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MIPR NUMBER 94MM4558

TITLE: Mechanism of Abnormal Cell-Extracellular Matrix
Interactions in Human Breast Cancer

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REPORT DATE: October 1996

TYPE OF REPORT: Annual

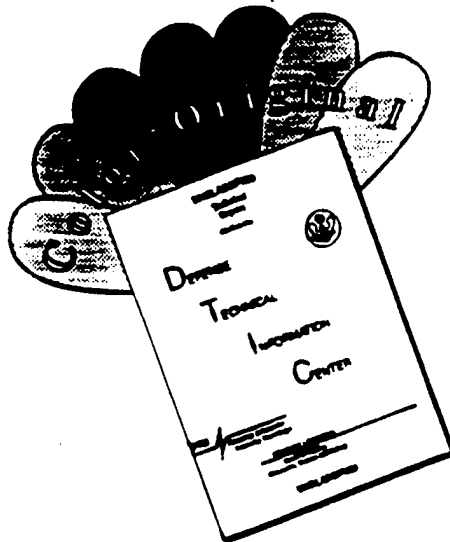
PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1996	3. REPORT TYPE AND DATES COVERED Annual (1 Oct 95 - 30 Sep 96)	
4. TITLE AND SUBTITLE Mechanisms of Abnormal Cell-Extracellular Matrix Interactions in Human Breast Cancer			5. FUNDING NUMBERS 94MM4558	
6. AUTHOR(S) Doctor Huei-Mei Chen				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Lawrence Berkeley Laboratory Berkeley, California 94720			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) Using a three-dimensional (3-D) reconstituted basement membrane (EHS matrix) assay, we have previously demonstrated growth and differentiation patterns of human mammary cells are regulated by cell-extracellular matrix (ECM) interactions and these signaling pathways are perturbed in malignancy. The impact of ECM on breast morphogenesis and tumorigenesis was investigated with a unique human breast cell series [S-1 (nonmalignant-"normal") and T4-2 (tumorigenic)] derived from an immortalized, nonmalignant human breast epithelial cell line, HMT-3522, which in turn was derived from reduction mammoplasty. Both S-1 and T4-2 cells grown within the EHS matrigel recapitulated the morphological behaviors seen in the breast tissue. Co-precipitation and cell-fraction extraction studies of E-cadherin and α - and β -catenins revealed that malignant conversion was associated with compromised adherens junction. ECM-directed transduction pathways via the integrins (ECM receptors) were clearly modified in the malignant breast cells, as T4-2 cells were refractory to the apoptotic signal induced by the β 1 integrin blocking antibody. Functions of classical tumor suppressor genes, both Rb and genes on chromosome 11, on the behavior of mammary epithelial cells in 3-D cultures is discussed. Future studies will focus on implementing molecular biology approaches to provide fundamental clues to the mechanisms underlying the breast tumor development.				
14. SUBJECT TERMS Breast Cancer, Extracellular-matrix, Basement membrane, Micro-environment, Suppressor genes, Metastasis, Integrins			15. NUMBER OF PAGES 19	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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ABSTRACT

Using a three-dimensional (3-D) reconstituted basement membrane (EHS matrix) assay, we have previously demonstrated growth and differentiation patterns of human mammary cells are regulated by cell-extracellular matrix (ECM) interactions and these signaling pathways are perturbed in malignancy. The impact of ECM on breast morphogenesis and tumorigenesis was investigated with a unique human breast cell series [S-1 (nonmalignant-"normal") and T4-2 (tumorigenic)] derived from an immortalized, nonmalignant human breast epithelial cell line, HMT-3522, which in turn was derived from reduction mammoplasty (1). Both S-1 and T4-2 cells grown within the EHS matrigel recapitulated the morphological behaviors seen in the breast tissue. Co-precipitation and cell-fraction extraction studies of E-cadherin and α - and β -catenins revealed that malignant conversion was associated with compromised adherens junction. ECM-directed transduction pathways via the integrins (ECM receptors) were clearly modified in the malignant breast cells, as T4-2 cells were refractory to the apoptotic signal induced by the β 1 integrin blocking antibody. Functions of classical tumor suppressor genes, both Rb and genes on chromosome 11, on the behavior of mammary epithelial cells in 3-D cultures is discussed. Future studies will focus on implementing molecular biology approaches to provide fundamental clues to the mechanisms underlying the breast tumor development.

INTRODUCTION

According to recent statistics, one of eight women in the United States will develop breast cancer in her life span. It is therefore crucial to define cellular and molecular mechanisms contributing to the development of breast cancer with the expectation that such knowledge can lead to more effective strategies for its prevention and/or treatment. Important clues as to how breast cancer is initiated and how it progresses can be gained by studying the normal mammary gland. During puberty, pregnancy, lactation, and involution and recovery periods, the breast naturally undergoes dramatic remodeling that requires the precise regulation of proliferation, invasion, cytological and functional differentiation, and eventual remodeling. Defining the factors and interactions that normally affect these changes may thus be the key to developing more effective intervention strategies for breast cancer. In addition to the well-defined endocrine pathways of hormone and growth factor signaling, it is now

clear that regulatory signals are provided by mammary epithelial cells (MEC) through local and reciprocal interactions with each other, the surrounding stromal cells, and through direct contacts with the extracellular matrix (ECM).

The ECM modulates breast tissue homeostasis *in vivo*, and has been shown to regulate growth, differentiation, and apoptosis of normal murine and human MEC in culture (2,3). Furthermore, breast and other epithelial cancers are associated with decreased, increased or perturbed expression of several integrins, the heterodimeric ECM receptors (4,5). Because altered cell-ECM interaction is a consistent feature of mammary tumors *in vivo* and in culture, it is possible that ECM-signaling pathways contain critical tumor suppressor checkpoints (3). If this were the case, i.e., if there were a cause and effect relationship between perturbed ECM-signaling, altered integrin function, and tumorigenesis, it should be possible to modify morphology and behavior of malignant cells by altering cell-ECM interactions.

It has been known that perturbed expression of cell adhesion molecules, i.e., E-cadherin, which are thought to have a central function in multicellular morphogenesis, is associated with malignancy (6). Cadherins are a multifunctional family of transmembrane glycoproteins and during recognition and adhesion between cells, cadherins regulate homophilic, Ca^{2+} -dependent interactions in epithelial cells. This initiates a cascade of events that leads to the structural and functional reorganization of the cells, including formation of junctional complexes, organization of the actin cytoskeleton at the apical junctional complex and assembly of the membrane cytoskeleton (7). Regulation of cadherin expression affects both normal development and oncogenic transformation. Abnormal expression of cadherins disrupts tissue morphogenesis and inhibition of cadherin function correlates with tumor cell invasion (8). Recent studies have identified three cytoplasmic proteins, α -catenin, β -catenin and γ -catenin (plakoglobin), which bind noncovalently to the cytoplasmic domain of cadherins (9). Formation of the cadherin/catenin complex is required for cadherin functions in cell-cell adhesion and cellular reorganization (10). Catenin molecules mediate the connection of cadherins to the actin filament network and are believed thereby to regulate the strength of cadherin-mediated adhesion (11).

In addition to genes that regulate structure, such as integrins and cadherins, which we feel could be considered suppressor genes, the function of classical suppressor genes such as retinoblastoma (Rb) need to be analyzed in 3-D culture. Among the different regulatory proteins which are known to be associated with the nuclear structure and which constitute checkpoints for the transition between differentiated and tumor phenotypes,

Rb is a good candidate to study in our cell model. Rb is the product of a tumor suppressor gene whose alteration of activity is found in various cancers, including breast cancer. Because of its ability to negatively regulate cell proliferation and its relationship with checkpoints in the cell cycle, Rb has been found often to be involved in differentiation processes (12). The active form of Rb protein associated with growth arrest (binding of E2F factor) and with differentiation (binding of specific transcription factors) has been shown to be hypophosphorylated (12). Interestingly, the hypophosphorylated form of Rb has been found to be associated with the nuclear matrix (13).

In breast cancer, as in most solid tumors, it is usually the metastatic disease rather than the primary tumor that causes death. Several studies have shown that metastasis is a molecular event distinct from initial tumor formation and that cells progress to metastatic capability after accumulating several genetic defects (14). Thus, characterization of the later molecular events in aggressive tumor cells can have several potential benefits. These include understanding the interplay between cancer suppressor genes and cell cycle genes like p53 and Rb, the contributions of genetic susceptibility, and the impact of environmental factors on the development of metastasis. Knowledge of genetic loci whose loss or inactivation contributes to metastasis development can help with decisions of treatment and prognosis of this disease.

Loss of heterozygosity studies have shown that regions on chromosomes 6 and 11 are frequently lost or mutated during the development of breast tumors (15). These genetic alterations suggest the presence of suppressor genes and the inactivation of which permits the development of tumor initiation and subsequent metastases. Many studies have demonstrated that the introduction of normal chromosomes into malignant cell lines may restore gene function and reverse the transformed phenotype. In fact, monochromosomal transfer of human chromosome 11 suppressed the tumorigenic phenotype of several tumor lines (16), including the MCF-7 (17) human breast cancer cell line. Interestingly, the same chromosome exerted a different effect on the highly metastatic MDA-MB-435 human breast cancer cell line. Recent studies in Drs. D. Welsh and B. E. Weissman's laboratories have shown that the metastatic potential of the human chromosome 11/MDA-MB-435 hybrid cell lines was suppressed in nude mice although the tumorigenicity remained (18).

In search of genes participating in the metastatic activity of chromosome 11, the gene KAI1 located at chromosome 11p11.2 was isolated and shown to suppress metastasis when introduced into rat AT6.1 prostate cancer cells (19). KAI1 belongs to a structurally distinct family of membrane glycoproteins (20) including ME491/CD63 (21) and MRP-1/CD9 (22) most of which have been identified as leukocyte surface

proteins. All these proteins have four transmembrane domains and a large extracellular N-glycosylated domain. Although the biological functions of these proteins are mostly unknown, their membrane localization and extensive glycosylation suggest that they function in cell-cell interactions and cell-extracellular matrix interactions (23), both of which are important in invasion and metastasis.

Although many models of cancer initiation and promotion have relied on the use of various xenobiotics including asbestos, benzopyrene, viral oncogenes, and radiation, several cancers, including breast cancer, are postulated to arise as a consequence of an endogenous hormonal imbalance (24). The main polypeptide cytokines that mediate the systemic endocrine signals in the breast microenvironment are transforming growth factor (TGF- β) and epidermal growth factor (EGF)/TGF- α (25). Recently, a malignant transformation of human breast epithelial cells was generated from an immortalized HMT-3522 by withdrawal of EGF from the chemically-defined culture condition (1). This spontaneous acquisition of tumor phenotype as a consequence of deprivation of one single growth factor has suggested that microenvironment cues are powerful factors in the induction of malignancy. In collaboration with Drs. O. Petersen and P. Briand in Denmark, we have conducted a series of studies (detailed in the following section) using these HMT-3522 derived cell lines. We believe that the data obtained from these studies will provide useful insight and understanding of breast cancer progression and the role of ECM and structural "suppressor genes" in the transformation process.

RESULTS/EXPERIMENTAL METHODS

In our previous annual report, we summarized the accomplishment made towards each of the three specific aims stated in the original grant proposal and we listed the corresponding manuscript and publication generated from those data. Here, we would like to reiterate the objectives of the grant proposal: to characterize the molecular mechanisms by which normal versus tumor breast cells interact with their ECM and the microenvironment, and to determine the functional significance of this interaction and to examine the role of ECM/cell signaling in tumorigenesis.

I. Studies with HMT-3522 human mammary epithelial cells:

We have gained access to a novel breast cancer series, HMT-3522, that was established under chemically defined culture conditions from fibrocystic human breast tissue (1). At passage 118, cells were adapted to

grow in medium without EGF and after additional 120 passages (a total of 238 passages) cells became spontaneously tumorigenic in nude mice. The parental line remained non-tumorigenic for greater than 400 passages and we have used these non-malignant cells at passage 50 (named S-1) in our study. In addition, the malignantly transformed cell line which has been through two mouse-culture passages (denoted as T4-2) was used as the tumor counterpart. These latter cells were shown to have a trisomy of chromosome 7p in addition to other genetic alterations such as p53 mutation and a *cmyc* amplification (1). These two cell lines, one originating from the other by spontaneous genetic events, therefore, provide a unique tool for addressing the specific mechanisms involved in malignant conversion in the breast.

A. To study the morphological changes in the malignant transformation by analyzing the expression and localization patterns of cell adhesion proteins:

--S-1 and T4-2 cells in a 3-D culture assay recapitulate the characteristics of normal and malignant breast tissue *in vivo*:

S-1 and T4-2 cells were grown to confluence as monolayers, trypsinized and embedded into EHS matrix (Matrigel, Collaborative Research) as single cells (8.5×10^5 cells/ml) and cultured in serum-free medium for 10-12 days as previously described (3,4) and reviewed in more detail by R.J. Blaschke et al., *Methods in Enzymology* **245**, 535 (1994). Although we have observed only small differences in morphology and growth rates between S-1 and T4-2 cells cultured on plastic support, profound differences became evident after only 4 days when they were grown within a 3-D reconstituted-basement membrane (BM) such as in EHS matrigel. Within ten days, S-1 cells formed organized acini reminiscent of those formed by cells from reduction mammoplasty, while T4-2 cells formed large, loosely disorganized colonies of cells similar to primary tumor cells previously tested in this assay (Fig. 1A and A'). In addition, S-1 cells were able to deposit and organize a BM, as shown by immunostaining of type IV collagen and laminin. T4-2 colonies, while staining for BM components, had no discernible organized BM (Fig. 1B and B'). Cell-cell adhesion was also grossly compromised as evidenced by the absence of lateral E-cadherin immunostaining (Fig. 1C and C'), an increase in cytoplasmic localization of E-cadherin (cell fraction and extraction studies) and reduced interactions of α and β -catenins with E-cadherin (co-precipitation, Fig. 1D). Nevertheless, the two cell types expressed essentially the same levels of the three cell adhesion proteins (Fig. 1E)

indicating that the malignant conversion was associated with compromised adherens junction assembly. We are currently investigating whether phosphorylation of these cell adhesion proteins may play a role in the process of malignant transformation. While S-1 cells were able to growth arrest and exit the cell cycle as demonstrated by thymidine incorporation and immunostaining for Ki-67, the tumorigenic T4-2 cells failed to growth arrest (Fig. 1F and G).

B. To identify the specific integrins (ECM receptors) that transduce the ECM signals to the cells by examining their expression patterns and to elucidate their functional role by disrupting the signaling pathways:

--Differential expression and localization patterns of integrins in S-1 and T4-2 cells:

A detailed integrin profile of S-1 and T4-2 cells was also analyzed. S-1 as well as T4-2 cells expressed integrins $\beta 1$, $\beta 4$, and $\alpha 6$, but their distribution patterns were significantly different in 3-D cultures (Fig. 2A and A' through D and D'). Both the total and the surface levels of $\beta 1$ integrins were higher in T4-2 than in S-1 cells (Fig. 2E and F). The surface $\beta 4$ levels, however, were much lower in T4-2 than in S-1 colonies (Fig. 2H) despite a higher total level of $\beta 4$ in T4-2 cells (Fig. 2G).

Since T4-2 cells had both a higher total level and elevated cell surface expression of $\beta 1$ integrin, we examined the consequences of treatment in 3-D with varying concentrations of a previously characterized rat monoclonal $\beta 1$ integrin antibody, which inhibits ligand binding. This antibody caused significant apoptosis in S-1 cells, as shown previously, while T4-2 cells were refractory (Fig. 2I). We are now in the process of analyzing the mechanisms underlying the differential response to the integrin blocking antibody in S-1 and T4-2 cells.

C. Analysis of the tumor suppressor gene Rb in HMT-3522:

We have first studied the expression level of Rb in S-1 and T4-2 cells grown on plastic. Rb is highly expressed in S-1 cells and is still expressed in T4-2 cells, however, at a lower level. Breast tumor cells (BT549 and MDA-MB-468) not expressing Rb and similar cells transfected with Rb were used as negative and positive controls in the Western blot analysis (26). Interestingly, besides the quantitative changes we detected qualitative differences in the size of Rb present in these two cells. It is postulated that such alteration may be due to different phosphorylation status of Rb.

We are currently conducting a similar analysis of Rb on cells grown in 3-D culture. Since the phosphorylation status of Rb has been associated with its presence or absence in the nuclear matrix, we will also look into the Rb expression level in the protein extracts from nuclear matrices as well as its localization using immunostaining technique in nuclear matrix preparations. One of the goals of the work is to identify a link between the phosphorylation status of Rb, its specific localization in the nuclear matrix, and alterations of nuclear matrix structural components which have been shown to participate in ECM signalling (27).

II. Search for additional tumor suppressor genes:

Interaction with basement membrane recaptures the inhibitory metastatic behavior of chromosome 11/MDA-435 hybrid cells:

In a double-blind study, we have analyzed 9 cell lines sent from Dr. D. Welch (Pennsylvania State Univ.) - 8 of which are microcell-transferred hybrid cells containing various pieces of normal human chromosome 6 and 11. The cells need to be initially adapted to serum-free medium, a very tedious and lengthy process. One cell line is the parental cell line. However, by examining the cell shape and morphology of these cell lines grown in monolayer and in 3-D culture, we have identified 2 lines that showed visually different phenotype from that of the parental cell line. Two of the hybrid cells that had received pieces of chromosome 11 exhibited colony of normal size and appeared much more epithelial-like as compared with the parental cells in monolayer culture and was much less invasive in 3-D culture. These observations are consistent with the in vivo behavior of the metastasis suppressor activity found in the nude mice inoculated with chromosome 11 microcell hybrid cells (18).

Further analysis of these hybrid clones revealed their significantly reduced growth rate and increased apoptosis levels. We are in the process of using the 3-D cell culture system to study the relevance of genetic information on chromosome 11 such as the possible involvement of KAI1 (the metastasis suppressor gene) in the development of metastatic breast tumors.

CONCLUSIONS

In this report, we have described the use of a 3-D culture assay and two closely related human mammary epithelial cell lines that have allowed us to examine the fundamental role of cell and tissue structure in functional differentiation and malignancy. The morphological disorganization of

tumorigenic T4-2 cells and the dispersed distribution and altered protein-protein interaction of an important cell adhesion molecule E-cadherin in 3-D culture clearly showed that some crucial messages directing the normal cell structure are compromised especially at the level of adherens junction assembly in malignancy. From the differential apoptotic response of nonmalignant and malignant cells to the treatment of function blocking β 1-integrin antibodies, we have begun to use our model system to understand the causal role of integrin in malignant conversion in human mammary epithelial cells. Our aims will be to link other regulatory pathways such as adherens junctions, cytoskeletal organization, cell growth rates, and basement membrane assembly to the integrin signals.

In addition, we have begun to explore the role of classical tumor suppressor genes, both Rb and genes on chromosome 11 on the behavior of mammary epithelial cells in 3-D cultures. The initial results are encouraging. In the coming year, we would hope not only to continue with the functional studies of suppressor genes, but also to isolate new suppressor genes in 3-D cultures using differential display technique.

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APPENDIX (publications and abstracts from research progress from Oct. 1, 1995 - September 30, 1996)

1. Chen, H.-M., Weaver VM, Petersen OW, and Bissell MJ (1996) Extracellular matrix (ECM) as a central regulator of function, growth and programmed cell death in breast cells of both mice and men: implications for therapy. *The Pezcoller Foundation Journal* 3, 7.
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3. Weaver VM, Clark S, Petersen OW, and Bissell, MJ (1995) Refractoriness to $\beta 1$ integrin antibody-induced apoptosis precedes malignant transformation in HMT-3522 mammary epithelial cells: a culture model of progressive human breast cancer. Abstract submitted to the American Society For Cell Biology.
4. Wang F, Weaver VM, Petersen OW, and Bissell, MJ (1995) Progression of HMT-3522 human mammary epithelial cells to a malignant phenotype is preceded by perturbed subcellular localization of E-cadherin. Abstract submitted to the American Society For Cell Biology.

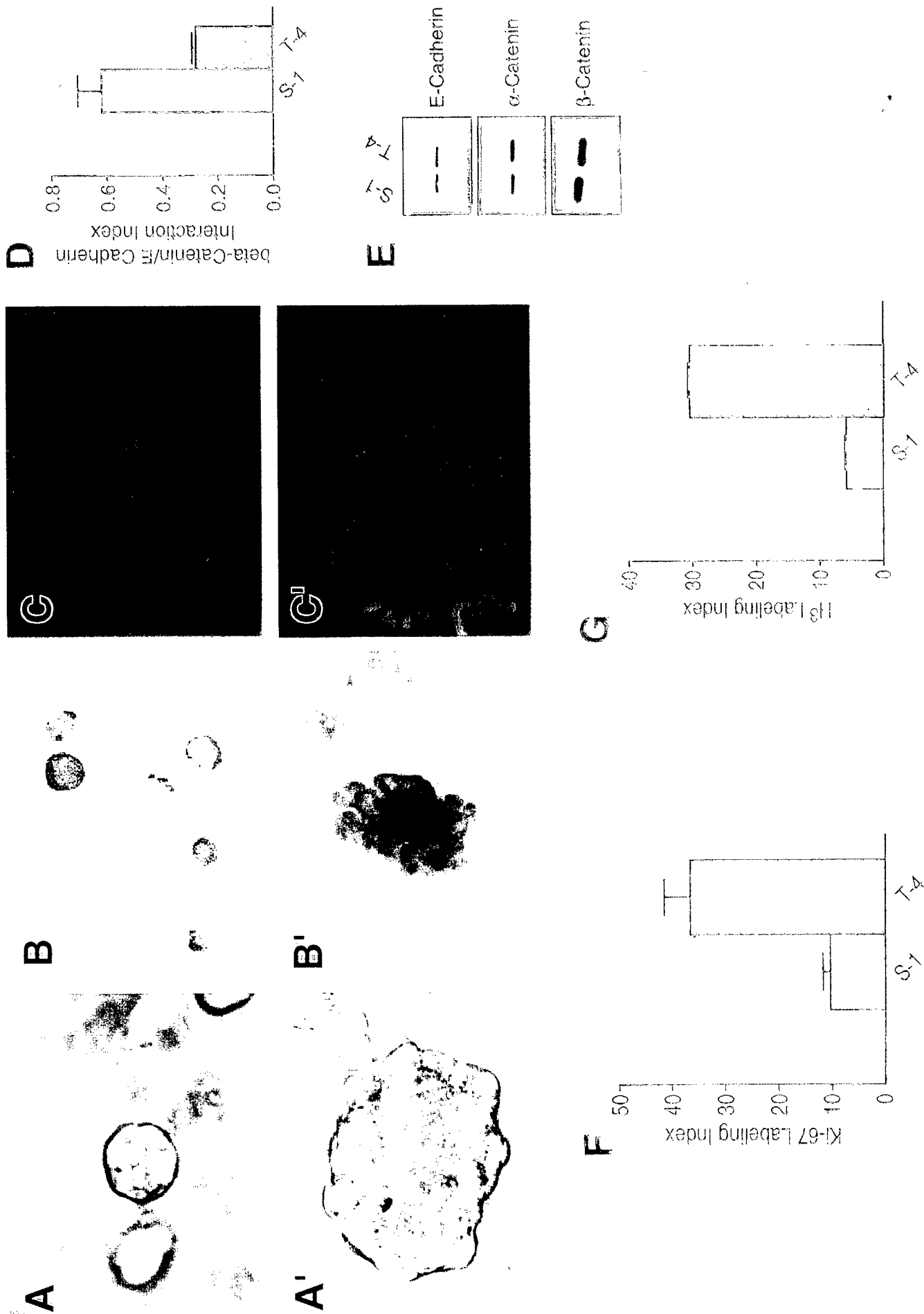
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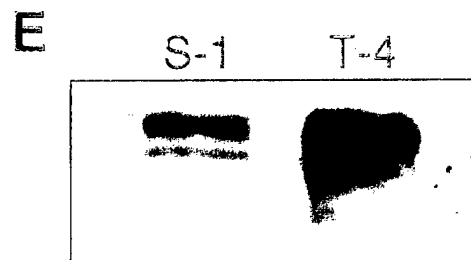
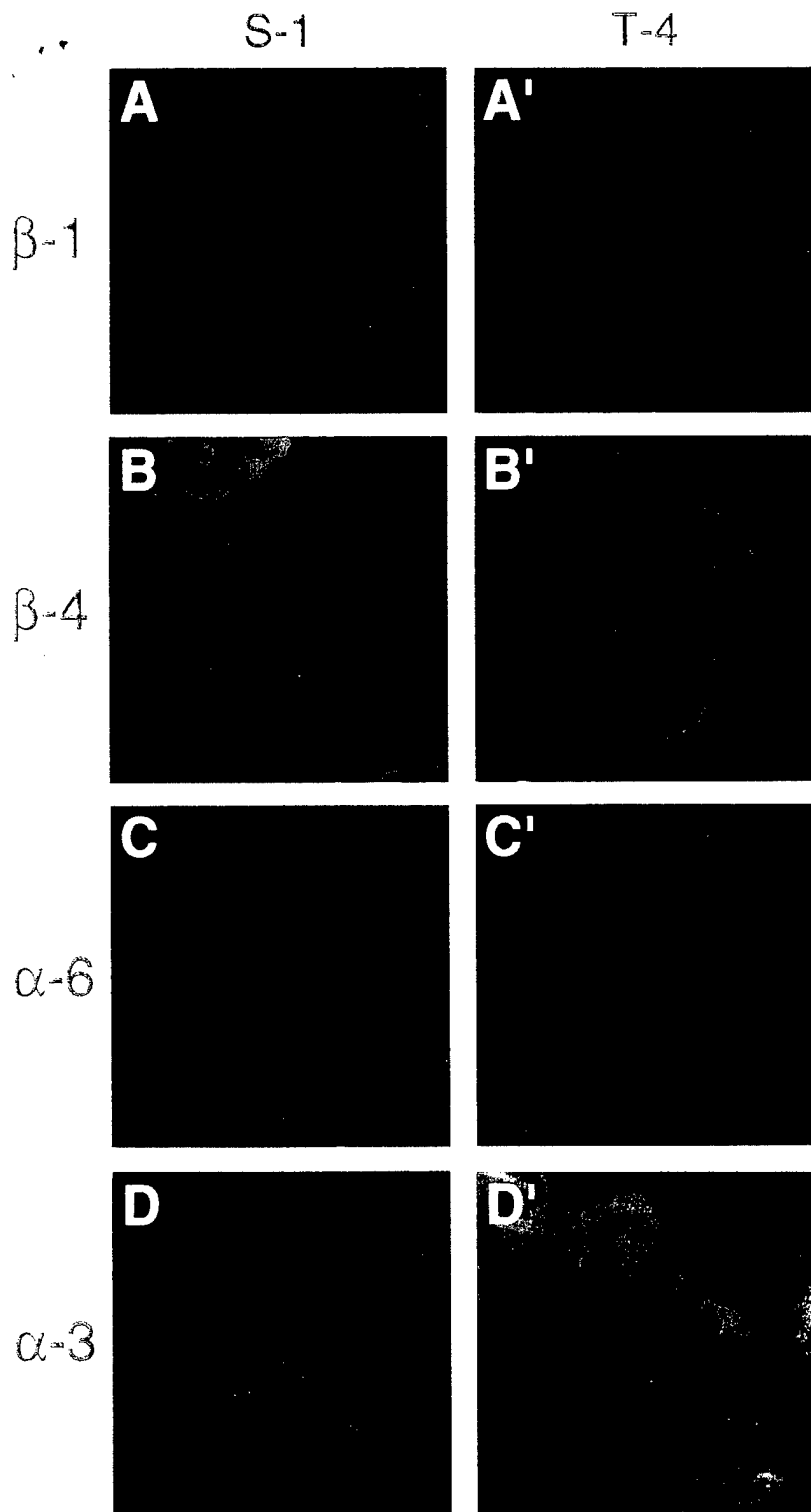
Fig. 1. Characterization of the HMT-3522 human breast cancer model.

(A,A') Phase contrast micrographs of non-malignant S-1 cells (A) and tumorigenic T4-2 cells (A') viewed directly inside EHS for morphology (x400). S-1 cells formed spherical structures reminiscent of true acini (A), whereas T4-2 cells formed large irregular colonies (A'). (B,B') Immunostaining for basement membrane components. Colonies were cryosectioned (5 μ) and immunoperoxidase stained for type IV collagen and hematoxylin counterstained for nuclei (x270). S-1 acini (B) stained for BM proteins at the cell-ECM junctions as expected, while BM deposition in tumor colonies (B') was clearly disorganized and no longer polarized. (Comparable results were obtained for laminin, not shown). (C, C') Confocal fluorescence microscopy of cryo-sectioned colonies immunostained for E-cadherin (x600). S-1 acini (C) stained for E-cadherin primarily at the cell-cell junctions as also observed in normal breast sections. The T4-2 colonies (C') showed punctate, peripheral cell expression and intracellular staining. (D) The β -catenin-E-cadherin interaction index. This was determined by immunoprecipitating E-cadherin from lysates of equivalent cell numbers followed by immunoblotting and densitometric analysis of adherens protein levels and expressed as a ratio of β -catenin to E-cadherin. There was a 50% decrease in β -catenin protein co-precipitating with E-cadherin in T4-2 cells as compared to S-1 acini. Data are expressed as the ratio of β -catenin to E-cadherin densitometric measurements from duplicates of 2 separate experiments. (Similar results were observed for α -catenin). (E) Immunoblots of total levels of E-cadherin, α -catenin and β -catenin. There were comparable levels of all 2 adherens proteins in S-1 acini and T4-2 colonies. (F, G) Percent of thymidine and Ki-67 labeling in S-1 acini and T4-2 colonies. Labeling indices were calculated by scoring 100-400 cells on cryo-sections immunostained for Ki-67 (F) or radiolabelled with thymidine (24 hrs. [3 H]TdR 20Ci/ml, NEN Research Products, Dupont) (G) from 3-4 separate experiments. All cultures were analyzed after 10-12 days inside EHS.

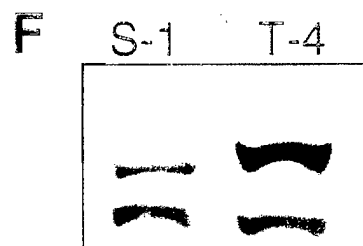
Fig. 2. Characterization of integrins in the HMT-3522 cells.

(A-D) S-1 acini and (A'-D') T4-2 colonies cryo-sectioned, immunostained and examined by confocal fluorescence microscopy for β 1-(A,A'), β 4-(B,B'), α 6- (C,C') and α 3-integrin (D, D') localization (x600). β 1, β 4, and α 6-integrins were targeted to the cell-ECM junction in the S-1 acini (A-C), but this polarized-basal distribution was lost in T4-2 colonies (A'-C'). S-1 acini exhibited baso-lateral α -3 integrins (D), whereas T4-2 colonies (D') demonstrated disorganized plasma membrane and cytosolic expression of this integrin. (E & G) Western analysis of β 1- and β 4-integrins. Total cell lysates of S-1 and T4-2 colonies showed elevated expression of β 1-(E) and β 4-integrins (G) in the tumor cell colonies. (F & H) Cell surface expression of β 1- and β 4-integrin heterodimers using biotinylation. Tumor colonies had higher plasma membrane levels of β 1-(F) but lower levels of β 4-integrin (H) heterodimers than S-1 acini. Surface expression was assessed by sulfo-NHS-biotin labeling (Pierce) of plasma membrane proteins, after dispase and trypsinization, either as colonies, or as single cells derived from colonies, followed by immunoprecipitation and immunoblot analysis. All cells were analyzed after 10-12 days inside EHS. (I) The apoptotic labelling index. This was determined by labelling the 3' OH DNA ends in cryosections of day 4 cells in EHS using an in situ apoptosis kit (Boehringer Mannheim). 150-200 cells, in each condition, from 2-3 separate experiments were scored for the presence of DNA nicks. Data are expressed as the number of nuclei labelled positive for DNA nicks divided by the total number of nuclei and multiplied by 100. Incubation with β -1 integrin function blocking antibodies induced greater than 60% cell death in S-1 cells whereas T4-2 cells were resistant to apoptosis induction.





β -1 integrin



β -4 integrin

