which protect them from cancer: tumor suppressor genes.

Retinoblastoma is the best-documented example of hereditary cancers which arise as a result of the inactivation of both alleles of a suppressor gene (refs. 1 and 2 and references therein). Evidence that the etiology of other cancers may also involve such loss of suppressor function is derived primarily from studies with somatic cell hybrids between normal and tumor cells (for review, see refs. 3 and 4). Suppression of tumorigenicity has been reported in crosses between normal and tumor cells of different species and tissue origin as well as in hybrids between distinct cancer types (ref. 3 and references therein). These observations support the idea that there are multiple mechanisms for tumor suppression (for review, see refs. 5 and 6), and further investigations have implicated tumor suppressor gene involvement in cell cycle control, signal transduction, regulation of angiogenesis, and development (reviewed in ref. 6).

We report here that cell hybrids produced by fusion of "normal" immortalized human mammary epithelial cells with MCF-7 human breast cancer cells are nontumorigenic. This result provides evidence for the existence of tumor suppressor genes in immortal breast epithelial cells. Other tumor cell traits are also suppressed, including growth factor independence, sensitivity to tumor necrosis factor (TNF), and expression of the breast cancer marker pS2 (7).

The transformation-suppressing capability of human chromosome 1 in human–hamster hybrids correlates with the production of thrombospondin (TSP), an inhibitor of angiogenesis (8, 9). Consistent with the potential link between inhibition of angiogenesis and tumor suppression (for discussion, see ref. 10), we find that both the immortal mammary epithelial cells and the hybrids have higher levels of TSP mRNA than do the tumorigenic MCF-7 cells.

Studies comparing gene expression patterns of similarly cultured normal and tumorigenic breast epithelial cells have shown that, in the absence of gene amplification, there is a remarkable equivalence in the steady-state levels of mRNAs for genes such as c-*myc*, Ha-*ras*, *erbB2*, retinoblastoma, and transforming growth factor β (TGF-β) (11). By employing subtractive hybridization techniques to probe a normal mammary epithelial cell cDNA library, it has been possible to identify sequences preferentially expressed in normal but not in tumor cells (12, 13). The expression of two of these genes, coding for keratin 5 (K5) and fibronectin, has been analyzed in the hybrid cells. Both the immortalized normal parent and the hybrids produce significantly higher amounts of these mRNAs than do the MCF-7 tumor cells. This result is consistent with suppression of the MCF-7 cell phenotype in these hybrids.

MATERIALS AND METHODS

Cell Culture. Benzo[*a*]pyrene-immortalized, nontumorigenic, breast epithelial cells (184B5) were obtained from M. Stampfer (14) and cultured in DFCI-1 medium (called D medium) as previously described (15). MCF-7 cells were maintained in α medium (15) and cultured in D medium for experiments as indicated. Hybrid cell populations were routinely cultured in D medium; all experiments reported here were performed with cells at approximately the same passage in culture as those used for the tumorigenicity testing.

Calcium Phosphate Transfection. 184B5 cells (plated at 10^6 cells per 100-mm dish) or MCF-7 cells (5×10^5 per dish) were transfected with 2 μg of pSV2hygro or 2 μg of pSV2neo and 8 μg of carrier pSP65 DNA, respectively, by the calcium phosphate coprecipitation procedure (16). Approximately 8 hr after transfection, cells were osmotically shocked with 15% (vol/vol) glycerol in solution A [a balanced salts solution

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Abbreviations: TNF, tumor necrosis factor; RFLP, restriction fragment length polymorphism; TSP, thrombospondin; EGF, epidermal growth factor; K5, keratin 5; TGF, transforming growth factor.

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(17)] for 4 min, washed twice with solution A, and fed fresh medium. Selection in hygromycin B at 50 μg/ml (184B5 cells) or G418 at 800 μg/ml (MCF-7 cells) was started 2 days later and clones (named B5hygro and MCF-7neo) were picked and expanded within 14 days.

Cell Fusion. A mixture of 10^6 cells each (per 100-mm dish) of B5hygro and MCF-7neo was plated in D medium. After an overnight incubation, the plates were washed once with D medium salts solution and exposed to 1 ml of 50% (wt/vol) polyethylene glycol ($M_r \approx 1000$; Baker) in D medium salts solution for 1 min. Upon removal of the polyethylene glycol, the cells were washed and fed with fresh medium. The following day, the cells were replated at 5×10^5 cells per 100-mm dish. Selection in hygromycin B (Calbiochem) at 50 μg/ml and G418 (GIBCO) at 400 μg/ml was initiated the next day and clones were picked into 24-well plates 9 days later. No clones were observed on control plates from fusions of MCF-7neo with MCF-7neo or B5hygro with B5hygro cells. Hybrid B5hygro–MCF-7neo and parental cell populations were negative for mycoplasma contamination as assayed with the GIBCO Mycotect kit.

Hybrid cell lines 4-2A and 4-10-1 were derived from hybrids 4-2 and 4-10 (passages 14 and 7, respectively) by selection for growth in α medium. 4-2A is the mixed cell culture which survived this selection and 4-10-1 is a subcloned population.

Cell Growth Studies. Parental cells (10^5 cells per Falcon T-25 flask) and hybrid cells (2×10^4 cells per T-25 flask) were plated in D or D−S medium [D medium minus supplementary epidermal growth factor (EGF), insulin, cholera toxin, triiodothyronine, hydrocortisone, and bovine pituitary extract]. Medium changes were the following day and every 2 days until harvest when the control flasks were approximately confluent. At that time, cells were trypsinized and counted in a Coulter counter.

TNF Sensitivity Determination. Cells were plated overnight in 60-mm dishes at the indicated cell densities in D medium and treated with TNF (Asahi Chemical Industries, Tokyo) for 96 hr. After this incubation period, cells were fed with fresh medium and cultured for 1–2 weeks with feeding on alternate days. Surviving colonies were fixed with 10% buffered Formalin, stained with 1% crystal violet (in 70% ethanol), and visually counted.

Subrenal Capsule Tumorigenicity Assay. Parental and hybrid cells ($5–10 \times 10^6$ cells from T-150 flasks in which the cells were 70–80% confluent) were trypsinized and washed three times in D medium before formation of a fibrin clot as described by Fingert *et al.* (18). Implantation of 0.5- to 1.0-mm fibrin clot fragments (containing an estimated $1–2 \times 10^5$ cells) under the kidney capsule of athymic, nude, female mice was performed essentially as described in the original Bogden protocol (19). All mice also received an estrogen pellet (0.72 mg, 60-day release; Innovative Research of America) inserted subcutaneously in the interscapular area. After 3–4 weeks, animals were sacrificed by cervical dislocation and the kidneys were inspected for tumor growth. Tumors and kidney sections for histological examination were fixed in 10% buffered Formalin and processed by routine procedures for hematoxylin/eosin staining.

Chromosome Counts. Logarithmically growing cells were treated for 2 hr with Colcemid (GIBCO) at 0.1 μg/ml, harvested by trypsinization, swollen for 30 min in 75 mM KCl at room temperature, and fixed in methanol/acetic acid (3:1, vol/vol). Cells were stored at 4°C until slides were prepared by standard techniques and Giemsa stained. The number of chromosomes in at least 10 good metaphase spreads was determined.

Northern and Southern Blot Analysis. Total cellular RNA was isolated from ≈70% confluent cultures of cells grown in D medium; Northern analysis was as previously described (11), except that the prehybridization and hybridization were performed in 50% (wt/vol) formamide (Fluka)/0.25 M sodium phosphate, pH 7.0/7% $NaDodSO_4$ (Bio-Rad)/5% dextran sulfate containing denatured sonicated salmon sperm DNA at 250 μg/ml.

For restriction fragment length polymorphism (RFLP) analysis, 10 μg of DNA was digested with *Taq* I, *Msp* I, or *Bam*HI as indicated and agarose gel electrophoresis, capillary transfer in 10× SSC (1.5 M NaCl/0.15 M sodium citrate, pH 7.0) to nylon filters (Zeta-probe, Bio-Rad), and hybridization were as previously described (11).

Source of Hybridization Probes. DNA probes for EGF receptor, pS2, and TGF-α were as previously described (11). Others are as follows: the *Hin*dIII 1.0-kilobase (kb) fragment from p9A7 (20) recognizes the D13S3 locus on chromosome 13; the *Pst* I 633-base-pair (bp) fragment from pEM36 (21) is specific for calcitonin; the 5.8-kb *Sst* I–*Kpn* I fragment from 6STXE (25) for TSP; the 250-bp *Xba* I–*Eco*RI fragment encoding the 3′ untranslated region of K5 RNA (13); and the 1.0-kb *Eco*RI fragment corresponding to the 3′ untranslated region of fibronectin (12).

Keratin Protein Analysis. Nearly confluent cultures of parental and hybrid cells grown in D medium in 60-mm plates were isotopically labeled with [^{35}S]methionine (Amersham) at 50 μCi/ml for 2 hr (1 Ci = 37 GBq). Extracts were prepared as described (13) and 100,000 cpm per lane was analyzed by $NaDodSO_4$/10% polyacrylamide gel electrophoresis.

RESULTS

Selection of Somatic Cell Hybrids Between 184B5 and MCF-7 Cells. Benzo[*a*]pyrene-immortalized breast epithelial cells (184B5) and MCF-7 breast tumor cells were transfected with plasmids bearing the bacterial genes encoding hygromycin and G418 (neo) resistance, respectively. Isolated clonal populations (B5hygro and MCF-7neo) were fused by using polyethylene glycol; selection in hygromycin/G418-supplemented D medium yielded stable somatic cell hybrids at a frequency of 7×10^{-6}. The hybrid cells are morphologically different from both parents, being considerably larger than the B5hygro cells and lacking the cuboidal appearance of the MCF-7neo cells (Fig. 1). All of the hybrids are resistant to concentrations of G418 (400 μg/ml) and hygromycin (50 μg/ml) which kill the parental cells (data not shown), indicating that the drug-resistance markers are present.

Chromosomal and DNA Analysis. Karyotypic analyses of the B5hygro and MCF-7neo cells revealed that the B5hygro

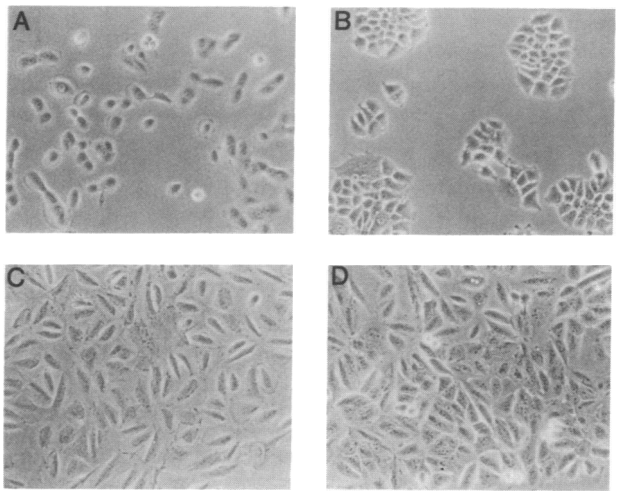

FIG. 1. Morphology of parental and hybrid cells. Light micrographs of B5hygro (*A*), MCF-7neo (*B*), hybrid 5-2 (*C*), and hybrid 4-10-1 (*D*). (Phase-contrast optics; ×40.)

cells are pseudodiploid (46 chromosomes, including several markers) and the MCF-7neo are aneuploid (75 chromosomes), in agreement with published reports (26, 27). Most of the hybrid cell populations contain the approximate number of chromosomes expected for a fusion between these two parents (e.g., 4-1, 4-2, 4-2A, 4-10, 4-10-1, 4-13, and 5-4); others have a somewhat greater chromosomal content (Table 1). Analysis of the DNA content by cytofluorography generated results consistent with the chromosomal counts (data not shown). Cytofluorimetry was therefore utilized to ascertain the relative stability of the hybrid cell populations over the course of the experiments presented here.

The hybrid nature of these cells was also confirmed by RFLP analysis of specific gene loci. Alleles which distinguish the parental cell chromosomes 11 and 13 [which harbor putative suppressor genes for breast cancer (28, 29)] as well as chromosomes 2 and 7 are present in the hybrid cells (Fig. 2). The hybrids shown here (4-1, 4-13, and 5-2) contain the alleles for the loci analyzed on both parental chromosomes 13 (D13S3), chromosome 11 (calcitonin) from the B5hygro parent, and chromosomes 2 (TGF-α) (30) and 7 (EGF receptor) (31) from the MCF-7neo parent. Other analyses (not shown) demonstrate that the allele from the B5hygro parent for the EGF receptor gene on chromosome 7 is also retained in these and the other hybrid cells.

Suppression of Tumorigenicity in Hybrid B5hygro–MCF-7neo Cells. The ability of the hybrid cell lines to form tumors in nude mice was compared with that of the parental tumorigenic MCF-7neo cells and nontumorigenic B5hygro cells in the subrenal capsule assay (19). In 11 of 13 cases (85%; Table 1), MCF-7neo cells formed nodules at the site of implantation which were confirmed to be neoplastic by histological examination (Fig. 3). Neither the B5hygro parent nor any of the hybrid cells produced tumors by gross or microscopic analysis of kidney sections taken from the implantation site.

Suppression of Tumor-Specific Traits in Hybrid Cells: Growth Factor Independence, TNF Sensitivity, and pS2 Expression. Both the normal immortalized and tumorigenic breast epithelial cells proliferate in the growth factor-enriched D medium (population doubling time 20 hr for B5hygro, 39 hr for MCF-7neo). Deletion of EGF, insulin, hydrocortisone, cholera toxin, triiodothyronine, and bovine pituitary extract generates a minimal medium containing only

Table 1. Characteristics of B5hygro–MCF-7neo hybrids

Cells	Chromosomes* Mode	Chromosomes* Range	Tumorigenicity,† tumors/sites	Growth‡ D	Growth‡ D–S
Parents					
B5hygro	46		0/4	+	–
MCF-7neo	75	69–77	11/13	+	+
Hybrids					
4-1	105	88–109	0/5	+	–
4-2	110	94–112	0/3	+	–
4-7	146	120–155	0/5	+	–
4-8	137	122–138	0/5	+	–
4-10	101	85–111	0/3	+	–
4-13	105	90–118	0/4	+	–
5-2	139	86–139	0/5	+	–
5-4	115	103–119	0/3	+	–
4-2A	105	69–109	0/5	+	–
4-10-1	107	97–111	0/4	+	–

*Chromosome counts from at least 10 metaphase spreads.
†Cell growth in the subrenal capsule was scored 3–4 weeks after implantation of $\approx 10^5$ cells. Tumors varied from 2 to 5 mm in diameter.
‡Growth factor requirement is tabulated as growth (+) or no growth (–) in two media: D, D medium; D–S, D medium minus supplements (EGF, insulin, hydrocortisone, cholera toxin, triiodothyronine, and bovine pituitary extract).

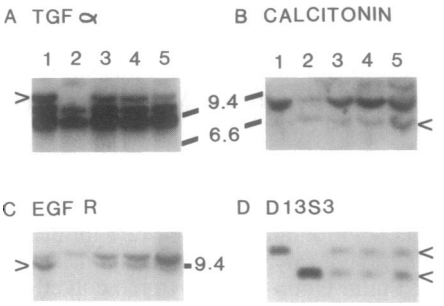

FIG. 2. RFLP analysis identifies parental chromosomes 2, 7, 11, and 13 in hybrid cells. Ten micrograms of DNA from MCF-7neo (lane 1), B5hygro (lane 2), hybrid 4-1 (lane 3), hybrid 4-13 (lane 4), and hybrid 5-2 (lane 5) was digested with *Bam*HI (*A*), *Taq* I (*B* and *C*), or *Msp* I (*D*) and analyzed by Southern blotting with the indicated probes (EGF R, EGF receptor). Arrowheads indicate RFLP specific to each probe (sizes of polymorphic fragments are *A*, 15 kb; *B*, 6.5 kb; *C*, 10 kb; and *D*, 1.45 and 1.0 kb). Sizes (in kb) of *Hin*dIII-digested λ phage DNA fragments (used as markers) are indicated.

1% fetal calf serum (D–S). The tumorigenic MCF-7neo cells grow somewhat better in this medium than in the complete medium (34 hr doubling time). By contrast, D–S medium does not support the growth of the B5hygro cells (Table 1), thereby demonstrating their requirement for growth factors not provided by this low concentration of fetal calf serum. The ability of the hybrid cell populations to grow in these two media was assessed. As indicated in Table 1, the hybrids are similar to the nontumorigenic parent in that they do not grow in D–S medium.

A wide range of tumor cell types are sensitive to the cytotoxic effects of TNF (32). Among the breast cancer cell lines tested, MCF-7 cells are particularly sensitive (33), whereas normal and nontumorigenic immortal breast epithelial cells are TNF resistant (V.B., P. Yaswen, and R.S., unpublished observation). As shown in Table 2, treatment with TNF had little effect upon B5hygro plating efficiency. In contrast, MCF-7neo growth is dramatically inhibited by this concentration of TNF. Analysis of several of the hybrid cell populations revealed that, like the B5hygro parent, they are all resistant to the cytotoxic effects of TNF (Table 2).

Sixty percent of all breast cancers which contain estrogen receptors also produce pS2 (7), a secreted protein with unknown function (34). MCF-7 cells synthesize large quantities of pS2 mRNA (24), whereas normal breast epithelial cells and immortal nontumorigenic cells show no detectable expression (Fig. 4*A* and ref. 11). As shown in Fig. 4*A*, expression of pS2 is extinguished in all of the hybrid cells examined.

Dominance of the B5hygro Phenotype: TSP, Fibronectin, and Keratin Gene Expression. Comparative analysis of the mRNA levels for K5, fibronectin, and TSP in the nontumorigenic immortal B5hygro cells and the tumorigenic MCF-7 cells

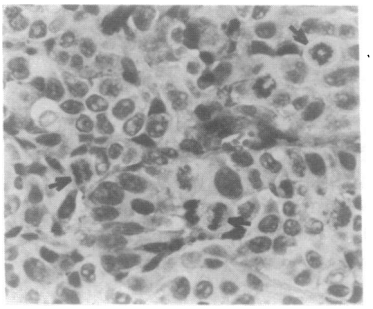

FIG. 3. Histology of MCF-7neo subrenal capsule tumor. Section reveals a poorly differentiated carcinoma with a high mitotic rate (see arrows). (×240.)

Table 2. TNF resistance of B5hygro and hybrid cell lines

Cells	Plating efficiency, %		+ TNF/ control, %
	Control	+ TNF	
Parents			
B5hygro	25	18	72
MCF-7neo	79*	0.05*/0.01	0.06*/0.01
Hybrids			
4-1	19	13	68
4-2	18	12	67
4-7	21	18	86
4-8	29	26	90
4-10	23	14	61
4-2A	15	10	67

B5hygro and hybrid cells were plated in triplicate in D medium at 200–500 cells per 60-mm dish. MCF-7neo cells were plated at 200 cells per dish (controls) and 5×10^5 cells per dish (+ TNF, 50 units/ml) in α medium (*) or D medium.

showed that they are all elevated in the B5hygro cells (Fig. 4 *B*, *C*, and *D*). To assess the potential significance of this differential expression for the carcinogenic process, the hybrid cells were screened for their expression of these genes.

TSP mRNA levels in the hybrids (Fig. 4*B*) vary from 8-(4-2A) to 75-fold (4-13) higher than the level found in the MCF-7neo cells. These data are for RNA isolated from cells ≈70% confluent. We have noted significant variation in relative TSP expression in different preparations of RNA. This presumably reflects differences in the growth state of the cells, since TSP expression is serum regulated (35) and cell density dependent (36) in some cell types, although variation due to chromosomal segregation cannot be ruled out. MCF-7neo production of this mRNA, however, was consistently lower than that found in any of the hybrid cells and did not fluctuate to the same extent (data not shown).

High expression of the mRNA for the extracellular matrix component fibronectin is observed in the B5hygro cells, whereas none is detectable in the MCF-7neo tumor cells. As can be seen from Fig. 4*D*, all of the hybrid cell lines produce this mRNA, although the levels vary.

A similar conclusion can be drawn for K5 mRNA expression. It is undetectable in the MCF-7neo cells, yet all of the hybrid cells produce significant amounts (Fig. 4*C*). Since post-transcriptional mechanisms have been shown to play a role in the control of keratin gene expression (37), the keratin

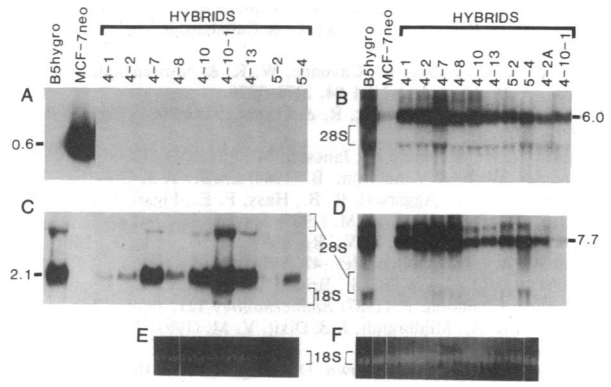

FIG. 4. Expression of pS2, TSP, fibronectin, and K5 mRNA in parental and hybrid cells. Northern blots of 20 μg of total RNA isolated from the indicated cells were hybridized with probes for pS2 (*A*), TSP (*B*), K5 (*C*), or fibronectin (*D*). The positions of the 18S and 28S rRNAs as well as the size (in kb) of the specific transcripts are indicated. Photographs of the ethidium bromide stained 18S rRNA bands are provided (*E* for blots *A* and *C*; *F* for blots *B* and *D*) to demonstrate equivalent sample loading and RNA integrity. These blots are representative of analyses of at least three separate RNA preparations for each cell line.

profile of the hybrid cells was compared with the profiles of the B5hygro and MCF-7neo parents (Fig. 5). The nontumorigenic B5hygro cells synthesize keratins characteristic of both basal (keratins 5 and 14; see ref. 38) and lumenal (keratins 8 and 18; ref. 38) epithelial cells (Fig. 5, lane 1). MCF-7neo cells produce lumenal cell-specific keratins 8, 18, and 19 (Fig. 5, lane 2); K5 expression is undetectable. K5 production is evident in many of the hybrid cell lines (Fig. 5, lanes 4, 5, 7, 8, 10, and 11) and roughly parallels the quantities of K5 mRNA found (compare Fig. 4*C* with Fig. 5). The variability in the amounts of K5 protein contrasts with the consistently low levels of keratins 8 and 18 in all of the hybrid cells, which mirror the low level found in the B5hygro parent. Both of these keratins are more highly expressed in the MCF-7neo than in the B5hygro cells. No consistent pattern of keratin 14, 17, or 19 expression is observed in the hybrid cells.

DISCUSSION

The presence in immortal nontumorigenic mammary epithelial cells (184B5) of gene products capable of suppressing the tumor-forming ability of a metastatic breast cancer cell line (MCF-7) has been demonstrated by somatic cell fusion of these two cell types. In contrast to the behavior of the MCF-7 cells, the hybrid cells do not form tumors when implanted under the renal capsule of athymic nude mice. In addition to tumorigenicity, other phenotypes manifest by the MCF-7 cells are also suppressed in all of the hybrid cells analyzed. These include growth factor independence, sensitivity to TNF, and expression of the estrogen-inducible pS2 gene, which is an estrogen receptor-positive breast cancer marker (7).

Like the nontumorigenic 184B5 parent, the hybrid cells have more stringent requirements for cell culture growth. We have found that the growth factors present in 1% fetal calf serum are insufficient for their proliferation, although the MCF-7 cells grow without further supplementation. Further studies will be necessary to identify the factors essential for hybrid cell growth, which may correlate with the tumor-suppressing activity of the 184B5 parent.

Resistance to the cytotoxic effects of TNF has been shown to be dominant by analysis of these hybrid cells. The genetic factors which determine cellular resistance to TNF are not understood, although it has been shown that some tumor cells which become resistant also produce TNF (39). The resistant hybrid cells provide additional material for experiments on the mechanism of TNF action.

The absence of detectable pS2 mRNA in the hybrid cells is most likely due to the extinction of estrogen receptor expression in these cells (D.A.Z. and R.S., unpublished results), since pS2 expression is controlled by estrogen receptor binding to pS2 promoter elements (40). It is intriguing to speculate that tumorigenicity of the estrogen-dependent MCF-7 cells may be suppressed in the hybrids through a mechanism which blocks the expression of this essential transcription factor. In estrogen-dependent tumor cells, the activity of genes critical to the process of tumor growth may

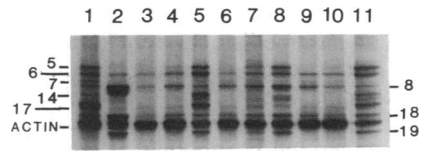

FIG. 5. Keratin protein profiles for parental and hybrid cells. $NaDodSO_4$/PAGE of [^{35}S]methionine-labeled extracts. Lane 1, B5hygro; lane 2, MCF-7neo; lanes 3–11, hybrids 4-1, 4-2, 4-7, 4-8, 4-10, 4-13, 5-2, 5-4, and 4-10-1. The position of specific keratin proteins and actin are indicated. The identity of these proteins has been verified by two-dimensional gel electrophoresis for the parents (13) as well as for several of the hybrid cell populations.

be totally dependent upon estrogens; without a receptor, these genes would be inactive in the hybrid cells.

The suppressed hybrid cells are phenotypically identical to the 184B5 nontumorigenic parent in a number of additional properties. Both 184B5 and hybrid cells produce significantly higher levels of the mRNA for TSP, an abundant glycoprotein in platelet cell α granules, which is also made by a variety of other cell types (see ref. 41 for review). A role for TSP in hemostasis and perhaps wound healing has been proposed on the basis of its probable involvement in platelet cell adhesion and aggregation and its mitogenic effects on fibroblasts (see ref. 41 and references therein). More recently, it has been identified as an inhibitor of angiogenesis and its presence has been shown to correlate with the suppression of transformation by human chromosome 1 in human hamster hybrids (8, 9). In this regard, it is interesting that chromosome 1 aberrations have been found in breast tumor biopsies by cytogenetic and RFLP analyses (for review, see ref. 22).

Morphological differences between normal and transformed cells most likely reflect alterations in genes controlling cell structure, extracellular matrix attachment, and cell–cell communication. Analysis of the expression of the genes for some of the proteins which may be important in determining these features (i.e., keratins, fibronectin, and possibly TSP) has demonstrated striking differences between normal and breast cancer cells (refs. 12 and 13 and this study). The normal immortalized 184B5 cells and the suppressed hybrid cells contain much higher levels of fibronectin mRNA than the tumorigenic MCF-7 cells. Also in support of the potential involvement of fibronectin regulation in tumorigenicity is the observation of Steel and Harris (23), who showed that expression of antisense fibronectin mRNA in suppressed mouse melanoma–fibroblast hybrids made them tumorigenic.

K5 gene expression is also elevated, relative to the MCF-7 cells, in the immortal and suppressed hybrid cells. The inverse situation is true for production of keratin 8 and 18 by both the 184B5 and hybrid cells, where reduced amounts accumulate in comparison with the tumor cells.

The dominance of the nontumorigenic 184B5 phenotype is particularly interesting in view of recent studies using *in vitro* cell culture models for breast cancer. Trask *et al.* (13) have shown that levels of K5 decrease in a stepwise fashion during the progression of a mammary epithelial cell through the phases of immortalization and acquisition of tumorigenic capabilities. The inverse correlation is true for expression of keratin 18, with production of this protein increasing along the pathway to cancer (13). Since changes in the cytoarchitecture itself may modulate gene expression and perhaps influence the transformation process (for review, see ref. 24), it is conceivable that one mechanism of tumor suppression may be through control of the type and quantity of intermediate filaments synthesized.

In summary, immortal nontumorigenic mammary epithelial cells express gene products capable of inhibiting the tumorigenic ability of MCF-7 breast cancer cells. A number of characteristics specific to the nontumorigenic parent have been retained by all of the hybrid cells. These include the production of high levels of mRNA for the angiogenesis inhibitor TSP, an intermediate filament keratin (K5), and the extracellular matrix component fibronectin. The relative amounts of keratins 8 and 18 also resemble those in the "normal" parent. In the hybrid cells, the pathways responsible for the cancer cell phenotypes of growth factor independence, pS2 expression, and TNF sensitivity are extinguished. These data suggest that the likely modifications in cell attachment, cytostructure, gene expression, and angiogenic potential dictated by the "normal" cell genome in these hybrids are important for suppression of tumorigenicity.

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