Origination of Organismal Form: Beyond the Gene in Developmental and Evolutionary Biology

edited by Gerd B. Müller and Stuart A. Newman

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7 Tissue Specificity: Structural Cues Allow Diverse Phenotypes from a Constant Genotype

Mina J. Bissell, I. Saira Mian, Derek Radisky, and Eva Turley

Two decades ago, based on the literature and her laboratory experience, one of us (Bissell) concluded that,

> if there is one generalization that can be made from all the tissue and cell culture studies with regard to the differentiated state, it is this: Since most, if not all, functions are changed in culture, quantitatively and/or qualitatively, there is little or no *constitutive* regulation in higher organisms; i.e., the differentiated state of normal cells is unstable and the environment regulates gene expression. (Bissell, 1981, p. 27; emphasis added.)

This concept, more recently referred to as the "plasticity" of the differentiated state, has gained some credence as literature has accumulated that differentiation may not be as terminal or fixed as was once thought—witness the cloning of Dolly, mice, and cows from restricted stem cells derived from adult tissues, or even from single somatic cells.

There is ample evidence that all cells retain the ability to modulate most, if not all, of their functions; even enucleated red blood cells still regulate their behavior depending on the context and what they encounter. It may be that cells never completely lose an intrinsic ability to morph from one cell type to another, and that they maintain a stable phenotype by integrating cues from the extra- and intracellular milieu. Indeed, there is also ample evidence to support the notion that, for a cell to continue functioning properly in a tissue-specific way, it must receive continuous signals to prevent growth or apoptosis and to maintain an appropriate structure and differentiation state, which is to say, cells must be directed at all times to remember how to behave within an organ. If these active signals are withdrawn from a resting, differentiated cell, or if a wrong signal is given (as is often the case in cell culture), it will do one of three things: die, start growing, or function inappropriately. What, then, are the cues in vivo that cause a cell to continue functioning in a manner that is specific for its tissue?

This and related questions raise a larger question that directly bears on the theme of this volume. Are mutations really the cornerstone of evolution through natural selection, or could radical changes in the microenvironment, even without spontaneous genomic mutations, allow an organism to evolve into a different form? The central tenet behind this reasoning derives its strength from the obvious miracle of development: we all began as a single cell and all our diverse tissues and organs contain the same DNA sequence. Thus we need to know not just how cellular differentiation is derived, but also how it is maintained against a constant DNA background.

Three-Dimensional Microenvironments

To address these complex biological questions experimentally, researchers must develop tractable model systems that pare the subject in question down to its most essential components. In higher organisms, this strategy has focused on using monolayer cultures of homogeneous cell populations propagated in vitro. Although this approach has been very successful in elucidating many of the basic principles of cell survival and growth, it has generally ignored the fact that within an organism, no cell is an island: each exists in the context of a complex microenvironment. In response to this limitation, inherent to two-dimensional (2-D) monolayer culture systems, cell culture strategies began to be redefined in the context of three-dimensional (3-D) microenvironments. In the seventies, Ellsdale and Bard (1974) and later Michaelopolis and Pitot (1975) and Emerman and colleagues (1977) grew cells on gels of collagen I, that were then floated; the resulting 3-D structures regained some of their original functions. Thus, when grown as monolayers on rigid substrata such as tissue culture plastic or attached collagen I gels, luminal epithelial cells extracted from mouse or human mammary glands did not differentiate structurally or functionally. Growing mammary epithelial cells on and in gels of extracellular matrix (ECM) materials similar in composition to the basement membrane (BM) associated with mammary epithelial tissues in vivo obviated the need for flotation and led to the formation of normal cellular architecture and to gene expression profiles characteristic of differentiated cells (Barcellos-Hoff et al., 1989; Petersen et al., 1992) (figures 7.1 and 7.2).

How does a gelatinous basement membrane with essentially insoluble proteins communicate with the nucleus? We believe that maintenance of tissue specificity involves an intimate and profound communication between the microenvironment around the cells and the organization of the nucleus. This concept, put forward two decades ago for ECM, is known as “dynamic reciprocity” (Bissell, Hall, and Parry, 1982; figure 7.1). Many of the essential players in the depicted signaling events (not then identified, and indicated by question marks in the insets) have since been characterized. Indeed, the number of proteins and protein modifications known to be involved in cell-ECM interactions is immense, but how the signals are integrated to permit organ formation is still far from clear.

Molecular Cues from the Extracellular Environment

The molecular mechanisms behind signaling from the extracellular matrix molecules through their receptors (largely integrins, but other receptors are being identified as well) have been intensely studied and elucidated (for reviews, see Clarke and Brugge, 1995; Guan and Chen, 1996; Yamada, 1997; Schoenwaelder and Burridge, 1999; Giancotti and Ruoslahti, 1999). How the signals are transduced to the nucleus and then propagated to

other cells and tissues is less obvious and is not well understood. We have known for some time that there are growth-factor- and hormone-response elements in the 5′ regulatory region of many genes. The discovery of the first ECM-response element was made possible through the development of transfectable mammary epithelial cells that could respond to ECM by making milk proteins (Schmidhauser et al., 1990). A reporter gene was cloned behind 1,600 bp of the 5′ sequence encoding the milk protein β-casein, and this construct was transfected into the functional mammary cell line. The reporter gene was from 50 to 150 times more active when cells were grown on ECM. Subsequent promoter deletion

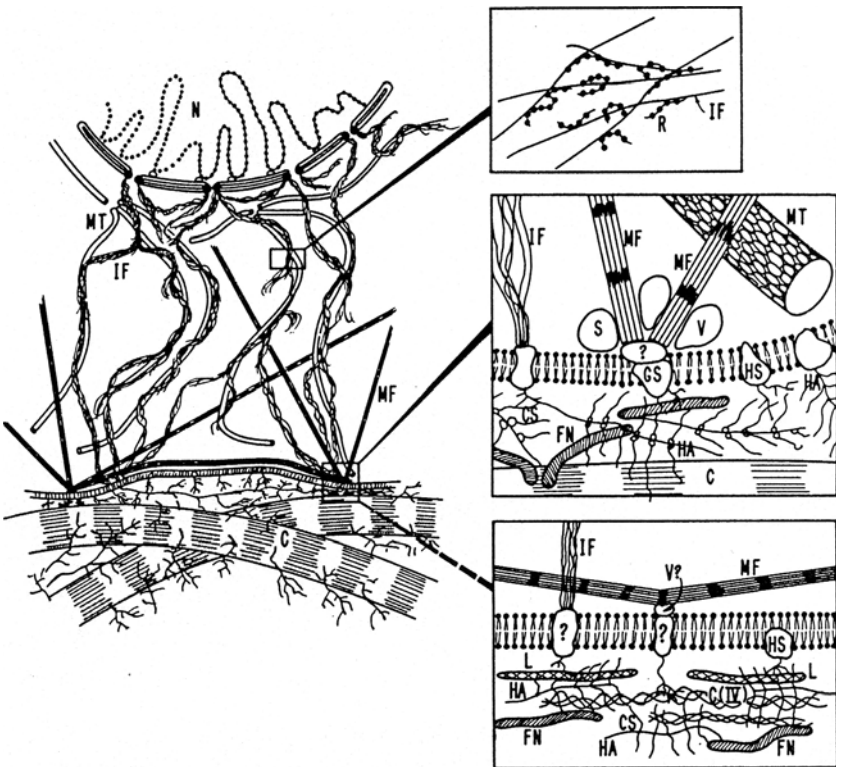

Figure 7.1
Dynamic reciprocity, the minimum required unit for tissue-specific functions. The postulated overall scheme for extracellular matrix–cell interactions. N, nucleus; MT, microtubules; IF, intermediate filaments; MF, microfilaments; C, collagen. (*Top inset*) Polyribosome attachment to cytoskeleton. R, ribosomes. (*Middle inset*) V, vinculin; S, *src* coded protein kinase; GS, Ganglioside (attaching fibronectin to membrane); FN, fibronectin; HA, hyaluronic acid; CS, chondroitin sulfate; HS, heparan sulfate. (*Bottom inset*) Possible attachment site to membranes in epithelial cells. L, laminin; C(IV), collagen type IV. (Reproduced with permission from Bissell et al., 1982.)

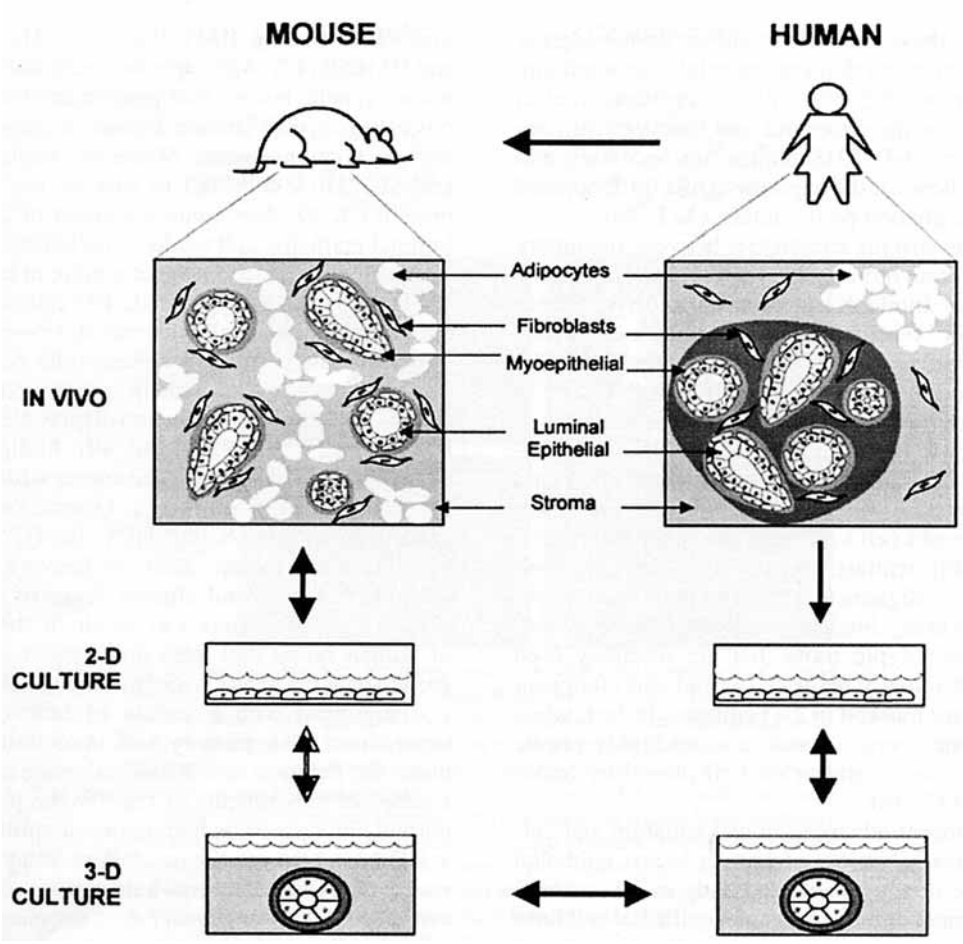

Figure 7.2
Consequences of culture in two versus three dimensions on tissue architecture. Modeling human breast function from studies of mouse mammary gland. As in humans, the mouse mammary tissue comprises multiple cell types, including luminal epithelial and myoepithelial cells, adipocytes, and stromal fibroblasts. Although mouse and human mammary tissue vary somewhat with respect to overall organization, the double-layered structure of the branching ducts and ductules is preserved in both organisms. In light of these fundamental similarities, it is not surprising that human and mouse epithelial cell types display similar behaviors in 3-D basement membrane (matrigel) cultures: both cell types undergo morphogenesis to form spherical structures that are similar to acini in vivo. (Reproduced with permission from Ronnov-Jessen, Petersen, and Bissell, 1996, and Schmiechel, Weaver, and Bissell, 1998, with minor modifications.)

analysis (Schmidhauser et al., 1992) identified a 160-nucleotide sequence that defined an ECM-response element in the regulatory sequences of this gene. Using site-specific mutagenesis, the response element was shown to be an enhancer and that C/EBPβ and STAT-5 transcription-factor-binding elements were essential for its activity (Myers et al., 1998). In this cell model, transcription factor binding was necessary, but not sufficient, to activate transcription in the absence of ECM. We now have found evidence that ECM signals can alter the histone acetylation/deacetylation status of chromatin, and that this change in chromatin structure is necessary to initiate the transcription of differentiation-specific genes (Myers et al., 1998; Boudreau and Bissell, 1998; Pujuguet et al., 2001).

Concurrent with the discovery of this ECM-response element, our laboratory and many others have investigated the ability of integrins, the largest and best studied class of ECM receptors, to activate signaling cascades (Streuli and Bissell, 1991; for reviews, see Clarke and Brugge, 1995; Yamada, 1997; Giancotti and Ruoslahti, 1999; Hynes and Zhao, 2000). Two important properties of these extracellular matrix receptors are essential to link the genome of a cell to its extracellular microenvironment, rendering the organism susceptible to evolutionary selection by epigenetic factors. The first is the ability of intracellular signaling molecules to modify the avidity of matrix receptors for their ligands, a property that in turn affects the subsequent intracellular signaling pathways that are activated. This two-way signaling across the membrane, referred to as "inside-outside" signaling, permits the cells to continuously interact with the extracellular microenvironment (Faull and Ginsberg, 1996; Brown and Hogg, 1996; Ruoslahti, 1997; Liu, Calderwood, and Ginsberg, 2000). The second important property is the ability of extracellular matrix receptors to functionally associate with growth factor receptors, thus linking the information conveyed by growth factors to the inside-outside paradigm (Hynes, 1992; Parsons and Parsons, 1997; Howe et al., 1998; Wang et al., 1998; Streuli and Edwards, 1998; Giancotti and Ruoslahti, 1999). In addition to this two-way flow of information mediated by extracellular matrix receptors, additional mechanisms permit extracellular matrix cues to be accessible to the genome. For instance, extracellular heparan sulfate trafficks to the cell nucleus (Bhavanandan and Davidson, 1975; Hiscock, Yanagishita, and Hascall, 1994; Isihara, Fedarko, and Conrad, 1986; Liang et al., 1997) as a complex with high molecular weight forms of extracellular basic fibroblast growth factor (bFGF; Amalric et al., 1994; Maciag and Friesel, 1995; Nugent and Iozzo, 2000); together, they coordinate specific intracrine functions within the nucleus (Nugent and Iozzo, 2000); bestowing the ability, for example, to grow in serum-deprived conditions (Arese et al., 1999). Conversely, intracellular, phosphorylated forms of bFGF are secreted by a Golgi–endoplasmic reticulum–independent pathway and these forms are preferentially delivered to the nuclei of neighboring cells (Guillonneau et al., 1998). Similarly, extracellular hyaluronan can be transported from either the extracellular matrix or intracellular pools into the cell nucleus (Collis et al., 1998;

Evanko and Wight, 1999), where it likely associates with intracellular hyaluronan-binding proteins (hyaladherins; Toole, 1990; Sherman et al., 1994). Some of these proteins are known to associate with intracellular signaling molecules (Zhang et al., 1998) and to stabilize their conformation (Grammatikakis et al., 1999). Interestingly, this group of "intracellular" hyaluronan-binding proteins, can, like bFGF forms, also traffic outside of the cell into the extracellular matrix and onto the surface of neighboring cells, where, together with integrins, they regulate growth factor receptor signaling into the cell interior (Zhang et al., 1998; see also www.glycoforum.gr.jp/science/hyaluronan/HA11/HA11E.html). Trafficking hyaladherins and β-FGF resemble a growing group of proteins originally considered to function strictly as nuclear or cytosolic proteins; referred to as "messenger proteins" (Prochiantz, 2000), these include transcription factors such as *engrailed* that are exported out of a cell, taken up by neighboring cells, and transported back into the nuclei of neighboring cells. A viral mimic of this class of proteins is the HIV protein TAT. These mechanisms exist to ensure constant communication between the extracellular matrix and the cell nucleus, blurring the boundaries that once demarcated the cell and its microenvironment.

Experimental Evidence for the Role of an Intact Microenvironment

From our studies comparing simple monolayer cultures to cells maintained in a three-dimensional, basement membrane–containing environment, we know that when a cell is deprived of extracellular matrix signals, it loses its tissue-specific differentiation (figures 7.3, 7.5). When cells are maintained in an appropriate 3-D environment, extracellular matrix receptors are correctly engaged, and a cell is able to *coordinate* subtle combinations of signals to permit morphogenesis and differentiation to higher orders of organization (figures 7.2–7.5). This principle applies equally to cancer cells: Weaver and colleagues (1997) have manipulated the interactions between malignant mammary epithelial cells and their microenvironment to effect a reversion to a functionally normal phenotype (figure 7.6; for an overview, see Bissell et al., 1999). It is important to note that, in this example, the genome retained its malignancy, yet form and function were normalized (figure 7.7).

A web of functional connections among thousands of signaling pathways sustains the organization that is necessary for differentiation. Because pathways and cells are now interconnected in three dimensions, perturbation of any connection will be detected as a change throughout the tissues and organs. We argue that the computing power of a tissue is greater than the sum of its component cells in much the same way that the collective properties of an ant colony are greater than those of its member ants (figure 7.8). Researchers have concentrated on aspects of a tissue that come under the general rubric of "perception, cognition, and generation of action." While current experimental methods focus on the relatively

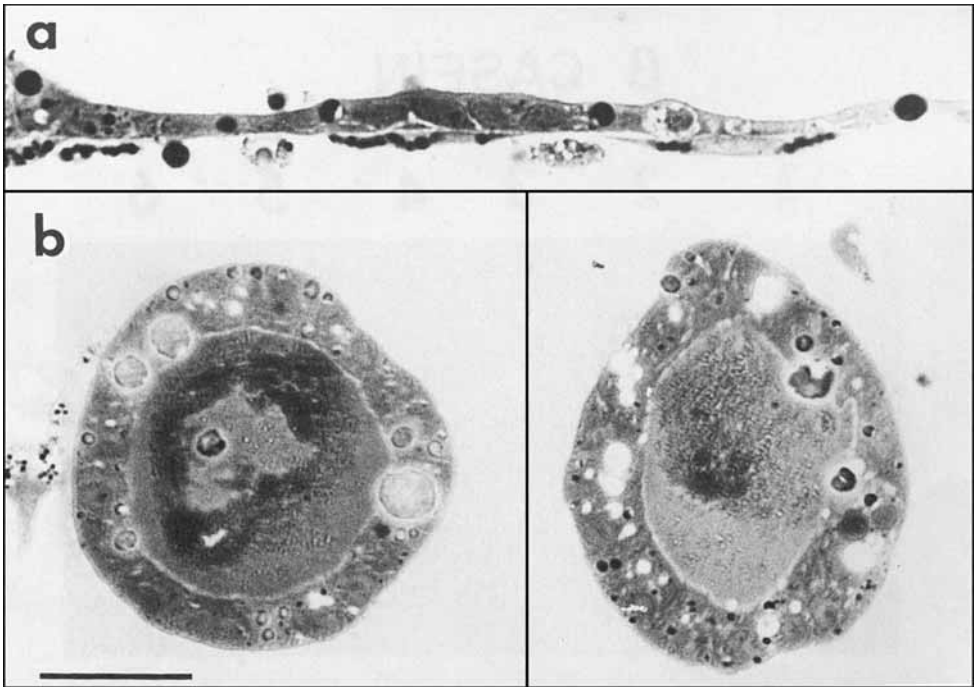

Figure 7.3
Electron micrograph of primary mouse mammary cells. Cross sections of primary mouse mammary epithelial cells on reconstituted basement membrane (matrigel). (*a*) Flattened cells on plastic. (*b*) Alveolar lumina formed by cells cultured on matrigel for 8 days in the presence of lactogenic hormones showing central lumen, minimal apical microvilli, and small lipid droplets, typical of cells in these cultures. Bar: 20 μm. (Reproduced with permission from Aggeler, Park, and Bissell, 1988.)

facile study of such aspects, robust techniques for quantitative and qualitative modeling of tissue evolution, reproduction, morphogenesis, and metabolism remain elusive.

Clearly, development of an organized extracellular matrix during evolution was an important step that enabled a collection of cells with individual features and characteristics to form a tissue capable of displaying aggregate behavior above and beyond those of its constituent cells. Knowing the essential attributes required to make this transition is akin to defining the minimal gene set necessary for cellular life. Thus we posit the need for a minimal tissue project whose goal is to elicit the necessary and sufficient features required to generate a functional tissue. The minimal genome project has estimated that 265–340 of the 517 genes of the bacterium *Mycoplasma genitalium* are essential for life (Hutchison et al., 1999). We propose that a minimal mammary gland tissue ecosystem includes luminal epithelial cells, myoepithelial cells, mesenchymal cells, lactogenic hormones, growth

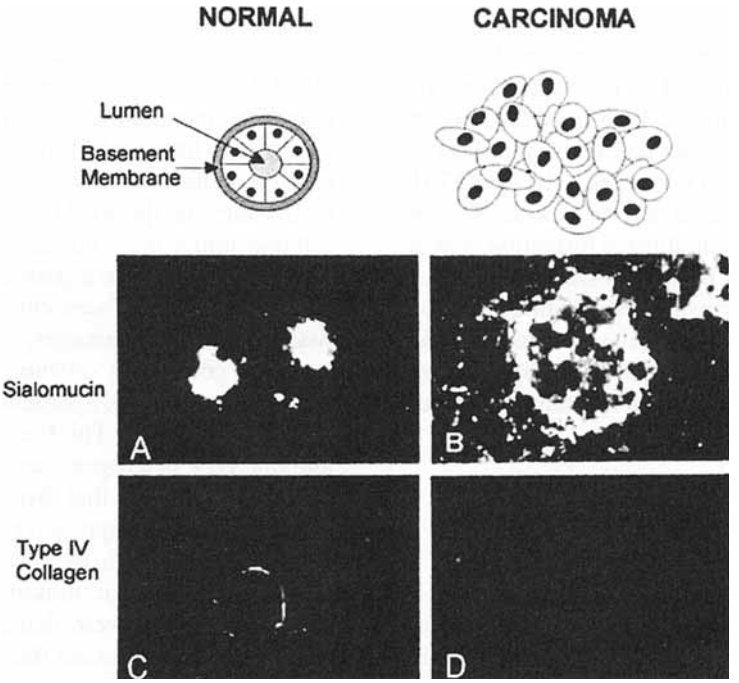

Figure 7.4
Three-dimensional basement membrane assay permits the expression of normal and malignant phenotypic traits by human breast cells. Primary cultures of normal breast epithelial cells (*A*, *C*) or breast carcinoma colonies were grown in 3-D matrigel for 7–10 days and were processed for immunofluorescence staining with antibodies directed against sialomucin (a marker for apical cell surfaces; *A*, *B*) or against type IV collagen (a marker for basement membrane; *C*, *D*). The staining here demonstrates that, whereas the normal breast epithelial cells grown in three dimensions are capable of forming organized spheres with central lumina and basally deposited basement membranes, their tumorigenic counterparts fail to undergo polarized morphogenesis and do not deposit endogenous basement membranelike material. (Reproduced with permission from Petersen et al., 1998, with minor modifications.)

factors, mesenchymal ECM, and basement membrane. Outstanding experimental challenges include determining the full repertoire of molecular and cellular components required to fabricate a minimal tissue ecosystem de novo.

Modeling the Role of Structural Cues

Current efforts to develop computational models of cells need to be accompanied by efforts to create computational models of tissues. The fundamental theoretical problems are how to create (1) mathematical formalisms with which to describe and define a tissue; (2) effi-

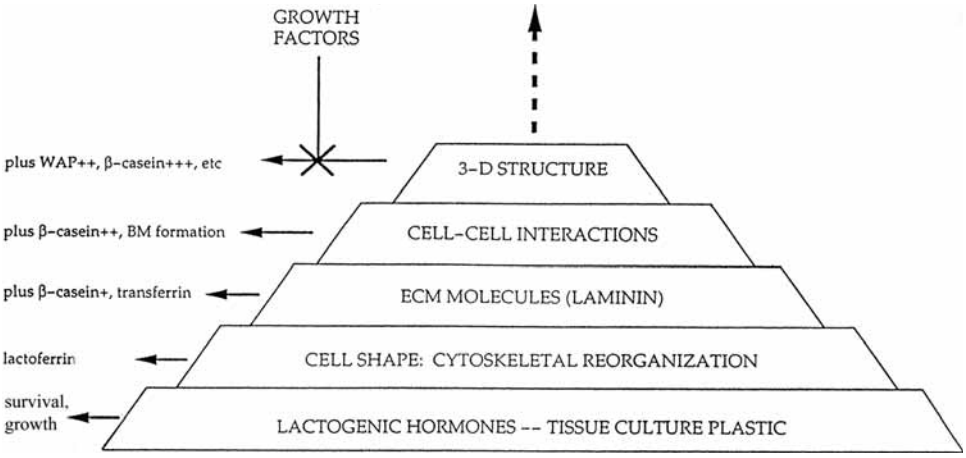

Figure 7.5
Hierarchy in regulation of mammary-specific gene expression in mouse epithelial cells. (Reproduced with permission from Bissell, 1997, with minor modifications.)

cient algorithms to estimate such models from incomplete, noisy, and heterogeneous data; and (3) techniques for generating nontrivial, accurate predictions at multiple levels of detail and abstraction. Candidate formalisms include stochastic process algebras (Hillston and Ribaudo, 1998) and graphical models (Jordan, 1998; but see also Britten and Rasskin-Gutman, chapters 5 and 17, this volume). The primary benefit of these approaches is their compositional nature: the components of a complex system and interactions between components can be modeled separately; the resultant models have clear structures, are easy to understand and can be constructed systematically by elaboration or refinement. Such techniques permit a library of reusable, hierarchical models to be developed and maintained. Because no single formalism will be adequate to represent all aspects of a tissue and no individual solution method will suffice to solve all models, an integrated approach will be necessary. With the completion of a draft human genome sequence in 2001, we are in a position to uncover some of the forces governing the interplay between the extra- and intracellular milieus that lead to formation and maintenance of a tissue.

A theory called "highly optimized tolerance" has been proposed to account for the tendency of interconnected systems to gain a measure of robustness against uncertainties in one area by becoming more sensitive in other areas (see the work of Doyle and colleagues at www.cds.caltech.edu/~doyle). If the unit of function in an organ (e.g., the mammary gland) possesses such a property, what are the common and designed-for uncertainties to which it is resilient? And what are the design flaws or rare events to which it is hypersensitive? Controlling and redesigning this highly optimized system so that transi-

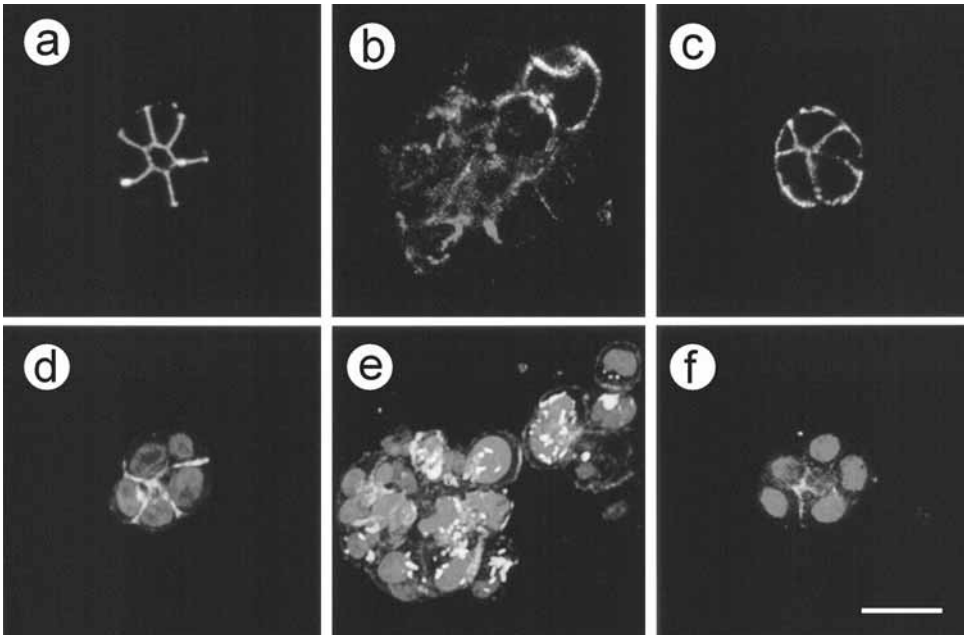

Figure 7.6
Reversion of mammary epithelial tumor cells. Treatment of T4-2 tumor cells with β1-inhibitory antibody leads to phenotypic reversion and acinar formation. Confocal immunofluorescence microscopy images of E-cadherin (FITC) and β-catenin (Texas red; dark gray) of phenotypically normal S1 cells (*a*), malignant T4-2 cells (*b*), and reverted T4-2 cells (*c*). In S1 (*a*) and T4-β1 reverted acini (*c*), E-cadherin and β-catenin were colocalized and superimposed at the cell junctions. In contrast, E-cadherin and β-catenin were often not colocalized in mock-treated T4 cells (*b*). (*d–f*) Confocal fluorescence microscopy images of F actin (FITC; light gray) and nuclei (propidium iodide; dark gray). Both the S1 (*d*) and the reverted T4-2 cells (*f*) showed acinar formation with basally localized nuclei (propidium iodide) and organized filamentous F-actin, whereas T4-2 mock-treated colonies had disorganized, hatched bundles of actin and pleiomorphic nuclei (*e*). Bar: 16 μm. (Reproduced with permission from Weaver et al., 1997, with minor modifications.)

tions to an aberrant state are minimized will require understanding its structure and behavior at many levels. One intriguing possibility is that the mammary gland exhibits the phenomenon known as "stochastic resonance" (SR), a mechanism whereby the presence of noise enhances the detection of weak signals; SR may be relevant to problems in sensory biology.

As a tissue evolves, it can adapt to, or learn from, its noisy environment. Whereas adaptation can be considered temporary with the system eventually resetting itself, learning involves a persistent and heritable change. Perhaps it makes sense to appropriate language from the machine learning community. Unsupervised learning, finding patterns or natural groups in data, might be the primary manner in which a collection of cells learns from

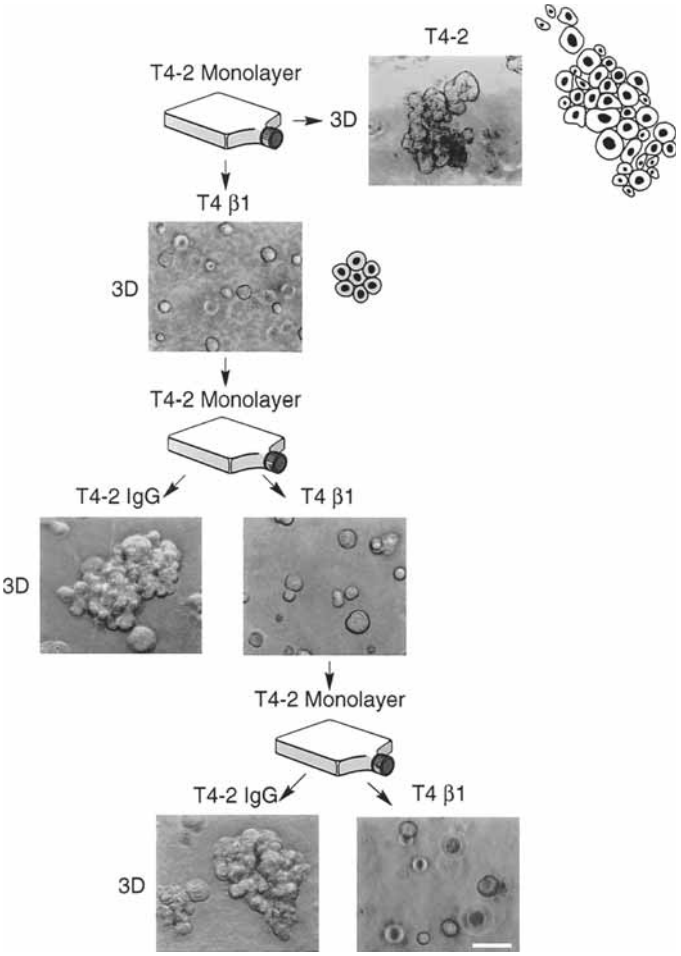

Figure 7.7
Evidence for phenotypic reversion rather than selection. Phase contrast micrographs of T4-2 (tumor) cells grown in matrigel and in the presence of $\beta 1$ function blocking antibody (T4 $\beta 1$), mock antibody (T4-2 IgG) or no antibodies (T4-2). Despite two rounds of treatment, these antibody reverted cells were able to resume their original tumorigenic phenotypes when cultured in the absence of antibody. (Reproduced with permission from Weaver et al., 1997, with minor modifications.)

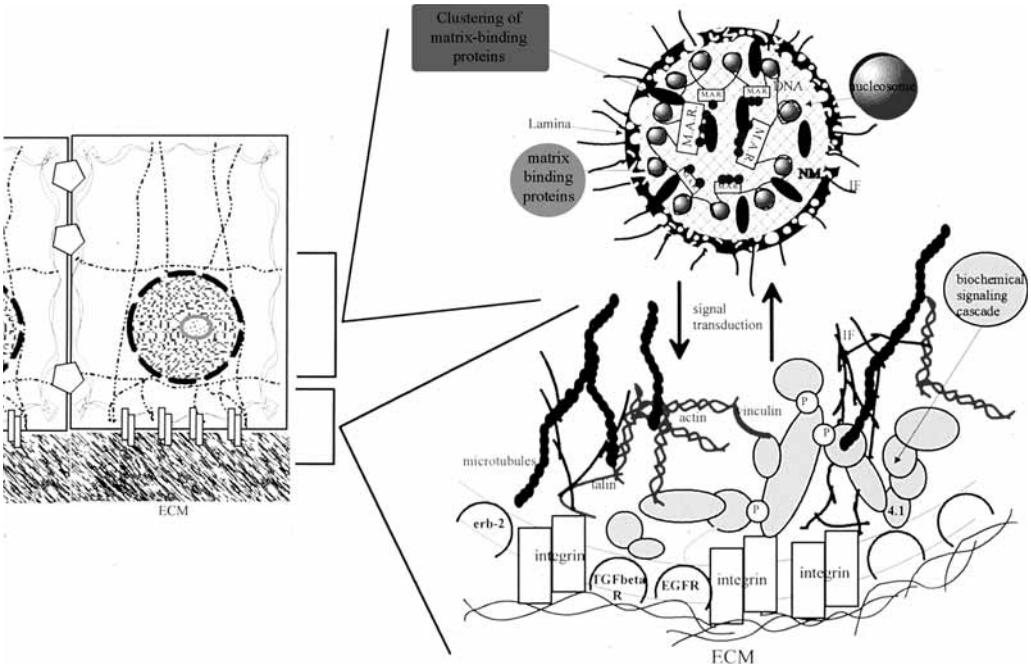

Figure 7.8
Central hypothesis: the bidirectional computing power of a tissue is greater than the sum of its component parts. Bidirectional flow of tissue-specific information is dependent on the nuclear and chromatin structures, the nature of membrane receptors, and environmental milieu.

its environment. Supervised learning necessitates the presence of a teacher to inform and guide data modeling and interpretation. For dynamic reciprocity, both the nucleus and ECM can play the role of teacher. Tissues might engage in what is known as "reinforcement learning." In the absence of a teacher, noisy feedback might serve to indicate how good an action was: different costs could be associated with alternative responses and this cost-benefit analysis of the stochastic environment might determine the actions implemented by the ECM or nucleus.

Cells inhabit an uncertain world. Signaling molecules that regulate intracellular and extracellular processes may be present in a few to a few hundred copies and display significant internal noise. Despite this, cells integrate and interpret myriad signals in a meaningful way, provided they are grown in natural tissue environments. Against a stable genome, cells differentiate into diverse phenotypes and associate into tissues, which in turn connect to form the entire organism. It is clear that understanding how these processes are

regulated will require studies that utilize tissues, organs, and tissuelike model systems. We have taken the first steps along this pathway, but a long and interesting journey lies ahead.

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