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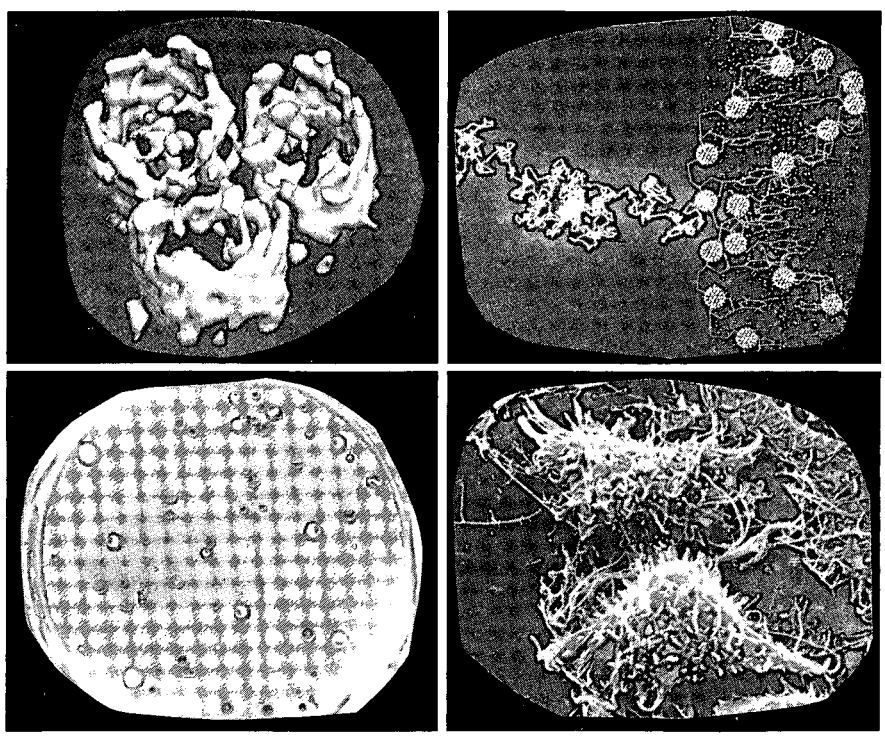

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AN IN VITRO HUMAN MAMMARY EPITHELIAL MODEL SYSTEM FOR STUDIES OF DIFFERENTIATION AND CARCINOGENESIS.

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Our laboratory has developed culture systems 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. One aspect of this work has been to derive cell types which may represent different stages in the progression from normal cells to malignant cells. Another aspect has been characterize these various cell types for their synthesis of and responses to different growth factors, as well as their expresson of differentiated properties. We have additionally utilized our normal and malignant cultures to identify new gene products which may be differentially expressed in these cells. Underlying this work has been the assumption that carcinogenesis involves aberrations in the normal pathways of proliferation and differentiation, and that while in vitro model systems may still not fully reflect the in vivo situation, they will nevertheless prove useful in advancing our understanding of the mechanisms of human carcinogenesis.

In collaboration with other groups, we have developed culture conditions which support the long term growth of HMEC derived from reduction mammoplasty, mastectomy and benign tissues (1-3). These tissues are digested to yield small epithelial clumps, termed organoids, as well as single cell populations which preferentially contain mesenchymal cells. This material can be stored frozen in liquid nitrogen, permitting multiple experiments utilizing cells from the same individual. Two main types of medium have been used to support growth of the HMEC, a serum containing medium, designated MM (4), and a serum-free medium, designated MCDB 170 (2). 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.

Cells grown in MM show active epithelial cell division for 3-5 passages before senescence. 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, a small number of cells with the cobblestone morphology maintain proliferative capacity and soon dominate the culture. These cells 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".

The post-selection HMEC 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. In order to relate the HMEC which maintain long-term growth in vitro to the different cell types identified in vivo, they have been examined for several phenotypes which have been studied using sectioned human breast tissues. Northern blot analysis of mRNA expression and immunohistochemical analysis of protein expression for keratins 5, 14, 8, 18, 19, vimentin, and the large polymorphic epithelial mucins have shown that the cells which initially proliferate in the serum-free MCDB 170 medium resemble mammary cells in the basal layer in vivo. However, post-selection cells begin to express some properties associated with the luminal cell type. Primary cultures of normal HMEC grown in MCDB 170 and early passage cultures grown in MM are heterogeneous. From these results we have proposed that the cells which display long term growth in the serum-free medium represent a multipotent stem cell population present in the basal layer of the gland. With increasing time in culture, these cells show a partial differentiation towards the luminal phenotype (5). Tumor cells in vivo and in vitro generally express the phenotype of the mature luminal cell (5).

Normal HMEC from specimen 184 have been transformed to immortality following exposure to the chemical carcinogen benzo(a)pyrene (BaP) (6, 7). 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, however, almost all of these 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 (8). 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) (6, 7).

Malignant derivatives of 184A1 and 184B5 have been obtained with the use of oncogene containing retroviral vectors and viruses. In the case a 184A1, a clonal derivative, A1N4, which showed reduced nutritional requirements, was exposed to the genes for SV40-T large antigen, v-H-ras, and v-mos singly and in combination (9). 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. v-H-ras or v-mos alone gave cells that produced tumors with reduced frequency and longer latency. SV40-T alone did not yield tumorigenic cells, but did effect the growth factor requirements for anchorage dependent and independent growth (10). 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 184B5 cell line has been exposed to v-K-ras (designated B5-K). This gene alone was capable of producing cells which were 100% tumorigenic in nude mice, with a short latency. However, these tumors did not grow beyond approximately 5cm diameter (7). Most of our studies on these cells have utilized a tumor resected from a nude mouse and placed in culture, leading to the culture designated B5KTu. 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. To perform these experiments, we first determined the requirements of 184A1 and 184B5 for the various growth factors present in MCDB 170. Spontaneous variants of 184A1 and 184B5 could be obtained that showed active

growth in the absence of EGF, insulin, hydrocortisone, or bovine pituitary extract, whereas normal HMEC grown without insulin, hydrocortisone, or bovine pituitary extract ceased growth after 1-3 passages. We next examined the effect of removal of multiple growth factors, and were able to define conditions which did not support the growth of any 184A1 or 184B5 populations.

Populations of 184A1 and A84B5 were then exposed to concentrations of N-nitroso-ethyl-urea (ENU) that yielded 80% growth inhibition, and the surviving cell populations were 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. However, none of the ENU treated cells showed an increase in AIG or formed tumors in nude mice. Thus, we have not yet been able to derive cells that showed tumorigenic properties following use of chemical carcinogens alone.

One main area 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. We have demonstrated that normal HMEC are growth inhibited by TGFβ, with the extent of inhibition increasing as cell are subcultured in vitro (11). All normal HMEC are ultimately growth arrested by TGFβ. In contrast, HMEC which have been transformed to immortality or malignancy may express sustained growth in the presence of TGFβ. 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 (12, 13). Synthesis of extracellular matrix associated proteins such as fibronectin, collagen IV, and plasminogen activator inhibitor 1 is increased upon TGFβ exposure.

Normal HMEC have a stringent requirement for EGF/TGFα for clonal growth. However, growth in mass culture proceeds without additional of exogenous EGF due to the significant level of endogenous production of TGFα (14). Addition of monoclonal antibody 225 IgG to the EGF receptor (MAb 225) prevents HMEC growth (15). Recent experiments have shown that MAb 225 produces a rapid, efficient, and reversible growth arrest in a Go or 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 the competent cells 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. Further studies are now addressing possible differences between normal HMEC of finite lifespan and the immortally transformed HMEC cell lines with respect to their response to MAb225 and their cell cycle controls.

Our HMEC culture system has also been used to identify genes preferentially expressed in normal vs malignant HMEC. Subtractive hybridization was performed between the normal 184 parental cells and the malignantly transformed B5KTu cell line in order to identify genes preferentially expressed in the normal parental cells. Using this technique, a 1.4 kb mRNA, designated NB-1, was found to be expressed in the 184 cells but was barely detectable in the tumorigenic B5KTu (16). 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 non-epithelial cells and tissues, or epithelial tumor cell lines. It's expression is decreased in the immortalized 184B5 cells and is undetectable in the immortalized 184A1 cells.

Sequence analysis of NB-1 revealed a 447 bp open reading frame with extensive similarity at the nucleic acid level to the three known intron containing human calmodulin genes. The NB-1 open reading frame displayed 70%, 71%, and 80% sequence identity with these three calmodulin mRNAs (17-19). The similarity between the translated amino acid sequence of NB-1 and human calmodulin was 85% over the length of the entire protein. The initial characterization of genomic DNA corresponding to the NB-1 transcript indicated the unexpected absence of introns. A literature search revealed the existence of a previously reported human calmodulin "pseudogene" hGH6, which shared identity with NB-1 cDNA (20). 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 (21).

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 precursor stem cell population in the basal layer of the gland.

HMEC plated on EHS, an extracellular matrix preparation derived from the Englebreth-Holm-Swarm murine sarcoma, showed decreased levels of NB-1 mRNA synthesis while forming structures with striking resemblance to endbuds in intact mammary gland tissue. EHS has previously been shown to support increased differentiated functions of a variety of cell types. Additionally, the non-proliferative differentiated luminal cells sloughed off into milk during lactation were negative for NB-1 expression by Northern or PCR analysis. The findings are consistent with the hypothesis that NB-1 is only expressed during certain stages of epithelial differentiation.

We have recently produced polyclonal antisera which can distinguish the NB-1 protein from vertebrate calmodulin, using full length recombinant NB-1 protein as an immunogen. The recombinant NB-1 protein, like calmodulin, binds phenyl-Sepharose in the presence of calcium. Initial studies have indicated that the relative abundance of the 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 normal breast fibroblasts.

The discovery of a new gene product which is homologous to a regulatory molecule as pivotal as calmodulin offers exciting possibilities in efforts to understand calcium regulation of intracellular processes. The strong homology between NB-1 and calmodulin suggests that the NB-1 gene product is a calcium binding protein with signal transduction capabilities. The NB-1 product may compete with calmodulin for calcium and bind with different affinity to cellular substrates. The pattern of expression exhibited by NB-1 in cultured epithelial cells and tissues suggests that NB-1 plays a differentiation specific role. 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 (22, 23). Loss of response to this calcium induced differentiation signal has been shown to correlate with the early stages of transformation in keratinocyte cultures (24). Downregulation of NB-1 expression observed after *in vitro* transformation of HMEC may reflect the fact that a particular state of

differentiation may be required for transformation or that the transformed state is incompatible with high expression of NB-1. Changes in signal transduction of growth or differentiation factors and their associated intracellular second messengers are often implicated in neoplastic transformation. Further analysis of the NB-1 gene product; its expression, function, and regulation, will undoubtedly lead to a more complete understanding of normal and abnormal epithelial cell growth and differentiation.

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