Because every cell within the body has the same genetic information, a significant problem in biology is to understand how cells within a tissue express genes selectively. A sophisticated network of physical and biochemical signals converge in a highly orchestrated manner to bring about the exquisite regulation that governs gene expression in diverse tissues. Thus, the ultimate decision of a cell to proliferate, express tissue-specific genes, or apoptose must be a coordinated response to its adhesive, growth factor, and hormonal milieu. The unifying hypothesis examined in this overview is that the unit of function in higher organisms is neither the genome nor the cell alone but the complex, three-dimensional tissue. This is because there are bidirectional connections between the components of the cellular microenvironment (growth factors, hormones, and extracellular matrix) and the nucleus. These connections are made via membrane-bound receptors and transmitted to the nucleus, where the signals result in modifications to the nuclear matrix and chromatin structure and lead to selective gene expression. Thus, cells need to be studied “in context”, i.e., within a proper tissue structure, if one is to understand the bidirectional pathways that connect the cellular microenvironment and the genome.

In the last decades, we have used well-characterized human and mouse mammary cell lines in “designer microenvironments” to create an appropriate context to study tissue-specific gene expression. The use of a three-dimensional culture assay, developed with reconstituted basement membrane, has allowed us to distinguish normal and malignant human breast cells easily and rapidly. Whereas normal cells become growth arrested and form organized “acini,” tumor cells continue to grow, pile up, and in general fail to respond to extracellular matrix and microenvironmental cues. By correcting the extracellular matrix-receptor (integrin) signaling and balance, we have been able to revert the malignant phenotype when a human breast tumor cell is cultured in, or on, a basement membrane. Most recently, we have shown that whereas β1 integrin and epidermal growth factor receptor signal transduction pathways are integrated reciprocally in three-dimensional cultures, on tissue culture plastic (two-dimensional monolayers), these are not coordinated. Finally, we have demonstrated that, rather than passively reflecting changes in gene expression, nuclear organization itself can modulate cellular and tissue phenotype. We conclude that the structure of the tissue is dominant over the genome, and that we may need a new paradigm for how epithelial-specific genes are regulated in vivo. We also argue that unless the structure of the tissue is critically altered, malignancy will not progress, even in the presence of multiple chromosomal mutations.

Introduction

“Science is built up with facts, as a house is with stones. But a collection of facts is no more science than a heap of stones is a house.”—Jules Henri Poincaré (1854–1912)

In adult organisms, cells must maintain the program of regulated gene expression that is instituted during development. What are the genomic rules that allow this program of gene expression to be selective? Even after the cells have arrived at their “destinations” within the tissues, they radically alter their patterns of gene expression, both quantitatively and qualitatively, as a result of systemic (hormones, chemokines, and environmental insults) and microenvironmental (cell-ECM, cell-cell, local growth factors, local injury, and others) signals. This is observed most dramatically when cells are isolated and cultured outside of the organism, most notably on tissue culture plastic (as 2D monolayers; 1). Along with loss of tissue-specific gene expression, the most striking change is in cellular and nuclear organization and architecture (2).

One of us proposed almost two decades ago that in addition to growth factors and hormones, ECM that surrounds tissues in vivo contains signaling molecules that are responsible for maintenance of tissue form and function (3), and furthermore, that there may be both mechanical and biochemical connections between the ECM and the cellular microenvironment, leading to changes in chromatin structure and gene expression. The combined work of many investigators, including our own, has confirmed the significance of the ECM in every tissue examined (reviewed in Refs. 4–6). In addition, the discovery of the ECM receptors, the most important family of which is the integrins (reviewed in Refs. 7 and 8), has elucidated a mechanism by which ECM signaling could be achieved across the cellular membrane.

In parallel, the work of structural and molecular biologists has unraveled important new information about the structure of the nucleus and the chromatin. The higher order structure of eukaryotic chromosomes consists of independent loop domains that are separated from each other by the attachment of specialized genomic sequences (matrix attachment regions) onto the nuclear skeleton, leading to changes in chromatin structure and gene expression. The combined work of many investigators, including our own, has confirmed the significance of the ECM in every tissue examined (reviewed in Refs. 4–6). In addition, the discovery of the ECM receptors, the most important family of which is the integrins (reviewed in Refs. 7 and 8), has elucidated a mechanism by which ECM signaling could be achieved across the cellular membrane.

The compaction of eukaryotic DNA into chromatin is thought to establish a specific pattern of gene expression. Heterochromatin, defined cytogenetically as regions of the genome that remain condensed throughout the cell cycle, is known to remain transcriptionally silent. Translocation of a euchromatic region of the genome to a site adjacent to heterochromatin often yields variable silencing of the translocated genes, as exemplified by the process of position effect variegation of the eye color gene brown, in Drosophila (14). Other examples of chromatin packaging associated with long-term transcriptional repression is the transcriptional silencing observed at the mating

The abbreviations used are: 2D, two-dimensional; 3D, three-dimensional; ECM, extracellular matrix; NM, nuclear matrix; MEC, mammary epithelial cell; EGF, epidermal growth factor; EGFR, EGF receptor.
type loci or at telomeres in yeast (15, 16). From these and many other studies, histones are emerging as substrates for activities controlling transcription.

Histone acetylation is a crucial and evolutionary conserved mechanism, allowing chromatin reorganization because it loosens up histone-DNA interactions by neutralization of the net charge of histone tails and disruption of nucleosome-nucleosome interactions (17, 18). Acetylated lysine residues in the NH2-terminal tail domains of nucleosomal histones allow us to distinguish euchromatin from heterochromatin, where silent heterochromatin is hypomethylated (19). The level of histone acetylation is determined by an equilibrium between histone acetyltransferases and deacetylases. The opportunity for chromatin structure to be precisely modulated through highly regulated reversible mechanisms offers the possibility of transcriptional silencing or activation by this mechanism.

**Synopsis of Previous Results and the Relevant Literature**

The above brief summary points to a vast, and as yet only minimally understood, area of how organization of chromatin and nuclear architecture may be regulated within specific tissues.

The following provides an abbreviated summary focused largely, but not exclusively, on the work of our laboratory and those of our collaborators to provide a background for our working hypothesis on the relation of tissue structure and normal and malignant behavior (Fig. 1):

(a) There is evidence that even “universal” regulatory pathways, such as apoptosis, are clearly tissue specific, e.g., radiation-induced apoptosis in vivo is p53 dependent in thymus and sphingomyelinase dependent in endothelium (20).

(b) It is now well established that ECM and its receptors critically affect tissue-specific structure and gene expression in all tissues; the mammary gland and MECs provide a well-studied example (reviewed in Ref. 5; Fig. 1).

(c) ECM affects both cellular “shape” and biochemical signaling (21, 22). Cytoskeletal shape-induced signaling is sufficient to modulate genes (such as lactoferrin; Refs. 22 and 23) and differential splicing of at least one protein examined thus far (band 4.1; 24).

(d) The magnitude of the ability of the ECM to influence gene transcription is exemplified by its ability to regulate the activity of some of the most potent transcriptional activators in eukaryotic cells, the viral enhancers (25, 26).

(e) Promoter sequences of both mammary-specific genes and growth factors contain ECM-responsive elements. /3-Casein gene promoter contains a 161-bp enhancer (BCE-1; see Fig. 1) that is induced strongly (15–150-fold) by ECM (27, 28). The transforming growth factor /3 promoter, on the other hand, is completely suppressed by ECM (29).

(f) The ECM-response element of the /3-casein gene (BCE-1) is active only within a chromatin context (this is true also for viral enhancers mentioned above). Furthermore, it now appears that contact with ECM alters the histone acetylation/deacetylation of the enhancer (30).
Abl Ref. 37). mammary tumor induction and invasion (31-36; briefly reviewed in overexpressed will override the ECM-induced morphogenesis the gap between studies in humans and mice and justifies the use of the mouse to model aspects of normal and malignant human breast function.

form spherical alveolar structures that are similar to acini and epithelial to mesenchymal conversion and may play a role in "acinus" formation) and push the cells back into the cell cycle. Branching ducts and ductules is found in both organisms. In light of these fundamental similarities, it is not surprising that human and mouse epithelial cells display similar behaviors in 3D basement membrane cultures; both cell types undergo morphogenesis to surface receptors such as EGFR (Ref. 41 and see below), which when changes in levels of cyclin D1, p21, and other growth parameters rise dramatically as a result of ECM ligation and changes in cellular shape (38, 39).

(i) ECM regulates transcription factors such as Id-1 (40) and cell surface receptors such as EGFR (Ref. 41 and see below), which when overexpressed will override the ECM-induced morphogenesis (i.e., "acinus" formation) and push the cells back into the cell cycle.

(j) By manipulating the cell surface, we can "revert" a disorganized and malignant human breast cell (HMT-3522, see below) to a quiescent, practically normal phenotype using inhibitory antibodies to β1 integrin (42). Phenotypic reversion is associated with dramatic changes in levels of cyclin D1, p21, and other growth parameters (such as the retinoblastoma susceptibility gene, Rb) both in culture and in vivo without a change in tumor genotype.

Modeling Normal and Malignant Mammary Gland in Culture

To address the question of how the bidirectional flow of information sets up the 3D structure of a tissue and how this in turn governs selective gene expression, we have used mammary epithelial cells and the mammary gland itself as our central model. Why the breast?

(a) It is one of the few tissues that can be induced to undergo dramatic shifts in structure and function as a result of extracellular cues, even during the adult life of the organism.

(b) Despite the inherent complexity in organization and function of any tissue, it is relatively simple (compared, for example, to the brain, lung, or liver) and thus can be modeled in culture.

(c) The breast is a highly sensitive target of radiation and environmental insults, and breast cancer is a devastating disease in need of diagnosis, prognosis, cure, and prevention.

(d) Breast cancer cells provide examples of loss of structure and altered gene expression and thus could be used as natural "mutants" for comparisons of genotype and phenotype.

(e) A number of mouse and human mammary epithelial cell models exist (43, 44). Together they provide tractable and complementary systems for the study of mammary gland function and tumorigenicity (Fig. 2). For example, results obtained from studies of mouse and human cells in culture can be verified by transplanting these cells back into mice. Comparisons between the behavior of the transplanted cells, both human and mouse, and that of the same cells in culture provide useful information that ultimately contributes to the understanding of breast functions in vivo in humans.

The HMT-3522 Breast Tumor Progression Series

One recently described human MEC model, the HMT-3522 series, has proven to be particularly useful in the study of human breast cancer progression (45). The HMT-3522 cell series originated from a purified luminal epithelial cell population recovered from a breast biopsy of a woman with fibrocystic breast disease. These cells, collectively referred to as S1, have been cultured under chemically defined conditions for >500 passages (46). Despite the fact that S1 cells from later passages are notably aneuploid and carried a mutation in the p53 gene, none of these cells have yet given rise to tumors in nude mice. Because growth autonomy has been cited as a prerequisite for malignant conversion, Brind and co-workers reasoned that removal of EGF from the HMT-3522 medium might eventually induce malignant transformation in S1 cells. Thus, to generate a tumorigenic HMT-3522 cell species, S1 cells (at passage 118) were grown in the absence of EGF. After ~120 passages, these EGF-free cultures gave rise to tumors in nude mice; cells cultured from these tumors were called T4-2 cells (47).

Although cells of the HMT-3522 series are hard to distinguish when cultured on plastic as 2D monolayers, phenotypic differences between the various HMT-3522 cell populations can be readily detected when these cells are cultured in a 3D reconstituted basement membrane (Fig. 3; Refs. 42 and 48). In this system, nonmalignant S1 cells form phenotypically normal structures reminiscent of terminal duct lobular units in situ, whereas their premalignant and tumorigenic counterparts form disorganized, continuously growing colonies. The following discussion will describe studies of S1 (passage 50) and T4-2 cells, exclusively.

Reversion of the Malignant Phenotype by β1 Integrin Inhibitory Antibody

Recent studies, using both in vivo and culture models, have demonstrated a role for cell-ECM receptors, or integrins, in human breast tumor progression (42, 48–51). Integrins are a class of heterodimeric transmembrane receptor proteins that mediate cell anchorage, influ-

(g) ECM and ECM-degrading enzymes have been shown to be central regulators of growth, apoptosis, branching morphogenesis, and epithelial to mesenchymal conversion and may play a role in mammary tumor induction and invasion (31-36; briefly reviewed in Ref. 37).

(h) Nuclear localization and half-life of important genes, such as Abl and p53, change dramatically as a result of ECM ligation and changes in cellular shape (38, 39).

(i) ECM regulates transcription factors such as Id-1 (40) and cell surface receptors such as EGFR (Ref. 41 and see below), which when overexpressed will override the ECM-induced morphogenesis (i.e., "acinus" formation) and push the cells back into the cell cycle.

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Mouse Mammary Cell Lines

Human Breast Cell Lines

In 3D

Fig. 2. Mouse and human mammary epithelial cell models provide complementary systems for the study of breast function. Although mouse and human mammary tissue vary somewhat with respect to overall organization, the double-layered structure of the branching ducts and ductules is found in both organisms. In light of these fundamental similarities, it is not surprising that human and mouse epithelial cells display similar behaviors in 3D basement membrane cultures; both cell types undergo morphogenesis to form spherical alveolar structures that are similar to acini in situ.

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ence cell shape, and propagate intracellular signals similar to those observed during growth factor receptor activation (7, 8, 52). Given that cell-ECM interactions are dramatically disrupted in breast cancer tissue, we compared the integrin profile of the “normal” nontumorigenic S1 cells to that displayed by tumorigenic T4-2 cells (42). The results showed that the levels of both total and surface-expressed β1 integrin proteins were significantly higher in the T4-2 cells than in the nontumorigenic S1 cells. To test the possibility that inappropriate integrin expression and activation contributes to the tumorigenic behavior of the T4-2 cells, a β1 integrin function-blocking antibody (monoclonal antibody AIIB2) was added to the 3D cultures of T4-2 cells. Upon β1 integrin inhibition, T4-2 cells underwent a striking morphological and functional normalization, characterized by reformation of actin with reassembled basement membranes, normal cell-cell contacts and cellular polarity, and a reorganized actin cytoskeleton (Fig. 4A). Furthermore, these “reverted” cells became growth arrested and were found to exhibit reduced tumorigenicity in nude mice (42). The program of pleiotropic changes that occur in T4-2 cells as a result of treatment with β1 integrin inhibitory antibody are summarized in Fig. 4B. These findings provide an independent demonstration that integrins, by virtue of their ability to sense and respond to cues from the ECM, can have profound effects on cellular perception of the microenvironment and therefore ultimately on cellular behavior. Furthermore, cells can harbor a myriad of chromosomal mutations, as is the case with T4-2 cells (determined by comparative genomic hybridization; not shown), but as long as the cell detects an appropriate cellular microenvironment that allows a cell to adopt a normal structure, the cell will display a normal phenotype. Our conclusions are supported by a recent report by Deng et al. (53), demonstrating that, although breast tissue found adjacent to neoplastic lesions display normal cellular morphologies in situ, cells within these lesions often harbor chromosomal rearrangements (i.e., loss of heterozygosity), identical to some of the mutations found in the adjacent tumor.

Fig. 4. Reversion of human breast cancer cells to a “normal” phenotype using a β1 integrin function-blocking antibody. A, confocal double fluorescence microscopy images of phenotypically normal acini (S1, left), tumorigenic disorganized colonies (T4-2, middle), and reverted, phenotypically normal acini after treatment with β1 integrin antibody (T4-2R, right). Colonies were double-stained for F-actin (arrows) and nuclei (propidium iodide; top) or E-cadherin and β-catenin (bottom). These data demonstrate that phenotypic reversion of HMT-3522 T4-2 cells is accompanied by a normalization of both cytoskeletal elements cell-cell junctional complexes. Images are reproduced in black and white from an original color figure by Weaver et al. (42). B, T4-2 cell phenotypic reversion was characterized by a number of assays summarized in this panel. These data, in combination with those in A, illustrate that the “reverted” T4-2 structures behave like their “normal” S1 counterparts, despite the fact that these cells harbor a number of chromosomal aberrations known to predispose cells to cancerous behavior (42).
The ultimate decision a cell makes to proliferate or differentiate is an integrated response to cues derived from both matrix molecules and growth factors within the tissue (5, 7, 54). Although the mechanism by which these extracellular cues are integrated inside the cell is largely unknown, growing evidence suggests that coordinated action of adhesion molecules and growth factors is dependent upon the precise coordination of the intracellular signaling events they induce. We have used the HMT-3522 cell series to explore the intracellular coupling of cell adhesion- and growth factor-dependent signaling and to determine how tissue structure influences signal integration.

EGFR overexpression is commonly associated with breast carcinomas and has emerged recently as a promising target for cancer therapy (55). In comparison with their nontumorigenic counterparts, T4-2 cells display a 10-fold increase in levels of surface expression of EGFR, a transmembrane tyrosine kinase receptor with well-established growth-promoting activity (56). Given the growth-promoting potential of EGFR and its elevated expression in T4-2 tumor cells, it was puzzling to find that inhibition of $\beta 1$ integrin function in T4-2 cells was sufficient to induce the growth-arrest characteristic of the phenotypic reversion. We therefore asked whether the phenotypic reversion observed upon treatment of 3D cultures with $\beta 1$ integrin inhibitors might be due to a reciprocal cross-modulation of $\beta 1$ integrin and EGFR activity: could inhibition of $\beta 1$ integrin function lead to inhibition of EGFR, and vice versa? In support of this hypothesis, we found that inhibition of EGFR function in T4-2 cells (using the function-blocking antibody, monoclonal antibody 225, or the chemical inhibitor, tyrphostin AG1478) resulted in phenotypic reversion that was identical to that observed with $\beta 1$ integrin inhibitors (not shown; Ref. 41). Each inhibitor, alone, was sufficient to revert the malignant phenotype in this 3D assay.

The observed phenotypic reversion was likely due to the coordinate attenuation of $\beta 1$ integrin and EGFR function at the level of protein expression and/or at the level of receptor activation. Using Western immunoblot analysis, we found that treatment of T4-2 cell cultures with a $\beta 1$ integrin inhibitor resulted in the down-modulation of both $\beta 1$ integrin and EGFR protein expression (summarized in Fig. 5); treatment with EGFR inhibitors caused a similar reduction in $\beta 1$ integrin and EGFR protein levels. In contrast, the levels of E-cadherin did not cause the down-regulation of $\beta 1$ integrin or EGFR proteins, nor did it result in concomitant attenuation of $\beta 1$ integrin or EGFR function (Fig. 5 and data not shown). Thus, the coordinate regulation of $\beta 1$ integrin and EGFR is dependent upon specific contributions from the 3D basement membrane, presumably in the form of either structural and/or biochemical cues. We interpret these results to mean that normal cells, in a 3D context, use the mechanism of receptor cross-modulation to establish a cellular signaling environment that promotes normal behavior, and that disruption of the mechanisms that regulate these processes can result in tumorigenic behavior.

### The ECM-Nuclear Matrix Connection

HMT-3522 cultures in 3D have been used effectively to demonstrate the importance of cell surface receptor coordination in normal and tumorigenic cellular behavior. In addition, this human mammary epithelial cell model has been used also to probe the relationship between nuclear structure and tissue architecture and function. When cultured in 3D basement membrane assays, HMT-3522 S1 cells exhibit a spectrum of cellular behaviors ranging from proliferative to growth arrested to differentiated. Given this behavioral range and the possibility that nuclear architecture itself may impart important behavioral cues (58), we investigated whether these progressive stages of S1 cell differentiation are accompanied by specific nuclear orga-
why a universal genetic lesion, for example in tumor suppressor genes such as BRCA-1 (60) and APC (61), would give rise to tumors only in specific tissues. A number of other laboratories are modeling tissues other than the mammary gland in 3D context. The combined effort will allow us to decipher how seemingly similar pathways and genes carry out different functions in different tissues. The information generated is complex and intriguing. As such, we will need to develop both the nuclear lamina protein, lamin B, remained unchanged, suggesting that this reorganization is specific for components of the internal NM.

Although these data indicate that NM proteins, such as NuMA, are significantly influenced by extracellular cues provided by the ECM, we suspected that NM architecture itself may also provide important behavioral cues. In support of this, we have demonstrated that treatment of permeabilized S1 acini with a NuMA antibody leads to disruption of the NuMA foci, alteration of histone acetylation, and perturbation of the acinar phenotype (59). These data provide the first evidence for the existence of a dynamic interplay between the ECM, the organization of the nucleus, and the epithelial phenotype. Thus, rather than passively reflecting changes in gene expression, nuclear organization may itself modulate the cellular and tissue phenotype.

Central Hypothesis and Future Directions

The studies summarized above support the hypothesis elaborated from our earlier predictions and set forth again in the abstract (Ref. 3, Fig. 7): the unit of function in higher organisms is neither the genome nor the cell alone, but the tissue itself. The context would determine how individual genes may operate in vivo. This concept could explain

Fig. 7. Dynamic reciprocity. Bidirectional flow of tissue-specific information is dependent on the nuclear and chromatin structures, the nature of membrane receptors, and the microenvironmental niche.

References


Dr. Bissell: We, in fact, have done something like this. For example, if we overexpress EGFR, we are able to disrupt normal function. I haven’t had time to show you a lot of the studies that we have done; I just had to choose, of course. But, the results are very interesting; if you put EGFR in phenotypically normal tissue, the structure is disrupted. The cells grow disorganized. I am not saying that they nec-

Dr. Bissell: We, in fact, have done something like this. For example, if we overexpress EGFR, we are able to disrupt normal function. I haven’t had time to show you a lot of the studies that we have done; I just had to choose, of course. But, the results are very interesting; if you put EGFR in phenotypically normal tissue, the structure is disrupted. The cells grow disorganized. I am not saying that they necessarily will make a tumor, but under these conditions, now β1 integrin will go up, but it will again go up only in three dimension. If you put the same cells on tissue culture plastic, there will be no detachable increase, despite the fact that EGFR is overexpressed. So, again, the important point here is the difference between how cells regulate themselves, i.e., whether they are in the proper context and in the proper shape, as opposed to being in a monolayer. But, yes, if you overexpress any of the genes involved in these pathways, you will override the structure, even inside a basement membrane, and you will have disorganized growth and possibly tumor at the end.
Second Speaker: I am interested particularly because I did two experiments, one about 10 years ago on lactic acid, and the experiment drew on two different tissue culture plastics. One is primaria, which is a slightly reduced negative charge, and the other is a normal tissue culture plastic. We found not only chromosomal differences but differences in immunohistochemistry, and then when I changed about 5 years ago to OB/GYN cancers, the problem was that we couldn’t find more than 75% success in the growth of these cells. So I transferred back to the other plastics, and by doing this we reached 97%, which is published in Cytogenesis in Ovarian Cancer. But, now I’ve started, you have baffled me; I’m confused whether my data are right, because, by culturing the two things, I made two different lots.

Dr. Bissell: I look forward to discussing this with you afterwards, and I think I have some answers from our data that would explain why you got the kind of data you did.

Third Speaker: Do you think the principles you described may be relevant to the development of the so-called liquid tumors, leukemias and lymphomas?

Dr. Bissell: You are raising a very interesting question, because I used to think that blood-borne tumors are an exception to the rule. But as you may know, differentiation of these blood cells, in fact, is very much dependent on the microenvironment in the bone marrow. More recently, people who have used retinoic acid to revert the leukemia or some of these lymphomas, in fact, show that what is affected is the level of β1 integrin. Therefore, it is the adhesion pathways that are being affected, so there may be a much wider applicability to this. In addition, there are examples of this in other kinds of epithelial tumors. So I think that if we really look hard enough, while there is a lot of specificity, the broader rules may apply in both blood-borne and solid tumors.
Tissue Structure, Nuclear Organization, and Gene Expression in Normal and Malignant Breast

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