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Authors
Stampfer, Martha R.
Yaswen, Paul

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M.R. Stampfer and P. Yaswen

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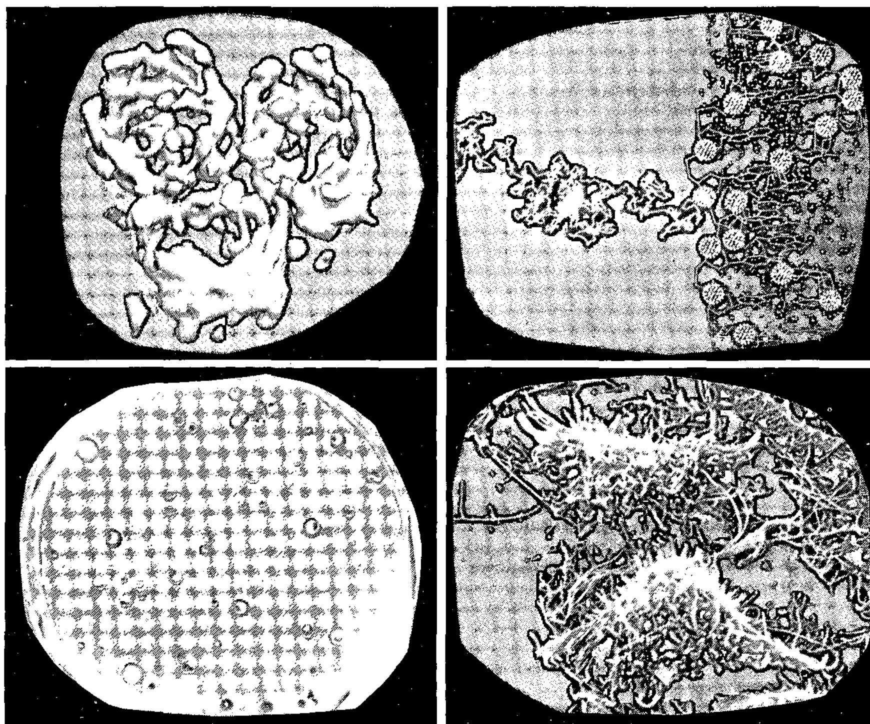

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Martha R. Stampfer and Paul Yaswen

Cell and Molecular Biology Division
Lawrence Berkeley Laboratory
University of California
Berkeley, CA 94720

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Factors Influencing Growth and Differentiation of Normal and Transformed Human Mammary Epithelial Cells in Culture.

Martha R. Stampfer and Paul Yaswen
Lawrence Berkeley Laboratory, Cell and Molecular Biology Division, Bldg. 934, University of California, Berkeley, CA 94720

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INTRODUCTION

The mammary gland is a very relevant organ system for carcinogenesis studies, since approximately one out of every nine women in the United States will develop breast cancer in her lifetime. The reasons for this high incidence of breast cancer are still unknown, necessitating a thorough analysis of the normal and abnormal processes of growth and differentiation which occur in this organ. The use of the human mammary gland as a model system for human carcinogenesis is facilitated by the abundant quantities of both normal and diseased tissues which are readily available as discard material from surgical procedures. These procedures include reduction mammoplasties, which supply tissues with normal epithelial cells from women of all ages; mastectomies, which supply primary tumor and non-tumor tissues from the same individual; effusions and secondary tumor sites, which supply metastatic cells; and gynecomastias, which supply benign male breast tissue. Normal mammary epithelial cells can also be obtained from lactational fluids. These cells and tissues provide valuable resources for in vitro studies on the factors which contribute to malignant progression.

Elucidating the interrelationship between differentiation and carcinogenesis is an important step in improving our understanding of malignant progression in epithelial cells. The mammary gland provides advantages and disadvantages for studies on this relationship. On the one hand, the variety of physiologic states of the mammary gland in vivo, i.e., normal cycling, pregnant, lactating, involuting, and post-menapause, each with distinct specialized functions, potentially provides many different markers of differentiation. On the other hand, the complexity inherent in such a range of phenotypes makes the mammary gland a difficult organ for delineating pathways of differentiation.

Our laboratory has developed a culture system utilizing human mammary epithelial cells (HMEC) in order to facilitate studies on the normal mechanisms controlling growth and differentiation in these cells, and to understand how these normal processes may become altered as a result of immortal and malignant transformation. Since cell culture systems provide the only means for systematic experimentation on cells of human origin, one of our goals has been to optimize the usefulness of this in vitro model system. We have approached this goal by (1) characterization of the cells grown in culture, particularly with reference to their relationship to cell types which exist in vivo, and (2) modifications of the culture system so the cells may better reflect the phenotypes and patterns of growth found in vivo. Underlying this work has been the assumption that carcinogenesis involves aberrations in the normal pathways of proliferation and differentiation, and that development of optimized culture systems to examine the behavior of normal HMEC will aid in our understanding of the mechanisms of carcinogenesis.

For the purposes of our characterization of HMEC in culture, we have defined two different kinds of differentiation. The first type, functional differentiation, refers to those properties of the mammary gland associated with its role in milk production. These include: the capacity for rapid proliferation in response to specific hormonal stimuli during the first half of pregnancy; the preparation of the gland for milk production in the second half of pregnancy; the synthesis and secretion of a variety of milk products during pregnancy (e.g.,

caseins, α-lactalbumin, medium chain fatty acids); and the remodeling of the gland, including protease activity and reduction in epithelial content, during involution. Since human mammary tissues, unlike tissues from animal model systems, can not readily be obtained in these functionally differentiated states, analysis of these properties in culture is extremely difficult. Maintenance of functional differentiation is difficult even in rodent mammary cultures obtained from pregnant and lactating glands; it has only recently been demonstrated in rodent tissues obtained from virgin animals [1]. However, mammary carcinoma cells do not exhibit functional differentiation. Thus, the absence of functionally differentiated cells in culture does not necessarily limit the usefulness of cultured HMEC for studies of carcinogenesis. A relationship that has been observed between functional differentiation and carcinogenesis is that women who have had a full-term pregnancy at a young age have a reduced incidence of breast cancer. This suggests that changes which occur during parity may influence the subsequent capacity of the mammary epithelial cells to become malignant. Since normal reduction mammoplasty tissues are available from women of differing ages and parity histories, experimental examination of this relationship can be made in vitro.

The second type of differentiation we have termed "maturation". This refers to the developmental history of a cell from a proliferative stem cell population to a cell with diminished reproductive capacity to a "terminally differentiated" cell no longer capable of division. The actual lineage of human mammary epithelial cells in vivo has not been fully defined. The mammary gland consists of pseudostratified epithelia, with a basal layer resting upon a basement membrane and an apical layer facing the lumen of the ducts and alveoli. The basal layer of cells does not contact the lumen, whereas the apical layer may contact the basement membrane as well as the lumen. Apical cells display a polarized morphology, with microvilli at the luminal side. The myoepithelial cells, which contain muscle-like myofilaments, and which contract upon appropriate hormonal stimuli to cause expulsion of milk, lie in the basal layer of cells. Based upon examination of keratin expression and other marker antigens, it has been proposed for the rodent mammary gland that a stem cell population capable of differentiating into both myoepithelial cells and the apical glandular epithelial cells, also resides in the basal cell layer [2].

Functional differentiation and maturation are two separate, though not necessarily independent processes in the mammary gland. Thus, a woman's epithelial cells may undergo lineage development from stem cell to a non-proliferative fully mature cell without ever becoming functionally differentiated, i.e., without ever undergoing the changes of pregnancy and lactation. In this sense, the mammary gland is unlike most of the other epithelial organs. The relationship of functional differentiation to stages of maturation is not known, that is, what cell type responds to the hormonal stimulus to proliferate during pregnancy, or what maturation stage responds to the hormonal stimuli to cease proliferation and start synthesizing milk products. These questions are of particular interest because, as will be described below, the phenotype of the cancer cells found in vivo, as well as that of most breast tumor cell lines in vitro, most closely resembles the phenotype of the normal mature apical cell in vivo.

Maturation, or terminal differentiation, may also follow a biological pathway distinct from cellular senescence observed in vitro. Normal human fibroblasts and epithelial cells in culture display a limited, fixed number of population doublings, which varies with cell type and culture conditions. For example, normal HMEC grown in the serum-free medium MCDB 170 will undergo 45-80 population doublings, depending upon the individual specimen donor, and then show no net increase in cell number. These non-dividing cells may maintain viability for months in culture. It is likely that the controls which limit the number of times a given cell may complete the cell cycle are distinct from those which lead to a mature, non-dividing and ultimately non-viable phenotype. Thus, a cell may senesce in culture without ever exhibiting the phenotype of the most mature or functionally differentiated cell type in its lineage.

In order to characterize the cell types grown in vitro, and to compare them to cell lineages in vivo, we have examined them for expression of potential markers of mammary epithelial cell maturation and differentiation. These include intermediate filaments (keratins and vimentin), the large polymorphic epithelial mucins, extracellular matrix associated proteins (fibronectin, collagen, laminin, proteases and protease inhibitors), and milk products (caseins and α-lactalbumin). In order to examine factors which control normal HMEC proliferation, we have measured the effects of a variety of potential growth stimulators and growth inhibitors on the growth and differentiation of cells which display long-term growth in a serum-free medium. In order to compare the properties of normal and transformed HMEC, we have utilized chemical carcinogens and oncogenes to transform normal HMEC from reduction mammoplasty tissue to cell lines displaying indefinite lifespan, reduced growth factor requirements, and tumor formation in nude mice.

GROWTH AND CHARACTERIZATION OF CELL TYPES IN CULTURE

Isolation and Growth of Normal HMEC

Our laboratory, in collaboration with other groups, has developed culture systems which support the long term growth of HMEC derived from reduction mammoplasty tissues [3-5]. Surgical discard material is crudely dissected to separate glandular from fatty and stromal tissue. This material is then digested with collagenase and hyaluronidase to yield epithelial cell clusters (termed organoids) free of stroma. The epithelial organoids are separated from single cells and small clumps via filtration, and both the epithelium, and the filtrate (which preferentially contains mesenchymal cells) can be stored frozen in liquid nitrogen. A single reduction mammoplasty can easily yield 10-60 frozen ampoules containing 0.1 ml each of concentrated epithelial organoids, permitting multiple experiments utilizing cells from the same individual.

We have used two main types of medium to support growth of the HMEC - a serum containing medium, designated MM [6], and a serum-free medium, designated MCDB 170 [4]. Both media contain a variety of growth factors, including insulin, hydrocortisone, EGF, and a cAMP stimulator. MM contains 0.5% fresh fetal bovine serum and 30% conditioned media from other human epithelial cell lines; MCDB 170 contains 70μg/ml bovine pituitary extract (BPE).

We have also used formulations of MM that lack the conditioned media (designated MM4).

Organoids placed in primary culture show initial cell migration followed by rapid division from the edges of the outgrowth. In MM, there is active epithelial division for 3-5 passages at 1:10 dilutions. The cells then acquire a mixed morphology, with larger, flatter non-dividing cells mixed with smaller cells growing with a cobblestone morphology. Some cobblestone appearing cells may maintain growth for an additional 2-5 passages, particularly in MM4, with overall slower growth of the cultures and the continued presence of non-dividing, flatter cells. In MCDB 170, there is initial active cell division for 2-3 passages of cobblestone appearing cells. These cells gradually change morphology, becoming larger, flatter, striated, with irregular edges, and reduced proliferative capacity. As these larger cells cease growth and die, a small number of cells with the cobblestone morphology maintain proliferative capacity. These smaller cells soon dominate the culture, and continue growing with a fairly uniform cobblestone appearance for an additional 7-24 passages, depending upon the individual reduction mammoplasty specimen. At senescence, the cells maintain the smooth edged cobblestone appearance, but become larger and more vacuolated. We have referred to this process, whereby only a small fraction of the cells grown in MCDB 170 display long-term growth potential, as self-selection. Self-selection can also be observed in primary cultures which are subjected to repeated partial trypsinization, a process wherein approximately 50% of the cells are removed and the remaining cells allowed to regrow. After about 10 partial trypsinizations, most of the cells remaining in the dish display the flat, striated, non-dividing morphology. However, nearly every organoid patch also gives rise to areas of the growing cobblestone cells, indicating a widespread distribution of the cell type with long-term growth potential.

Most of our current studies on normal HMEC biology utilize the post-selection cells which display long-term growth in MCDB 170. These cells have doubling times of 18-24 hrs, and will grow clonally with 15-50% colony forming efficiency. Large batches of post-selection cells can be stored frozen, permitting repetition of experiments with cells from the same frozen batch, as well as from the same individual. We have carefully followed the growth of each cell batch until senescence [7] so that the remaining reproductive potential of a given cell batch is known. All reduction mammoplasty derived cells thus far examined have shown a normal karyotype [8-9], however we have not examined cells near senescence.

In Vitro Transformation of HMEC

Normal HMEC from specimen 184 have been transformed to immortality following exposure to the chemical carcinogen benzo(a)pyrene (BaP) [7,10]. Primary cultures were grown in MM and exposed 2-3 times to 2μg/ml BaP. Selection for transformed cells was based on the ability of BaP treated cells to continue growing past the time that the control cells senesced. Treated cultures typically contained cells with an extended lifespan compared to controls. These extended life cultures were very heterogenous with respect to morphology and growth potential. Often, they represented the outgrowths of individual patches or colonies. However, almost all of these extended life cells eventually ceased

growth. In only two instances have we observed escape from senescence, leading to cell lines with indefinite lifespan. The two resulting cell lines, 184A1 and 184B5, each show specific clonal karyotypic aberrations, indicating their independent origins from single cells [9]. Some of the karyotypic abnormalities found in 184B5, e.g., 1q22 breaks and tetrasomy for 1q, are also frequently observed in cells obtained from breast tumors [11]. Upon continued passage in culture, these two lines show some genetic drift, but it is relatively minimal compared to that observed in most human breast tumor cell lines. Thus, the vast majority of the cell population would be expected to remain karyotypically stable when studied over the course of a few passages in culture, yet the presence of some genetic drift could give rise to rare variants in the cell population. Although 184A1 and 184B5 are immortally transformed, they do not have properties associated with malignant transformation. They do not form tumors in nude mice and they show very little or no capacity for anchorage independent growth (AIG).

Malignant derivatives of 184A1 and 184B5 have been obtained with the use of oncogene containing retroviral vectors and viruses. In the case of 184A1, A1N4, a clonal derivative with reduced nutritional requirements (i.e., growth in MM4), was exposed to the genes for SV40 large T antigen, v-H-ras, and v-mos, singly and in combination [12]. The combination of H-ras and SV40-T led to cells (designated A1N4-TH) which formed progressively growing tumors in nude mice and showed AIG. Exposure to v-H-ras or v-mos alone led to cells that produced tumors with reduced frequency and longer latency. SV40-T alone did not yield tumorigenic cells, but did affect the growth factor requirements for anchorage dependent and independent growth [13]. In all cases of oncogene exposure, the resultant cells were capable of proliferation in media that did not support the growth of the parental A1N4 cells. The karyotype of the A1N4 cells is aneuploid, near triploid, with one additional clonal chromosomal aberration beyond the three present in the parental 184A1 cells. A1N4-TH has a near tetraploid karyotype, which is missing the A1N4 chromosomal marker and contains only one additional clonal chromosomal aberration relative to 184A1. Thus the malignantly transformed cell line, containing v-H-ras, does not show an unstable karyotype in terms of chromosomal aberrations.

The 184B5 cell line has been exposed to v-K-ras (cell line designated B5-K). In 184B5, the K-ras gene alone was capable of producing cells which were 100% tumorigenic in nude mice, with short latency. However, these tumors did not grow beyond approximately 5cm diameter [7]. Most of our studies have utilized the culture designated B5KTu, a cell line originating from a tumor resected from a nude mouse and placed in culture. B5-K and B5KTu do not display AIG.

We have also conducted a series of experiments to attempt to obtain malignantly transformed derivatives of 184A1 and 184B5 following additional exposure to chemical carcinogens. The design of these experiments was based on the observation that the oncogene derivatives of A1N4 showed reduced nutritional requirements, as well as on the existing literature on the reduced nutritional requirements of many transformed cells. To perform these experiments, we first determined the requirements of 184, 184A1, and 184B5 for the various growth factors present in MCDB 170 for short term culture (Table 1)

and for continued passage in culture. In the short term experiments, 184A1 and 184B5 showed a few differences from each other and normal HMEC in their growth requirements. Both were more dependent upon EGF for growth in mass culture than the normal cells. 184A1 showed little effect upon removal of HC. In the long-term experiments, removal of hydrocortisone (HC) or BPE from mass cultures of normal HMEC led to cessation of growth over the course of 1 to 3 passages. Removal of insulin (I) did not prevent continued proliferation, but led to slower growth, a less healthy appearing culture, and earlier senescence. Removal of I from 184A1 and 184B5 also did not prevent continued growth. There was an initial reduction in growth rate, but the cell populations resumed active growth within 2 passages. However, removal of BPE or EGF led to cessation of growth for the vast majority of 184A1 and 184B5 cells. Nonetheless, a few cells could be observed which maintained growth without addition of BPE or EGF. The growth rates of these variants slowly increased upon continued subculture for 4-6 passages, leading to selected subpopulations which maintained active growth in the absence of BPE or EGF.

We next examined the effect of removal of multiple growth factors, and were able to define conditions which did not support the continued growth of 184A1 or 184B5, e.g., removal of I and EGF, I and BPE, or EGF and BPE for 184A1, and removal of I and EGF, or I and BPE for 184B5. Populations of 184A1 and 184B5 were then tested for colony forming ability in the presence of the direct acting carcinogen, N-nitroso-ethyl-urea (ENU). Concentrations of ENU that yielded 80% inhibition were chosen for further experiments, i.e., 1500 μg/ml for 184A1 and 750 μg/ml for 184B5. Two T-75 flasks each of treated and control cells were exposed to ENU or solvent alone for 2 or 3 consecutive passages. The resulting cell populations were then tested for their ability to grow in the restrictive media and for AIG. Under some conditions the ENU treated cells were capable of sustained growth whereas the untreated cell lines quickly ceased growth. The resulting growth factor independent variants, such as A1ZNEB, which does not require either EGF or BPE, and B5ZNEI, which does not require EGF or I, may represent a further step in malignant progression. However, none of the variants showed AIG, nor did they form tumors in nude mice. Thus, we have not been able to derive cells that showed tumorigenic properties following use of chemical carcinogens alone.

Characterizaton of Normal HMEC Grown in Culture

In order to relate the HMEC which maintain growth in vitro to the different cell types identified in vivo, we have examined the normal and transformed cells for phenotypes which have been characterized using sectioned human breast tissues. A variety of studies have defined properties which can be used to distinguish basal vs. luminal breast cells, and which change during the course of lactation [14-16]. In general, mammary basal cells, similar to basal cells in stratified tissues such as the skin, express keratins 5 and 14. Some reports have indicated that a subpopulation expresses the common mesenchymal intermediate filament, vimentin [15,17]. Expression of α-actin has also been localized to the basal cell layer. Luminal cells express the keratins 8 and 18 found in simple epithelia like the lung. Keratin 19 shows variable expression in the luminal cells. The keratin 19 positive cells display low proliferative potential in culture, suggesting that they may represent the least proliferative, or most

mature luminal cell type in vivo [18-19]. Expression of specific epitopes of a polymorphic epithelial mucin (PEM) has been localized to luminal cells in vivo. Cells in the resting gland are weakly mucin postitive, whereas cells from lactating glands may express higher levels of specific mucin epitopes [14]. High expression of specific PEM epitopes has also been correlated with a low proliferative potential in normal HMEC in vitro [20]. Only a small fraction of normal mammary epithelial cells in vivo are estrogen receptor positive, and this positive population is preferentially localized in the non-basal layer [21-22]. It is not clear whether the mammary gland contains cells which are terminally differentiated such as those in the most mature layers of stratified epithelium, since even keratin 19, mucin positive cells can show a limited capacity for cell division in vitro.

In collaboration with others [16], we have examined the HMEC grown under our culture conditions for expression of certain phenotypic markers by Northern blot and by immunohistochemical analysis. Figure 1 shows some of the data on antigen expression; Figures 2 and 3 include some of the data on mRNA expression. Primary cultures of normal HMEC grown in MCDB 170 and early passage cultures grown in MM are heterogeneous. Some cells have the basal phenotype - keratin 5/14 positive, mucin negative, and a-actin positive; other cells show the luminal phenotype - keratin 5/14 negative, keratin 8/18/19 positive, mucin positive (Figure 1). The cells which initially proliferate in the serum-free MCDB 170 medium are those with the basal phenotype. However, post-selection cells begin to express some properties associated with the luminal cell type, i. e., keratins 8 and 18 and some mucin epitopes. Expression of these luminal properties increases with continued passage in culture, such that the senescent cells uniformly express these markers. At the same time, expression of the basal keratins 5 and 14 is not lost. We have not been able to detect keratin 19 in any post-selection population. Vimentin expression is found in both the pre- and post-selection cells which proliferate in MCDB 170.

The above results led us to propose that the cells which display long term growth in the serum-free medium represent a multipotent stem cell population initially present in the basal layer of the gland. With increasing time in culture, these cells show a partial differentiation towards the luminal phenotype. Based on phenotypic expression of keratins and other markers, a multipotent basal, stem cell population has also been proposed for the rodent mammary gland [223]. However, we can not be sure that culture conditions have not induced some artifactual phenotypic expression. In particular, growth of cells on impermeable plastic substrates prevents the normal cell-extracellular matrix contacts and precludes the normal development of cellular polarity. The lack of normal cell polarity may in turn effect the cells' phenotypic expression in culture. Cells which are positive for keratins 5, 14, 8 and 18 are not commonly observed in vivo, although coexpression of keratin 8 and vimentin has been reported [17].

We consider it important to continue to characterize the populations growing in vitro and to optimize the culture conditions to permit better approximation of in vivo like conditons. However, we also believe that the HMEC culturd under the current conditions are useful for certain studies in molecular biology, biochemistry, and carcinogenesis that require large number of cells and

may not require stringent fidelity to the in vivo situation. The HMEC grown in MCDB 170 provide large standardized pools of normal human epithelial cells for such experiments.

Characterizaton of HMEC Transformed In Vitro

When cells from breast tumor tissues or tumor cell lines are examined, they display the maturation phenotype found in the mature luminal cell population. They rarely express keratins 5 and 14, and nearly uniformly express keratins 8, 18 and 19 [16]. Most tumor cells have high levels of expression of PEM, including the epitopes found in the differentiated lactating cells [14]. Around 70% of breast tumor tissues also display high levels of the estrogen receptor. However, vimentin expression, normally confined to basal cells in vivo, has been observed in a subset of estrogen receptor negative breast tumor cell lines and tissues [24]. Thus breast tumor cells in vivo and in vitro display a phenotype which, in normal HMEC, is associated with low proliferative potential in vitro.

Some of our cell lines which have been transformed in vitro to immortality and tumorigenicity have been examined for their expression of differentiation markers, including intermediate filaments, mucins, the extracellular matrix protein fibronectin, and a newly isolated gene designated NB-1 (see below) (Figures 2,3). The immortalized cell lines 184A1 and 184B5 maintain some expression of keratins 5 and 14, but Northern blot analysis shows that the level of keratin 5 mRNA is decreased in 184B5, and even further decreased in 184A1, while expression of keratin 18 mRNA is increased relative to normal HMEC. These lines also differ from the normal cells in their barely detectable levels of vimentin mRNA. 184B5 expresses the luminal PEM antigens, including one epitope, recognized by the antibody HMFG-2, which is found in tumor cells (data not shown). The tumorigenic transformants, A1N4-TH and B5KTu have very low levels of keratin 5 and increased levels of keratin 18 mRNA. While B5KTu remains vimentin negative, the A1N4-TH cells show re-expression of vimentin. We have not been able to detect keratin 19 mRNA in any of these lines. These results suggest that the transformed cells, particularly 184B5, have a phenotype which is closer to the luminal phenotype than that seen in the normal HMEC, but do not fully resemble breast tumor cells.

Fibronectin represents one of the major proteins secreted by normal HMEC in culture [25]. In many transformed cells, the level of fibronectin mRNA and protein synthesis is decreased. Our transformed cell model system is similar in that expression of fibronectin is reduced in the transformed cells (Figure 3, unpublished results). Nonetheless, even where fibronectin synthesis is low or barely detectable, as in the case of the 184A1 cell line, its expression can be upregulated by exposure of the cells to TGFβ.

The normal and transformed HMEC have also been characterized with respect to both their growth patterns and their gene expression when placed on reconstituted basement membrane material derived from the Englebreth-Holm-Swarm (EHS) murine sarcoma. Growth on EHS has previously been shown to support increased differentiated functions of a variety of cell types. Normal HMEC are capable of forming three-dimensional structures with striking resemblance to endbuds in intact mammary gland tissue (Figure 4A,B) whereas

184A1 displays only less developed structures and 184B5 forms only small clusters (Figure 4C,D). The A1N4-TH cells show even less structure formation than 184A1 (Figure 4E), whereas the B5KTu cells resemble 184B5 (data not shown). Although we do not know the underlying basis for these differences in growth patterns, transformation appears to be correlated with a loss of the capacity to form glandular-like three dimensional structures on EHS. We have examined several genes to determine if their expression is effected by culture on EHS (Figures 2,3). The most consistent effect thus far observed is a downregulation of keratin 5 and NB-1 mRNA.

Another approach we have taken to characterize differences between our normal and transformed HMEC cultures has been to identify genes which are expressed in the normal HMEC, but which are downregulated in the immortal and malignantly transformed cells. Towards this end, selected normal HMEC cDNAs were identified and cloned using probes enriched by subtractive hybridization between the normal 184 cell cDNA and both the B5KTu and the 184B5 cell mRNA [26]. Several genes preferentially expressed in normal 184 cells were isolated by this method, including those for fibronectin, keratin 5, and vimentin. Additionally, one 350 base pair cDNA fragment was isolated which initially showed no similarity to any sequence reported in GenBank. This cDNA hybridized specifically to a 1.4 kb mRNA, designated NB-1, which was expressed in the normal HMEC, but was downregulated or undetectable in the transformed cell lines (Figures 2,3). Sequence analysis of a full length NB-1 clone revealed a 447 bp open reading frame with extensive similarity (70%, 71%, and 80%) at the nucleic acid level to the three known human genes coding for the ubiquitous calcium binding protein, calmodulin. The similarity between the translated amino acid sequence of NB-1 and human calmodulin was 85% over the length of the entire protein.

Using Northern and PCR analysis, NB-1 mRNA has been thus far found only in normal epithelial cells and tissues from human breast, prostate, cervix, and skin. It has not been found in normal epithelial cells other than those from stratified or pseudo-stratified tissues, nor is it detectable in non-epithelial cells and tissues, or in epithelial tumor cell lines. Human breast cells obtained from lactational fluids were also negative for NB-1 expression by PCR analysis.

Although NB-1 mRNA is easily detectable by Northern analysis in total RNA from cultured normal HMEC, it is less abundant in total RNA from organoids and unprocessed reduction mammoplasty tissue. Such differences are unlikely to be due to variations in proliferative state since expression of NB-1 mRNA is not significantly decreased when cells are growth arrested by exposure to anti-EGF receptor antibodies or in senescing cells where proliferation is minimal, and it is increased in cells growth arrested by TGFβ. One possible explanation is that, unlike calmodulin, NB-1 expression may be limited to a particular state of epithelial cell maturation, and thus be confined to certain subpopulations of epithelial cells in vivo. Since NB-1 mRNA levels are high in the post-selection normal HMEC population which displays active long-term growth in MCDB 170, and which has attributes of multipotent stem cells, it is possible that expression in vivo may be limited to a stem cell population in the basal layer of the gland. The findings that NB-1 expression is reduced when HMEC are grown on EHS, and is absent in the non-proliferative differentiated luminal cells sloughed off

into milk, are consistent with the hypothesis that NB-1 is only expressed during certain stages of epithelial differentiation.

Using full length recombinant NB-1 protein as an immunogen, we have recently produced polyclonal antisera which can distinguish the NB-1 protein from vertebrate calmodulin (unpublished results). Initial studies have indicated that the relative abundance of the corresponding 16kD protein reflects relative NB-1 mRNA levels in various cell types, being most highly expressed in normal HMEC, lower or undetectable in the immortally transformed cell lines, and virtually undetectable in tumorigenic breast and prostate cell lines as well as in normal breast fibroblasts.

The initial characterization of genomic DNA corresponding to the NB-1 transcript indicated the unexpected absence of introns. All three vertebrate calmodulin genes contain five similarly placed introns [27]. A literature search revealed the existence of a previously reported human calmodulin "pseudogene" hGH6, which shared identity with NB-1 cDNA [28]. This gene was designated a pseudogene since the authors were unable to demonstrate the existence of a corresponding mRNA. Our evidence of expression of NB-1 at both the mRNA and protein levels suggests that NB-1 may be a rare example of an expressed retroposon [29].

External calcium concentration has been shown to affect the proliferative potential and differentiated states of some cultured epithelial cells, including keratinocytes and mammary epithelial cells [30-32]. Loss of response to the calcium induced differentiation signal has been shown to correlate with the early stages of transformation in keratinocyte cultures [33]. The downregulation of NB-1 expression observed after in vitro transformation of HMEC may reflect the fact that a particular state of differentiation is required for transformation, or that the transformed state is incompatible with high expression of NB-1.

GROWTH FACTORS CONTROLLING HMEC PROLIFERATION AND DIFFERENTIATION

A main focus of our research has been to study the effect of growth factors on normal HMEC proliferation, and compare these data with growth control of the transformed HMEC. In particular, we have examined the effects of TGFβ and EGF/TGFα. One long-term objective of these studies is to understand the parameters influencing cell cycle progression in normal, finite lifespan human epithelial cells, in the hope that this information may facilitate elucidating the aberrations which occur when cells attain immortality and malignancy. As part of this objective, we have tried to obtain conditions that would permit an efficient and readily reversible cell-cycle synchronization of normal HMEC.

Initial studies examined the effect of TGFβ on normal and transformed HMEC. We have demonstrated that normal HMEC are growth inhibited by TGFβ, with the extent of inhibition increasing as cells are subcultured in vitro [34]. All normal HMEC are ultimately growth arrested by TGFβ. Analysis by flow activated cell sorting indicates that cells are arrested in the G1 phase of the cell cycle (data not shown). This growth inhibition is at least partially reversible,

although the extent of reversibility decreases with cell passage in vitro, and is relatively asynchronous. Normal HMEC show distinctive morphologic changes in the presence of TGFβ, characterized by an elongated, flattened appearance. HMEC which have been transformed to immortality or malignancy have altered growth responses to TGFβ. Although varying degrees of growth inhibition and morphologic changes are observed in 184A1 and 184B5, both lines contain populations that maintain active growth in the presence of TGFβ [34]. However, even though TGFβ may not inhibit their growth, the immortalized HMEC lines retain receptors for TGFβ and, like the normal HMEC, express specific differentiated responses such as increased synthesis of the extracellular matrix associated proteins fibronectin, collagen IV, and plasminogen activator inhibitor 1 [35] (manuscript in preparation). Thus, TGFβ effects on cell growth can be dissociated from the effects on differentiated cell properties. The tumorigenic cell lines A1N4-TH and B5KTu maintain growth in the presence of TGFβ. This HMEC model system therefore resembles the situation observed with other normal and transformed epithelial cells in that loss of TGFβ induced growth inhibition accompanies the carcinogenic progression.

While these studies using TGFβ have illustrated differences in growth control between normal and transformed HMEC, TGFβ growth inhibition does not appear to be a useful method for studying cell cycle effects in HMEC. The degree of reversal of growth inhibition is variable and the cells are not arrested in a resting state. Indeed, the pronounced changes in normal HMEC protein synthesis and secretion, which result in an increased level of protein synthesis per cell, suggest that TGFβ induces a particular state of cell differentiation. The relationship of this state to normal epithelial cell homeostasis or wound healing remains to be determined.

Studies on the effects of EGF/TGFα on normal HMEC have indicated a stringent requirement for this growth factor class for clonal growth. However, growth in mass culture proceeds without addition of exogenous EGF due to the significant level of endogenous production of TGFα [36]. As mentioned earlier, the transformed cell lines may display a progressive loss of this EGF/TGFα requirement. Since both normal and transformed cells are capable of TGFα production and synthesis, this aspect of the autocrine loop can not, by itself, account for growth control differences between normal and tumor cells. It is possible that other changes, such as production of other EGF/TGFα related ligands, changes in the intracellular signal transduction pathway, or alterations in the cells' normally polarized pathways for secretion, may be responsible for the altered behavior of mammary tumor cells.

Addition of monoclonal antibody 225 IgG to the EGF receptor (MAb 225) prevents HMEC growth [37]. Recent experiments (manuscript in preparation) have shown that MAb 225 produces a rapid, efficient, and reversible growth arrest in an early G1 phase of the cell cycle. Protein synthesis remains depressed in the presence of the antibody, and DNA synthesis is sharply decreased by 24hr. Removal of MAb 225 leads to a rapid increase in protein synthesis. DNA synthesis increases only after 10hr and peaks around 18hr. A 1hr exposure to EGF after MAb 225 removal is sufficient to allow the majority of cells capable of cycling to subsequently enter S phase. High levels of synthesis of mRNA for the early response genes c-myc, c-fos, and c-jun are observed within 1hr of antibody

removal. Synthesis of TGFα mRNA, which is inhibited in the presence of MAb 225, is detected by 2hr after antibody removal. It thus appears that blockage of EGF receptor signal transduction is sufficient by itself to cause normal HMEC to enter a Go-like resting state similar to the Go state described in fibroblasts. Future studies will now be able to address possible differences between normal HMEC of finite lifespan and the immortally transformed HMEC cell lines with respect to their response to MAb 225 and their cell cycle controls.

DISCUSSION

Values and Limitations of Human Mammary Cell Culture Systems

Although much valuable information on the carcinogenic process has been obtained using animal model systems, ultimately, it is necessary to ascertain the nature of these processes in the cells of greatest interest, i.e., human cells. While the rodent model systems commonly employed in mammary cancer research may clarify fruitful areas of investigation, the existence of known differences between humans and rodents in mammary physiology, in response to etiologic agents, and in properties of transformation, emphasizes the uncertainty in transfer of information gained in model systems to the human situation. Direct study of human cells is the only way to determine the parameters of normal and aberrant human mammary cell biology. Yet, experimentation with human cells entails problems not encountered with rodent model systems. In vivo experimentation is not possible, and, in the case of the mammary gland, it is nearly impossible to obtain abundant quantities of cells in functionally differentiated states.

The advantages and limitations of human cell experimentation underscore the importance of developing human culture systems that can reflect, as closely as possible, the in vivo situation. Obviously, this goal represents an ideal; the achievable reality is to develop ever closer approximations. Our laboratory has been working to develop an "approximate" culture system for normal HMEC biology as well as a model system for the carcinogenic process. At this stage, the normal HMEC system can provide large, standardized quantities of actively proliferating cells from individual specimen donors. The short doubling time, the large number of doublings possible before senescence, the serum-free medium, the clonal growth capability, and the ease of large-scale growth, offer significant advantages for certain kinds of studies, e.g., molecular and biochemical analyses requiring large, uniform, proliferating cell populations. On the other hand, these cell populations represent only a limited range of the spectrum of mammary epithelial cell types found in vivo. To observe a full range of normal HMEC behavior, different culture conditions are required. For example, early passage cells, cells from lactational fluids, cells grown in different medium (varying in serum and growth factor content, calcium concentration, and presence of differentiation inducing agents), cells grown on substrates other than plastic (on or in collagen gels, extracellular matrix material, permeable membranes, hollow fibers) will display varying phenotypes. The extensive studies with rodent mammary epithelial cells [1,38] strongly suggest that these differing conditions will prove necessary in order to obtain the variety of cell lineages and differentiated phenotypes observed in vivo. The crucial importance of achieving truly representative human cell culture systems will

hopefully provide continued momentum for studies on optimizing these culture systems.

The model system for human mammary carcinogenesis which we have developed likewise has its values and limitations. We have available cells from one individual which display a progression of changes which correlate with changes observed during carcinogenic progression - extended lifespan, immortality, growth factor independence, and tumorigenicity. The immortal cell populations retain most of the growth factor requirements of the normal HMEC, display a more luminal phenotype than the normal HMEC grown in MCDB 170, and show minimal genetic instability. While any immortalized cell line can not be considered to represent normal cells, lines of indefinite lifespan are in many instances more convenient to use than finite lifespan cells. The retention by 184A1 and 184B5 of many normal characteristics makes them useful substrates for some areas of experimentation in normal cell physiology. On the other hand, the fact that they have acquired some aberrant properties relative to normal HMEC, especially their indefinite lifespan, makes them useful substrates for determining the potential capacity of additional factors (e.g., chemical and physical carcinogens, oncogenic viruses, transfected genes) to induce malignant transformation.

Cell lines immortalized by chemical carcinogens, like the rare cell lines which have immortalized spontaneously [39-40] may be more appropriate for some uses than lines which have been immortalized by the use of specific viral oncogenes which are not associated with mammary transformation in vivo or which commonly result in lines with gross genetic instability. However, the rarity of spontaneous and chemically induced transformation of human epithelial cells has meant that few immortal cell lines, reflecting a limited range of phenotypes, currently exist. Immortal transformation using SV40-T or papilloma virus transforming genes occurs more frequently, allowing one to target specific cell phenotypes, such as in the recent report of immortal transformation of milk derived cells expressing a mature luminal phenotype [41]. An unanswered question in the use of immortalized lines in a model of tumor progression relates to whether or not immortality is truly a requirement for malignancy. While it is clear that only cells from malignant tissues reproducibly yield immortal cell lines, this does not mean that immortalization is a necessary step in carcinogenesis. In fact, only rare cells from a small percentage of human breast tumors show indefinite lifespan in culture. It is possible that the extended lifespan seen when many epithelial cells are exposed to carcinogenic agents and viruses may be a more accurate reflection of the growth control derangements present in the majority of primary breast tumor cells.

The use of ras containing retroviruses for induction of malignant transformation raises similar issues to the use of SV40-T and papilloma virus oncogenes for immortal transformation. None of these viruses are known etiologic agent for human breast cancer. There is, in fact, considerable data showing the absence of ras mutations in human breast cancer. Yet, these oncogenes have thus far provided the only consistent means of obtaining malignant transformation of human mammary epithelial cells. Our efforts to achieve malignant transformation by chemical carcinogens alone have not been successful. Possibly, future studies on the effect of genes known to be involved in

breast cancer, such as c-erbB-2, p53, and the retinoblastoma genes, may provide insights that will enable development of more efficient and relevant malignant model systems. An alternative method for achieving a model system of malignant progression would be to obtain cell strains and immortal lines from the non-tumor and tumor tissues of one patient, although cells from the non-tumor tissues could not be assumed to be fully normal. A model system has been described which provides some of the steps of malignant progression through the development of cell lines from primary and metastatic tumor tissues of one individual [42].

Relationship of Transformation and Differentiation

A relationship between transformation and differentiation is suggested by the fact that cancer cells are often found to reflect specific stages in the differentiation pathway of the organ system from which they arise, and that loss of response to differentiation inducing agents is one of the earliest observed growth control aberrations in epithelial cell transformation. In order to understand the nature of this relationship, we need to know more about the pathways of funtional differentiation and of maturation in epithelial organ systems. In addition to performing organ-specific specialized functions, epithelial cells display a maturation lineage starting from a proliferative population located next to the basement membrane leading to a more mature population with little or no proliferative potential. In some organ systems, such as the epidermis, the pathway of maturation coincides with that of functional differentiation. In simple or pseudostratified epithelia, the maturation lineage may be more difficult to define since it is not delineated by obvious positional information. In these tissues, the pathways of functional differentiation and maturation do not necessarily coincide. In the mammary gland, the situation becomes even more complex because the gland is not usually in a functionally differentiated state. This variety in the physiologies of the different epithelial organ systems suggests that there may also be variety in the specifics of the relationship between transformation and differentiation among the different epithelial tissues.

In the case of the mammary cells, the somewhat surprising observation is that the tumor cells nearly uniformly express a phenotype which most closely resembles that of the normal mature luminal cell - the cell type which shows the least proliferative potential in culture. Clearly, the tumor cells have acquired some derangement in normal growth control since they readily proliferate even though displaying this "mature" phenotype. We have no definitive explanation of this phenomenum. It is possible that cells in a particular state of maturation are more susceptible to carcinogenic transformation. On the other hand, it is possible that the transformed state is either incompatible with the basal cell phenotype, or requires some aspect of the mature luminal cell phenotype, resulting in changes subsequent to transformation. We are particularly interested now to examine whether the presence or absence of the NB-1 protein plays a causal role in effecting the mammary cell's capacity to transform or to express a transformed phenotype.

The explanation for the distinctive phenotype of breast tumor cells will require a more complete understanding of both the normal pathways of growth

and differentiation in this cell type, and how the state of differentiation effects the cells' capacity to acquire and maintain a transformed state. This information, in turn, may allow us to develop more efficient protocols for in vitro transformation of HMEC. For example, it may be possible to define and develop specific culture conditions which permit cells to be in a differentiated state in which immortal or malignant tranformation is more likely to occur. Conversely, this information may allow definition of conditions which will interfere with the maintenance of the transformed phenotype, opening up possibilities for novel methods of clinical intervention.

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Table 1. Growth Factor Requirements of Normal and Transformed HMEC in MCDB170

	Percentage of Control Cell Growth					
	184		184A1		184B5	
Medium	MC	CFE	MC	CFE	MC	CFE
Complete MCDB170 +IP	100	100	100	100	100	100
minus I	49	47	11	18	26	73
minus HC	36	32	84	88	18	61
minus EGF	86	2	20	0	12	0
minus BPE	15	21	21	24	16	75

[a] Abbreviations used: I, insulin; HC, hydrocortisone; EGF, epidermal growth factor; BPE, bovine pituitary extract; IP, isoproterenol; MC, mass culture growth; CFE, colony forming efficiency. Cells from specimen 184 (passage 11), and cell lines 184A1 and 184B5 (passages 17-20) were grown in complete MCDB 170 with isoproterenol. For mass culture, cells were subcultured into duplicate 35mm dishes (5 x 10^4 per dish) in the indicated media. When control cultures were subconfluent or just confluent, all the cultures were trypsinized and the cells counted by hemocytometer. For clonal cultures, single cells (100-1000) were seeded into triplicate 100mm dishes. After 10-14 days, cells were stained with Giemsa and colonies greater than 30 cells counted.

Figure Captions

Figure 1. Keratin staining of normal HMEC. HMEC specimen 184 was grown in MM as decribed [5], and second passage cultures stained by indirect immunoperoxidase assay [16], using anti-keratin antibodies monospecific for (A) keratin 14; (B) keratin 18; (C) keratin 19. The results demonstrate the heterogeneous populations present in MM medium. Cells growing in tight patches show the luminal phenotype of keratin 14 negative, keratins 18, 19 positive, whereas the more loosely growing cells show the basal phenotype of keratin 14 positive, keratins 18, 19 negative.

Figure 2. Modulation of mRNA expression in normal human mammary epithelial cells by EHS extracellular matrix and by TGFβ. (A) Normal mammary epithelial strain 184 (passage 14) was plated at 5.0 x 10^6 (Hi EHS) and 1.0 x 10^6 (Lo EHS) cells per 100 mm dish on EHS derived extracellular matrix material. Control (184 and 184B5 passage 26) and TGFβ treated cultures were plated at 0.5 x 10^6 cells per 100 mm dish directly on tissue culture grade plastic dishes. The cells were harvested for RNA at 96h (EHS) or at subconfluence (Control and TGFβ). 10 ug of total cellular RNA was fractionated on 1.3% agarose/formaldehyde gels and transferred to nylon filters as described [26]. The filters were sequentially probed with cDNA to (A) NB-1; (B) keratin 5; (C) vimentin; (D) fibronectin; (E) keratin 18; (F) c-myc. (G) shows total RNA in the original gel stained with ethidium bromide.

Figure 3. Modulation of mRNA expression in transformed HMEC lines by EHS extracellular matrix and by TGFβ. Cells (184B5 passage 26, B5KTu passage 18, 184A1 passage 47, and A1N4TH) were plated and examined as decribed in Figure 2.

Figure 4. Normal and transformed HMEC grown on EHS matrix. Normal human mammary epithelial cell strain 184 (passage 14) was plated at high density (5 x 10^6/100mm dish) on EHS. The cells organized into structures resembling ducts and endbuds normally present in vivo (A x32; B x128). In contrast, 184A1 (passage 36) and 184B5 (passage 26), when plated at the same cell density on this matrix material, made poorly organized structures (C and D, respectively x32), while A1N4-TH was incapable of structure foramtion (E, x32).

CBB 901-253

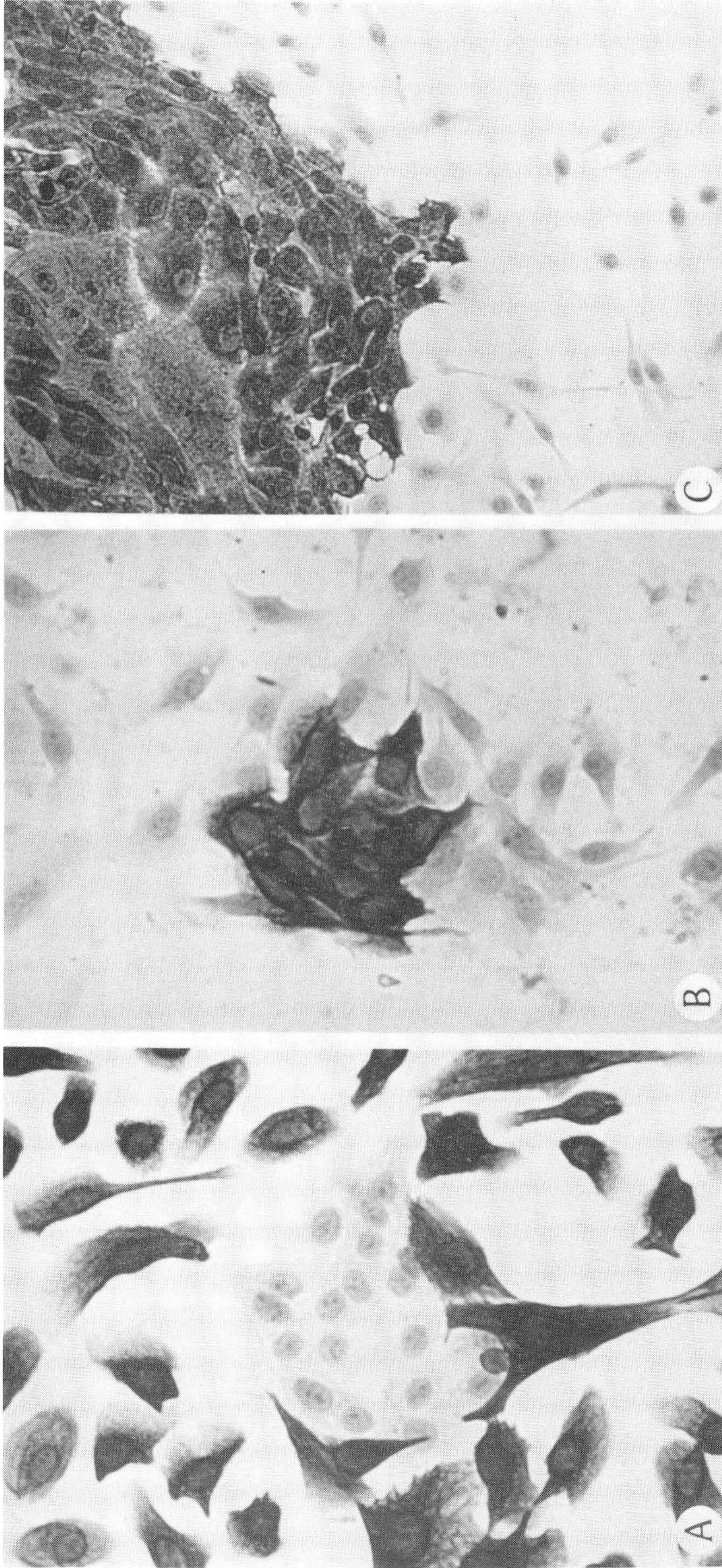

Figure 1

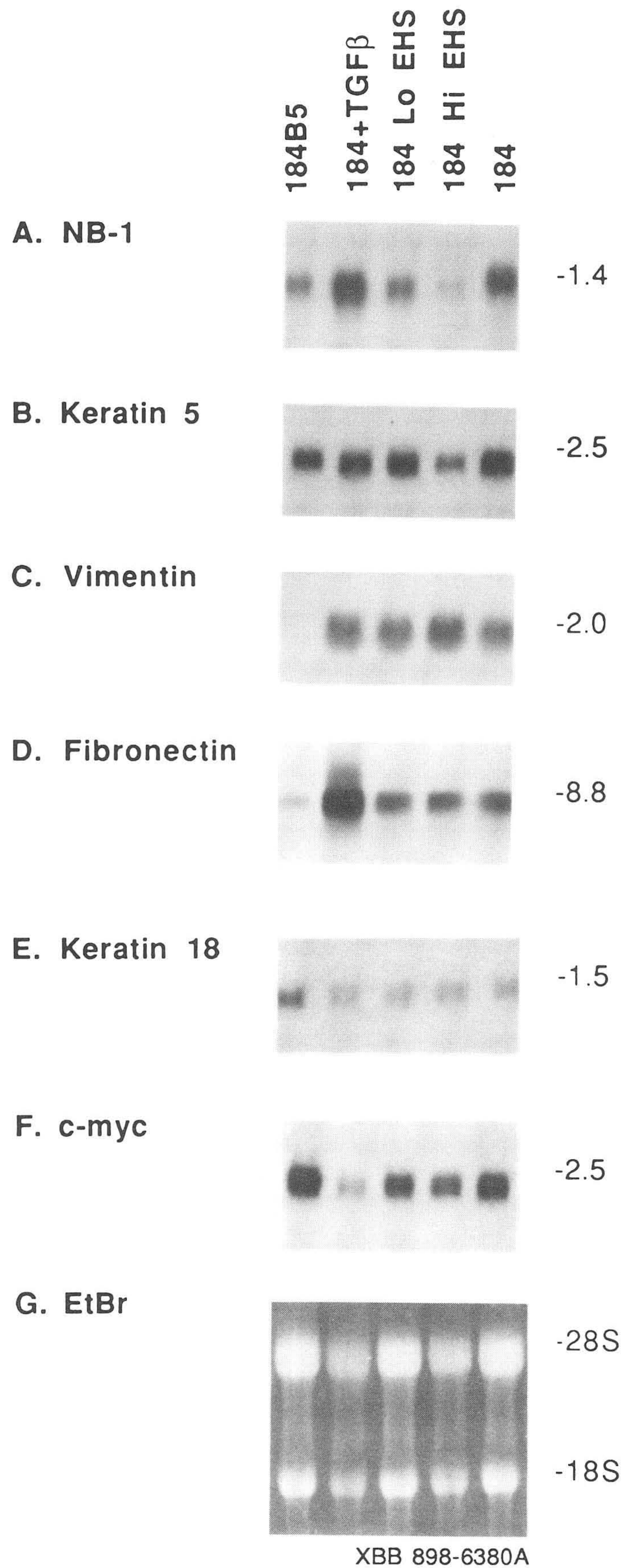

Figure 2

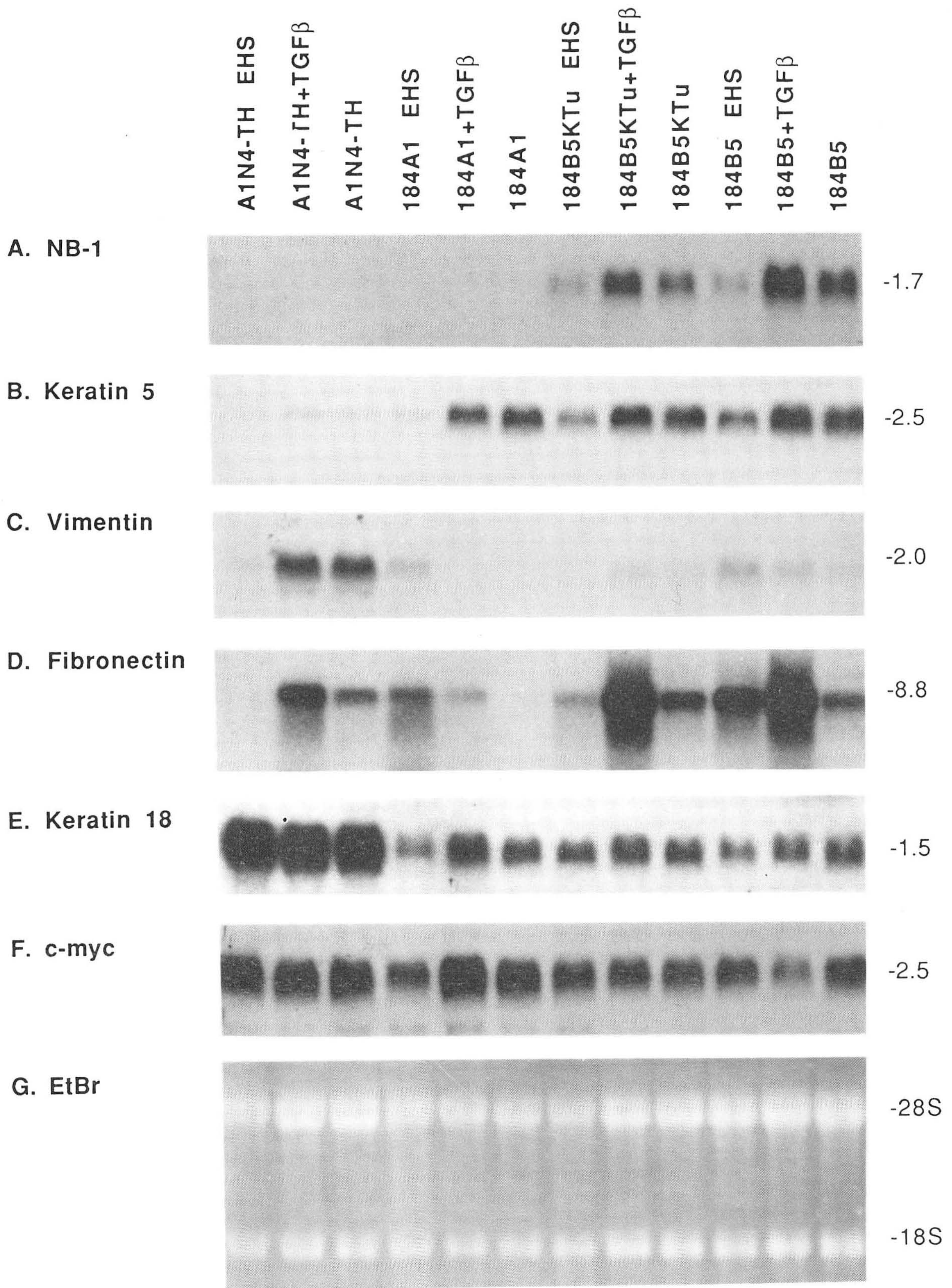

Figure 3

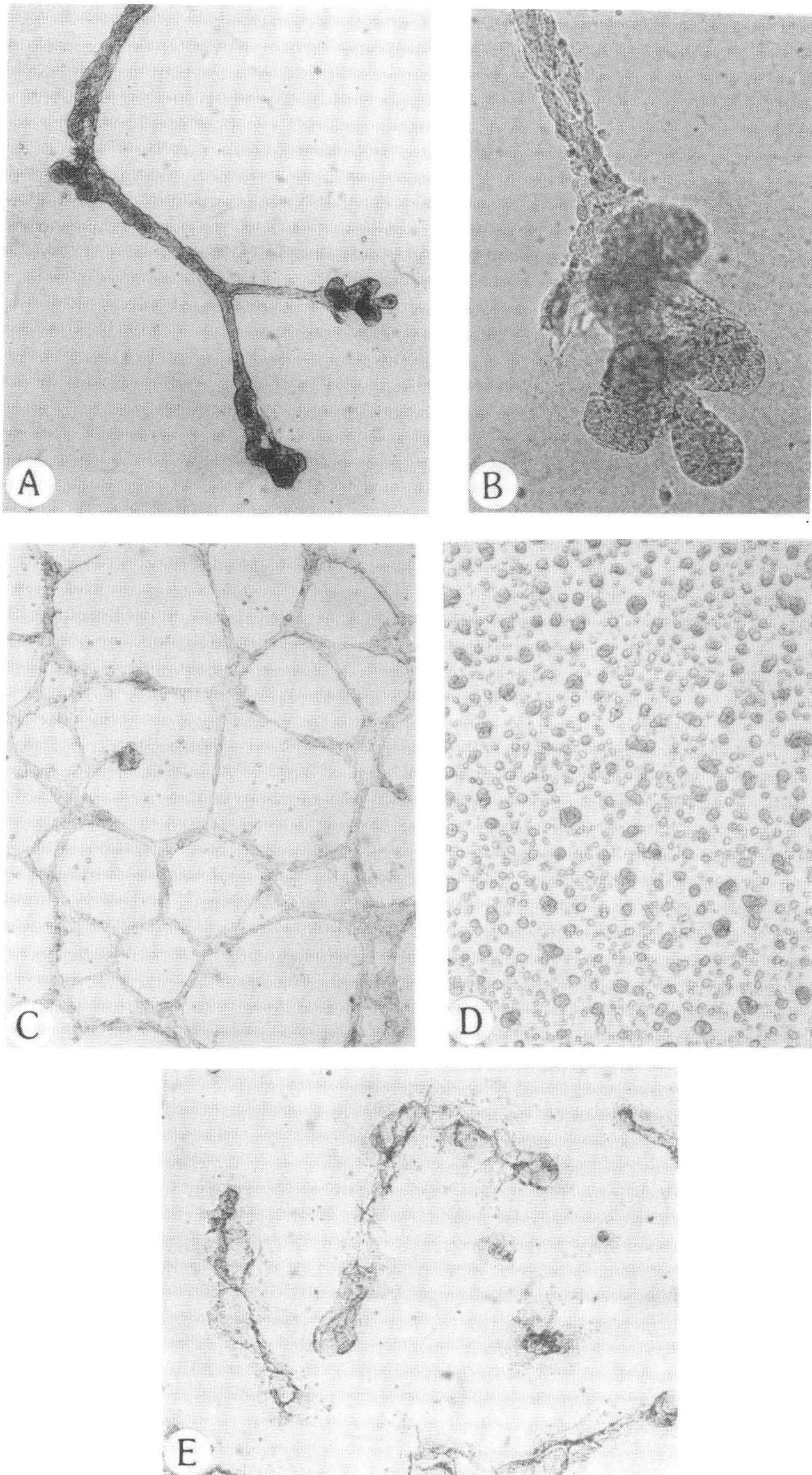

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Figure 4

LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
INFORMATION RESOURCES DEPARTMENT
BERKELEY, CALIFORNIA 94720