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Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells

(extracellular matrix/rapid transformation assay/breast cancer/tissue structure and function)

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ABSTRACT **Normal human breast epithelial cells show a high degree of phenotypic plasticity in monolayer culture and express many traits that otherwise characterize tumor cells *in vivo*. Paradoxically, primary human breast carcinoma cells are difficult to establish in culture: most outgrowths arise from the normal tissue surrounding the tumor. These characteristics have posed major obstacles to the establishment of simple reliable criteria for mammary epithelial transformation in culture. In the present study, we show that a reconstituted basement membrane (BM) can be used to culture all normal human breast epithelial cells and a subset of human breast carcinoma cells. The two cell types can be readily distinguished by virtue of the ability of normal cells to reexpress a structurally and functionally differentiated phenotype within BM. Twelve specimens of normal breast tissue and 2 normal breast epithelial cell lines (total 14 samples) embedded in BM as single cells were able to form multicellular spherical colonies with a final size close to that of true acini *in situ*. Sections of mature spheres revealed a central lumen surrounded by polarized luminal epithelial cells expressing keratins 18 and 19 and sialomucin at the apical membrane. Significantly, two-thirds of normal spheres deposited a visible endogenous type IV collagen-containing BM even though they were in contact with exogenously provided BM. Growth was arrested completely within the same time period. In contrast, none of 6 carcinoma cell lines or 2 cultures of carcinoma from fresh samples (total 8 samples) responded to BM by growth regulation, lumen formation, correct polarity, or deposition of endogenous BM. These findings may provide the basis of a rapid assay for discriminating normal human breast epithelial cells from their malignant counterparts. Furthermore, we propose that the ability to sense BM appropriately and to form three-dimensional organotypic structures may be the function of a class of "suppressor" genes that are lost as cells become malignant.**

Development of human breast neoplasia is believed to occur through multiple steps of genotypic and phenotypic alteration in the luminal epithelial cells of terminal duct lobular units (1–3). Normal terminal duct lobular units are lined by a single layer of polarized luminal epithelial cells showing apical expression of a variety of mucus glycoproteins (sialomucins) (4, 5). The basolateral surface rests on a basement membrane (BM) and a discontinuous layer of myoepithelial cells (2). Studies in the rodent have shown that the extracellular matrix regulates the growth and differentiation of normal mammary epithelial cells *in vivo* and in culture (6–13).

Thus far, efforts to study early events in human breast tumor development have been hampered by the lack of adequate means to distinguish between normal and transformed cells in culture and have relied on the criteria of senescence and immortalization (14–19)—phenomena that as yet do not have clear counterparts *in vivo* and take a considerable amount of time to establish.

We asked whether normal human breast epithelial cells could respond to BM and recapitulate certain aspects of their normal growth and differentiation program as has been shown using rodent models (8–12). We show that human mammary epithelial cells can indeed express a normal pattern of growth and differentiation when cultured within a reconstituted BM derived from the Englebreth–Holm–Swarm (EHS) tumor. Furthermore, in response to exogenous BM, the cells basally deposit an endogenous BM. In contrast, carcinoma cell lines and biopsies from primary breast tumors were not capable of responding appropriately to BM nor were they able to deposit an intact endogenous BM.

Other investigators have also used an EHS matrix to study human cell lines, including metastatic breast tumor cells (20–25). These studies have focused on morphology of the cells on an EHS matrix. By including primary tissues and addressing growth as well as three-dimensional architecture in normal and malignant cell lines and primary cultures, we have been able to exploit cell–extracellular matrix interaction as an assay not only to distinguish between normal and malignant breast cells but also possibly to "grade" atypia and malignancy. In addition, the system can be used to define differentiation markers and to delineate early changes in transformation assays or preneoplastic lesions.

MATERIALS AND METHODS

Cell Culture. Primary breast epithelia were prepared from 12 reduction mammoplasties and three breast carcinoma biopsies (two primaries and one lymph node metastasis). The primary specimens were selected, disaggregated, and cultured in serum-free CDM3 medium (26) without Hepes or phenol red in the basal medium (Dulbecco's modified Eagle's medium/F12) and with triiodothyronine at 10 nM. We used two normal breast epithelial cell lines [HMT-3522 (27) and MCF-10A (28)], another "normal" line [HBL-100 (29)], and six tumorigenic breast cell lines. These included HMT-3909S13 (30, 31), MCF-7 (including subline 9), ZR75, T47-D, and BT-20 (32, 33), and CAMA-1 (34). These were cultured as described initially except for MCF-10A, which was cultured in the same medium as HMT-3522. The normal cells were either passaged into monolayer culture or into 300 μl of

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Abbreviations: BM, basement membrane; EHS, Englebreth–Holm–Swarm.

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EHS matrix (7–10 μg/ml, obtained as Matrigel; Collaborative Research) as single cells at 2.5×10^5 cells per well of a 24-well plate (Nunc). In some experiments, cells were plated on top of the EHS matrix (150 μl per well). Carcinoma cell organoids were harvested after 1–2 days of suspension culture in CDM3 and embedded in the EHS matrix at 250–900 organoids (10–50 cells per organoid). A lower layer of 100 μl of EHS matrix was gelled before adding the organoid/EHS matrix suspension, to avoid organoid contact with plastic. For replating, the gels were rinsed in 150 μl of dispase (Collaborative Research) and further incubated for 1–2 h at 37°C in 500 μl of dispase. Dispase was inactivated by dilution. The medium was changed (1 ml per well) every second or third day and cultures were kept in a humidified atmosphere of 5% CO_2/95% air.

Cell Growth Determination. Cell growth was determined by the following criteria. (*i*) The size of growing spherical structures was measured with an eye piece equipped with a micrometer spindle. Normal spheres were selected based on the presence of a central lumen. For tumors the 20 largest colonies were measured because of continual colony turnover. (*ii*) [^{3}H]Thymidine incorporation over a 24-h period (20 Ci/mmol; 2.5 μCi/ml; 1 Ci = 37 GBq; NET-027X, NEN) was measured. Immunoperoxidase cytochemistry (F3006 and 115D8, see below) was performed on monolayer cultures and frozen sections prior to autoradiography to quantitate luminal epithelial cells (35). (*iii*) The final number of cells either in normal spherical profiles or carcinoma colonies in sections of EHS gels was counted.

Immunocytochemistry. Frozen sections (5 μm) were prepared from cultures in the EHS matrix. Sections were stained for cytokeratin K18 (F3006, Sanbio, Am Uden, The Netherlands) (15) and K19 (M772, Dakopatts, Glostrup, Denmark). Milk fat globule membrane antigen was detected with monoclonal antibody 115D8 (15). BM type IV collagen was detected with three antibodies, M785 (Dakopatts), PHM-12 (AMD, Armaton, NSW, Australia), and COP (Medac, Hamburg, F.R.G.) (15). Double labeling (35) was performed with COP and fluorescein-coupled goat anti-rabbit IgG (6200, Tago) plus either antibody 115D8 or antibody F3006 and Texas red-coupled anti-mouse IgG (1030-07, Southern Biotechnology Associates, Birmingham, AL). Control sections were stained with second antibodies only.

RESULTS

Sensitivity of Normal Breast Epithelial Cells to the EHS Matrix. We first defined the behavior of normal breast epithelial cells in the EHS matrix. Normal organoids from 12 reduction mammoplasties (Fig. 1 *a* and *b*) in primary monolayer cultures grew exponentially as described (15, 26). The cells were passaged at high density to ensure complete disruption of the structural organization of the organoids and to enrich for luminal epithelial (as opposed to myoepithelial) cells (Fig. 1*c*). In the third passage, cells were either replated in monolayer culture or embedded in the EHS matrix as single cells. Within 7 days of cultivation, the cells within the EHS matrix had formed a population of almost uniform spherical colonies (Fig. 1*d*) that enclosed a small lumen of <20% of the total diameter (Fig. 1*e*). Mean diameter of spheres and [^{3}H]thymidine labeling index were measured in 3 of the 12 primary cultures as a measure of growth. The initial growth rate in the EHS matrix was higher than in parallel monolayer cultures (Fig. 2*a*). Cultures in the EHS matrix grew exponentially until day 7 and then arrested abruptly. In contrast, cells grown in monolayer did not form spheres and grew continuously. The lack of growth after 7 days within the EHS matrix was not due to a lack of mitogen access since cells plated on top of the EHS matrix also formed small spheres (data not shown). Similarly, regulation

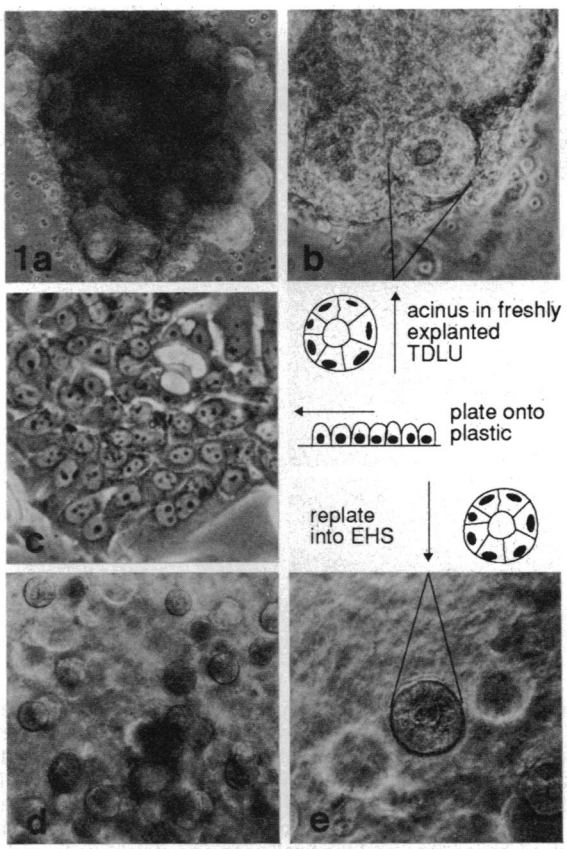

FIG. 1. Phase-contrast micrographs of normal primary breast epithelial cells. (*a*) Freshly explanted terminal duct lobular unit at low magnification. (*b*) Single acinus in focus at a higher magnification. (*c*) Second-passage cells in monolayer culture. (*d*) Fully developed spheres in the EHS matrix. (*e*) Single sphere at higher magnification. (*a* and *d*, ×100; *b*, *c*, and *e*, ×250.)

of growth was not due to inhibitory factors in the EHS matrix, since cells in a monolayer overlaid with EHS matrix grew at the same rate as in the monolayer alone. The final mean diameter varied from 38.7 ± 3.8 μm to 50.3 ± 7.2 μm, depending on the biopsy of origin (Fig. 2 *a* and *b*). For comparison, the mean diameter of acini from freshly explanted tissue was 48.6 ± 19.3 μm. Resemblance to acini *in vivo* was further confirmed by the appearance of larger ducts (Fig. 2*c*), small connecting ductules between the spheres (Fig. 2*d*), lumen formation in sectioned spheres (Fig. 2*e*), and the expression of luminal epithelial cell keratins K18 (Fig. 2*f*) and K19 (data not shown). The mean number of cells in equatorial sections of spheres was 8 ± 1.2 (Fig. 2*e*).

Distinctive Growth Regulation of Breast Carcinoma Cells vs. Normal Cells in the EHS Matrix. In view of the capacity of the EHS matrix to arrest the growth of normal breast epithelial cells, we analyzed the pattern of breast carcinoma cell growth under similar conditions. [^{3}H]Thymidine incorporation in two normal primary cultures and two normal cell lines was compared with that in three primary carcinoma cultures and six established breast carcinoma cell lines at days 6 and 12 (Fig. 3*a*). Both normal primary cultures and normal cell lines formed spheres and were growth-arrested at day 12. The growth of cell lines in the absence of the EHS matrix is shown in Fig. 3*a*. Normal cell lines formed spheres of similar size and cell number to the normal primary cells (Fig. 3 *b* and *c*). The growth-arrested cells in the EHS matrix were not terminally differentiated and had not degenerated since it was possible to recover exponentially growing cultures after dispase treatment (data not shown). We also analyzed an additional cell line (HBL-100) that is considered normal,

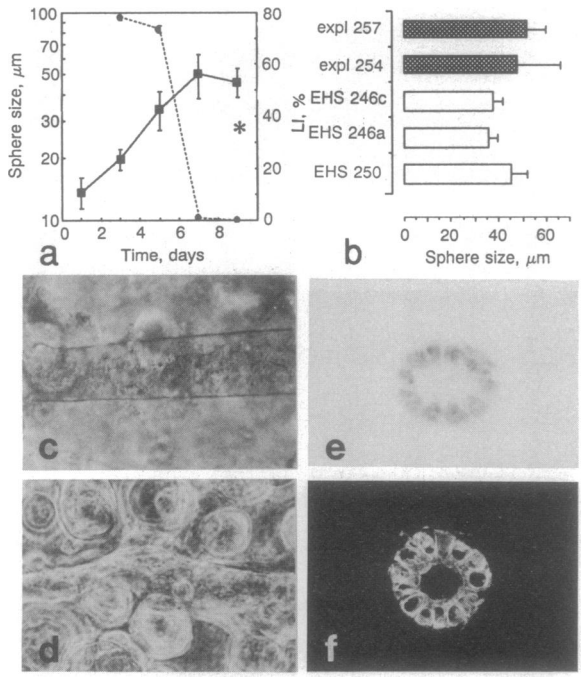

FIG. 2. Kinetics of sphere formation by normal breast epithelial cells in the EHS matrix. (*a*) Growth curve of diameter of spheres starting from single cells inside the EHS matrix. The dotted line indicates the concomitent [^{3}H]thymidine labeling index (LI). The asterisk indicates the LI in parallel subconfluent monolayer cultures (SD <10%). (*b*) Mean size of freshly explanted acini from two biopsies (shaded bars) and the final mean size of spheres from three normal primary cultures in the EHS matrix (open bars). (*c* and *d*) Phase-contrast micrographs of normal primary cultures in the EHS matrix showing formation of large (*c*) and small (*d*) duct-like structures. (*e*) Hematoxylin-stained frozen section of sphere showing basally located nuclei and a small lumen. (*f*) Immunofluorescence showing cytokeratin K18 in the cells of a sphere. (*c–f*, ×200.)

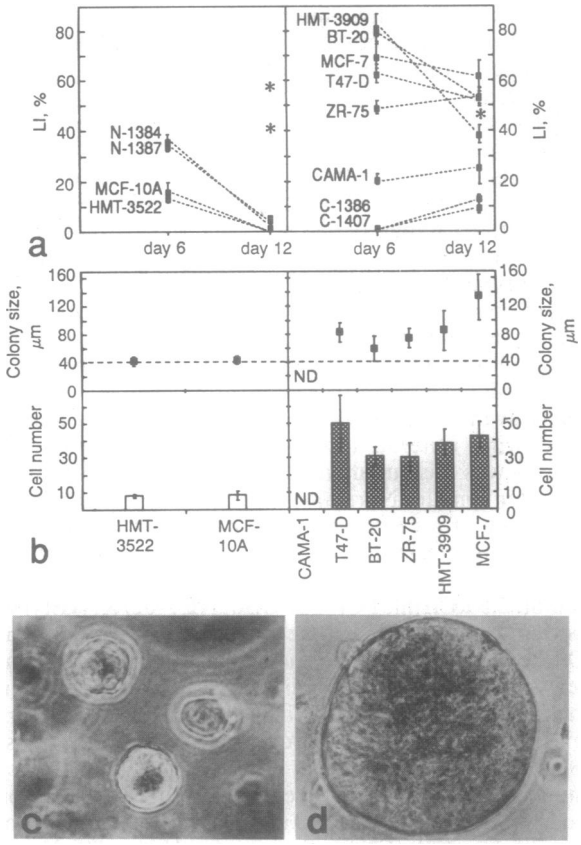

FIG. 3. Differential response of normal and carcinoma cells to BM. (*a*) [^{3}H]Thymidine labeling index (LI) on days 6 and 12 in normal breast epithelial cells (*Left*) and carcinoma cells (*Right*) (n = 200–400 cells per point). The asterisks indicate level of thymidine incorporation in monolayer cultures at day 6. Left, upper asterisk, HMT-3522; lower asterisk, MCF-10A. Right, MCF-7. For primary carcinoma cells, day 6 refers to the LI in traditional monolayer culture instead of in the EHS matrix to visualize the improvement obtained by EHS matrix culture. (*b*) (*Upper*) Colony size diameter; n = 20 colonies per point. (*Lower*) Maximal cell number per colony profile (n = 10 profiles per point). Data are from normal cell lines (*Left*) and carcinoma cell lines (*Right*). (*c* and *d*) Phase-contrast micrographs of maximal sphere size in cultures of cell lines HMT-3522 (*c*) and MCF-7 (*d*). (×200).

although it contains simian virus 40 and is highly aneuploid (36), and found that it expressed an intermediate phenotype in that it formed dense interconnected sphere-like clusters that did not growth-arrest by day 12 (data not shown). In contrast to the normal cultures, two of three primary carcinomas and all carcinoma cell lines continued to proliferate within the EHS matrix at a time when the normal cells ceased growing (Fig. 3*a*). Carcinoma cell lines in the EHS matrix formed spheres with significantly higher final colony diameter and cell number (Fig. 3 *b* and *d*). Although two of three carcinoma biopsies grew in the EHS matrix, all three parallel samples in monolayer culture were either nonadherent or nongrowing as was the case for many previous biopsies (O.W.P., unpublished results).

Differentiation of Normal and Carcinoma Cells in the EHS Matrix. The behavior of normal and carcinoma cells in the EHS matrix was further related to known patterns of differentiation *in situ*. All normal luminal epithelial cells, identified by expression of keratins K18 and K19 in normal primary cells and K18 in normal cell lines, showed a polarized apical expression of sialomucin, as is the case *in vivo* (Fig. 4*a*). Cell lines and normal primary cultures differed somewhat in that in MCF-10A sialomucin was distributed generally in luminal cells suspended within the lumen of the spheres rather than localized at the apical membrane, and that in HMT-3522 sialomucin was coexpressed on both the lateral and apical membranes. Also, the expression by normal cell lines was less frequent than for normal primary spheres (12% for HMT-3522 and 37% for MCF-10A; Fig. 4*d*). The normal cell lines deposited a continuous layer of BM around all of the spheres that stained for human type IV collagen (Fig. 4*e*). The intermediate line HBL-100 also deposited a type IV collagen containing BM around the cell clusters. A continuous BM was formed around 66% (n = 225) of spheres formed by normal primary cells as shown by double labeling with keratins (Fig. 4 *b* and *c*), although staining was stronger around occasional myoepithelial cells (data not shown).

Primary carcinoma or carcinoma cell lines also expressed sialomucin. However, in contrast to normal cultures, the sialomucin was either unpolarized, as seen in lines CAMA-1, BT-20, and two primary carcinomas; expressed at the basal surface of cells in contact with the EHS matrix as seen in one primary carcinoma (Fig. 4*a'*) and lines MCF-7, HMT-3909, and ZR-75 (Fig. 4*d'*); or showed luminal, basal, or unpolarized expression, depending on the cell in question in line T47-D (data not shown). Furthermore, neither of the primary carcinoma colonies nor carcinoma cell lines HMT-3909, ZR-75, and MCF-7 deposited endogenous BM-like material (Fig. 4 *b'*, *c'*, and *e'*). Some small diffuse or fragmented spots of BM-containing material were occasionally observed in CAMA-1, T47-D, and BT-20 colonies (data not shown).

DISCUSSION

The search for tumor-specific markers that could be used for the development of diagnostic and therapeutic strategies,

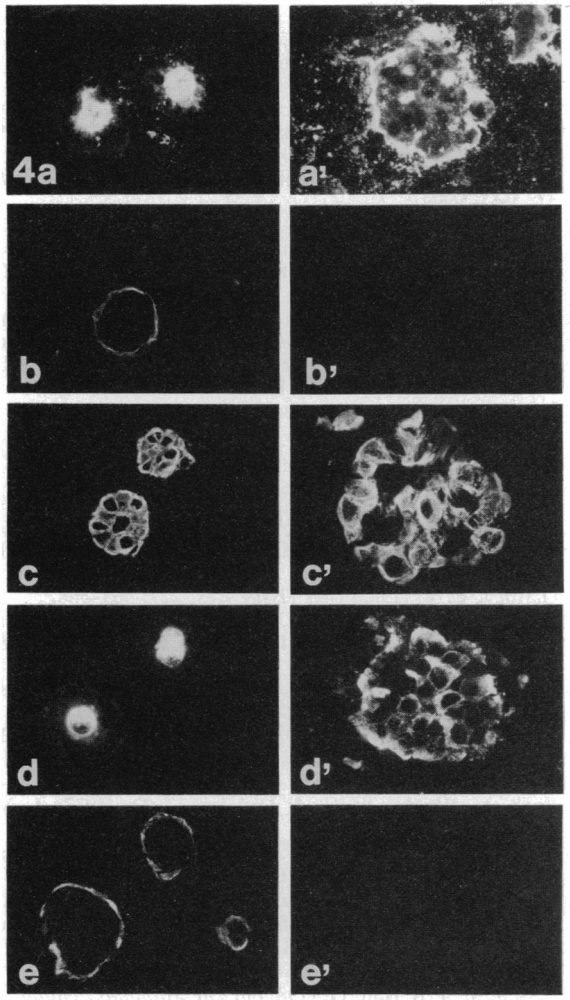

FIG. 4. Immunofluorescence of normal primary spheres (*a–e*) and carcinoma colonies (*a'–e'*) in the EHS matrix. (*a* and *a'*) Sialomucin staining. Note the apical accumulation of sialomucin in normal spheres (*a*) and basal expression in primary carcinoma cultures (*a'*). (*b*) Human type IV collagen staining around fully developed normal spheres. (*b'*) No BM is present around carcinoma cells. (*c* and *c'*) Double labeling for keratin K18 on same sections as in *b* and *b'*. Double labeling for sialomucin (*d* and *d'*) and human type IV collagen (*e* and *e'*) in normal MCF-10A spheres (*d* and *e*) and MCF-7 colonies (*d'* and *e'*). Note the similarity between the pattern of differentiation for cell lines and the primary cultures. (×170.)

especially for breast cancer, has been intense. Unfortunately, many tumor markers identified thus far are also found in cultured breast epithelial cells, in fetal cells, and in cells participating in physiological processes such as wound healing or hormonally mediated growth and involution. We have argued in the past whether our failure to define a cancer cell may stem also from our inability to define a normal cell in culture (37). To address this problem, the nature of the microenvironment needed to maintain normal differentiation and morphogenesis for lactating rodent breast epithelial cells has been defined (for review, see refs. 38–40).

As a logical extension of these studies, here we demonstrate the ability of a reconstituted BM to direct morphogenesis and differentiation of normal human breast epithelial cells. We can thus define markers for normal breast tissue in culture. We also show, in contrast, the consistent inability of breast carcinoma cells to respond appropriately to this microenvironment.

Other workers have used the EHS matrix to distinguish between normal and malignant endometrial cell lines by morphologic criteria (20) or between various human breast carcinoma cells (24, 25). To our knowledge, however, this is the first demonstration of the ability of normal primary breast epithelial cells and biopsied carcinoma cells to recapitulate in culture the growth behavior as well as structural and functional differentiation characteristics of these cells *in vivo*.

The ground breaking work of others had established methods for culturing normal human mammary cells (41) and had formulated serum-free culture conditions for normal breast epithelial cells (19). Petersen and van Deurs (15, 26) had described a medium for culturing both luminal epithelial cells and to some extent carcinoma cells. However, the problems of how to distinguish normal from tumor cells in culture and how to culture breast tumor cells more reliably remained to be solved.

An essential first step toward the development of our assay system was the establishment of almost pure populations of luminal epithelial cells by maintenance and passage of cultures at high density—conditions that do not favor the proliferation of myoepithelial cells. Misleading results would be obtained with cultures contaminated with myoepithelial cells since the latter can form relatively large solid colonies in the EHS matrix with a central core of cornified squamous cells surrounded by an endogenous type IV collagen-containing matrix. The culture medium (H14) was found to adequately favor the formation of luminal epithelial-like spheres over squamous differentiation for the cell line MCF-10A (unpublished data).

All 12 normal cultures from reduction mammoplasties and the two normal cell lines tested showed distinct patterns of acinous differentiation. One normal cell strain cultured on the EHS matrix had also been observed (25) by gross morphology to resemble ducts. What is most exciting here, however, is when luminal epithelial cells are seeded in the EHS matrix, the quiescent state of normal breast epithelial cells, but not carcinoma cells (both primary cells and cell lines), is restored after only 7 days. This dramatically reduces the time needed to test cells for "normal" behavior. Survival of freshly biopsied carcinoma cells, albeit in this very small sampling of tumors is nevertheless a vast improvement over monolayer culture on plastic where only rare tumor cells grow from few biopsies. In our assay, all carcinoma cell lines and those primary carcinomas that grow can be readily distinguished from normal cells. Previous studies have found that luminal breast epithelial cells differ from tumor cells by the expression of filamentous proteins otherwise restricted to myoepithelial cells, that is, cytokeratins K5 and K14 and vimentin (18, 42). On the other hand, normal luminal cells often lose the ability to express cytokeratin K19 whereas many tumor cells do not (42). In this study normal primary cells in the EHS matrix continued to express K18 (and K19) whereas spheres formed from normal cell lines expressed K18 only (data not shown).

Experiments with human cells on floating collagen gels have resulted in organization of tubular structures with much less fidelity to acini than those we observed in the EHS matrix (43, 44). Also, apparently, there is no signal transduction in collagen I gels to limit the growth of human breast epithelial cells (43). Thus far, a visible endogenous BM has not been demonstrated for rodent mammary epithelial cells in EHS culture (12). The ability to use human-specific antibodies for BM components has allowed us to demonstrate that the collagen IV-containing BM around normal human breast spheres in the EHS matrix culture is of human and not mouse origin.

The coordinated loss of BM around tumor cells and the lack of membrane polarity have been reported for *N*-methyl-*N*-nitrosourea-induced rat mammary tumors (45). In this study all normal luminal cultures showed basal expression of BM components and the normal primary cultures showed

apical membrane localization of sialomucin. In the normal cell lines MCF-10A and HMT-3522, sialomucin expression was somewhat less restricted. In contrast, the breast tumor cells were either inversely polarized or not polarized at all. The inside out orientation of a luminal type apical membrane toward the EHS matrix may facilitate release of degradative enzymes to the environment. This is not incompatible with recent observations of enhanced tumorigenicity elicited by tumor cells coinoculated with EHS matrix in nude mice (46–49).

Finally, the use of BM to elicit a hidden structural potential in normal breast epithelial cells and the loss of this potential in some tumor cells should facilitate the definition and identification of a class of tumor suppressor genes (47)—those involved in cell–cell and cell–extracellular matrix interaction and communication.

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