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Tissue architecture: the ultimate regulator of breast epithelial function

Commentary

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Abbreviations

2D	two-dimensional, or monolayer cultures
3D	three-dimensional cultures of cells embedded in extracellular matrix components
BCE-1	bovine casein element 1
BM	basement membrane
ECM	extracellular matrix

Introduction

A problem in developmental biology that continues to take center stage is how higher organisms generate diverse tissues and organs given the same cellular genotype. In cell and tumor biology, the key question is not the production of form, but its preservation: how do tissues and organs maintain homeostasis, and how do cells within tissues lose or overcome these controls in cancer? Undoubtedly, mechanisms that maintain tissue specificity should share features with those employed to drive formation of the tissues. However, they are unlikely to be identical. At a simplistic level, developmental pathways may be thought of as a series of extremely rapid short-term events. Each new step depends on what came before, and the outcome is the organism itself at birth. All organs, with a few notable exceptions, such as the mammary gland and the brain, 'arrive' together and are complete when the organism is born. In mice and humans, these events occur in a mere 21 days and 9 months respectively. The stability of the differentiated state and the homeostasis of the organism, on the other hand, will last 40–110 times longer. How does the organism achieve this feat? How are tissues maintained? These questions also relate fundamentally to how tissues become malignant and, although not discussed here, to aging.

While there is much literature on differentiation — loosely defined as the gain of a single or a series of functions — we know much less about the forces and the pathways that maintain organ morphology and function as a unit. This may be partly because it is difficult to study a tissue as a unit *in vivo* and there are few techniques that allow maintenance of organs *in vitro* long enough and in such a way as to make cell and molecular biology experiments possible. Techniques for culturing cells in three-dimensional gels (3D) as a surrogate for tissues, however, have been steadily improving (for a recent review of current models, see [1]) and the method is now used by several laboratories.

In this commentary we discuss the following: first, how our laboratory came to develop a model of the mammary gland acinus; second, what this model has told us about mechanisms that govern tissue specificity and malignancy; and third, possible directions for future studies. We summarize the evidence for the central role of ECM signaling in the maintenance of mammary function in culture and (more briefly) its role in tumorigenesis. This is followed by a discussion of the role that tissue architecture and tissue polarity (as opposed to cell polarity) may play in these processes.

In an elegantly written and reasoned essay [2], Kirschner *et al.* coined the new science of developmental biology 'molecular vitalism'. They framed new concepts for self-organization as well as schemes for information flow in biological organization. Rao *et al.* [3••] reviewed and elaborated on differential-equation-based models of biochemical reaction networks and intracellular noise, with emphasis on bacteria and phage. Similarly, Hartwell *et al.* [4] discussed the synergy between experiment and theory in elucidating 'modules' — collections of interacting molecules — and in unraveling how these modules collaborate to perform cellular functions such as signal transduction. We believe that many of these ideas will also be applicable to the maintenance of tissue specificity. As much as we agree with Kirschner *et al.* [2] regarding the limitations of the machine analogy to biological systems, we conclude with thoughts on how we may proceed to model the complex tissue networks that govern breast tissue architecture. We suggest that our understanding of the structure and function of breast tissue would benefit from examining recent techniques for modeling large complex networks such as the World Wide Web and the Internet backbone among others [5,6••].

What constitutes a unit of function in metazoa?

Single cells are units of function for the single-celled organism. The following instructive question may be asked: what is meant by a unit of function in higher organisms? The hierarchical nature of biological form and function argues for an operational definition, one that depends upon context and desired outcome. Thus, single non-malignant mammary cells are 'functional' in that if they can attach to a substratum, they can proliferate, or at least survive and metabolize for a substantial length of time. Tumor cells often lose even the requirement for attachment and can grow as single cells, at least in culture. As such, single cells in metazoa can be a unit of function if growth or metabolism is the designated end point. If, however, function is defined to mean tissue-specific function, then we know that individual cells on tissue culture plastic are not functional units. In this context, it was clear even in the 1970s that normal cells lose functional differentiation when isolated and placed on tissue culture plastic. On the basis of the existing literature, as well as observations in the laboratory, it was posited that context in general, and the extracellular matrix (ECM) in particular, play crucial roles in maintenance of tissue specificity [7]. Evidence from many laboratories provides convincing support for these ideas. Given that cells in culture lose tissue-specific function (reviewed [8]; [9]), what are the determinants of tissue-specificity *in vivo* and what molecular mechanisms are involved in these processes?

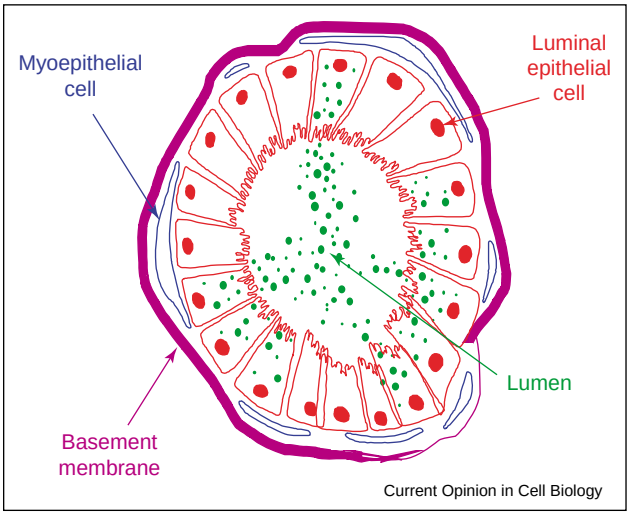
In the mid-1970s, malleable gels prepared from rat-tail collagen (essentially collagen I) were shown to be effective in allowing some epithelial cells to maintain or restore several differentiated functions in culture [10–13]. Experiments using these floating gels, and work on modulation of collagen levels in chick tendons in culture [14,15], resulted in the proposition that 'designer microenvironments' needed to be formulated to study tissue-specificity [16]. The rationale was the desire for tractable systems amenable to experimental manipulation: cells that could be cultured to become functional or made to lose function at will. Rather than the genetic engineering of mice, a technology not available at the time, the goal was the civil and environmental engineering of tissues and organs. Integrating our observations with other studies that had documented the influence of tissue interactions, the stroma and the ECM in the development and functional regulation of tissues, we postulated that the 'unit of function' in higher organisms was not a single cell, but the cell plus its surrounding ECM. Furthermore, it was conjectured that the ECM could signal to the nucleus and vice versa, and that this process was both dynamic and reciprocal [7]. If the operational unit of function is larger than a cell, experimental systems involving dissociated cells in tissue culture are inadequate to meet the challenging task of investigating the mechanism of tissue specificity, although they are useful tools for answering many other questions. The goal was not only to model and thus recapitulate a unit of functional differentiation in culture, but also to understand how this system becomes dysfunctional in breast tumors.

The mammary acinus as an experimental organism

To systematically explore the mechanisms behind tissue specificity in an epithelial model system, we chose to study the mammary gland, and more specifically the mammary acinus, as an experimental 'organism' (see Figure 1). The mammary gland is one of very few organs in which substantial development occurs only after an animal is born; it also undergoes cycles of growth, differentiation, apoptosis, regression and remodeling during the lifetime of the organism. As such the mammary gland is a versatile experimental model for studying how form and function unite to bring about functional differentiation. There are sufficient examples from other laboratories (reviewed in e.g. [1]) to support our assertion that insights gained from the acini of the mammary gland should be applicable to other glandular organs — if not in fine detail, at least in terms of broad concepts.

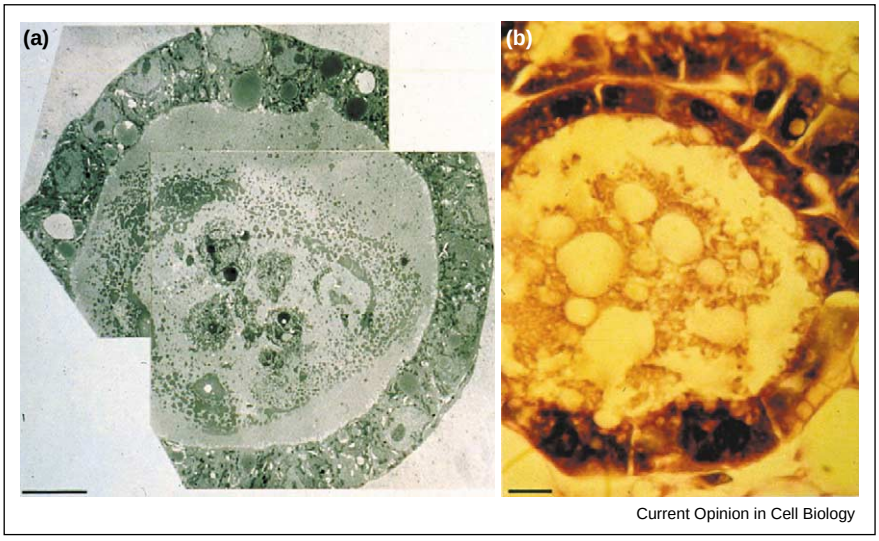
The collagen gel assay could be used to induce *de novo* tissue-specific functions — not only the expression of milk proteins [17,18], but also global, tissue-specific metabolic patterns [19]. We showed that the signals directing the synthesis of milk proteins emanated from the basement membrane (BM), as the BM and some of its

Figure 1



The mammary acinus as an experimental animal. Schematic presentation of a 3D acinus in basement membrane. Questions currently being addressed include the following. How is an acinus formed? How does it maintain polarity? How does it become disordered in malignancy? What molecules and signaling pathways are involved?

Figure 2



The structure resulting from acinus formation in 3D BM cultures resembles an *in vivo* mammary acinus [21]. **(a)** A low magnification transmission EM of an acinus formed in culture. **(b)** A light microscope picture of an acinus from a section of a gland *in vivo*.

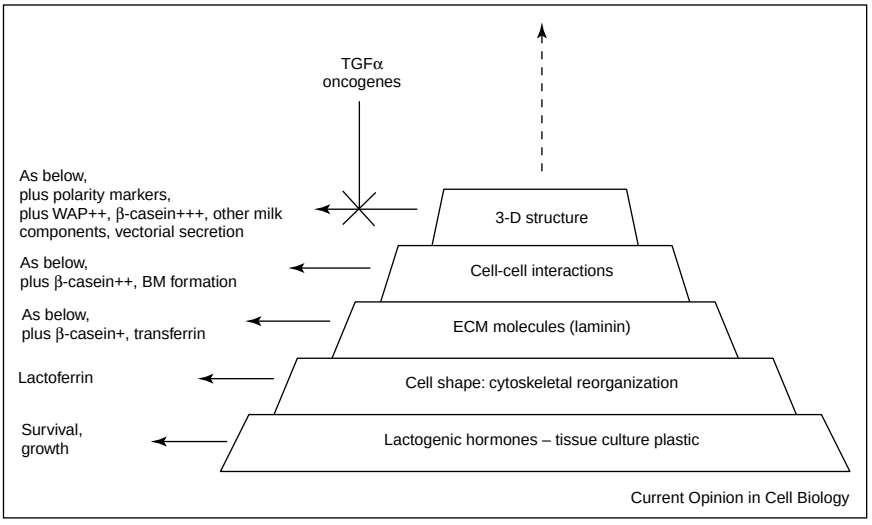
components could substitute for the floating collagen gels in inducing milk protein expression [20]. In contrast to other culture conditions, culturing cells on top of a malleable laminin-rich BM resulted in a remarkable degree of both morphological and functional differentiation [18,21] (see Figure 2). The reason functional differentiation occurred in floating collagen gel was shown to be the deposition of endogenous BM under these conditions [22]. However, differentiation did not occur if the BM was cross-linked or if a thin layer of BM was applied to the dish. Under these conditions, the cells could not deform the gel and therefore could not become polarized. In this way, the requirement for BM molecules, cell shape change [23] and substratum malleability was recognized.

Can single cells in contact with the ECM become differentiated to express milk proteins? The answer is a qualified yes. Cells will secrete the milk protein β-casein if the gel is made of BM but not if it is composed of collagen I [24], indicating a requirement for specific BM components. Inhibiting β1-integrin–cell interactions could interrupt the signaling that induces β-casein expression, suggesting a requirement for β1-integrin ligands [24]. The BM component that interacted with β1-integrins proved to be laminin-1 [25], a molecule initially reported to be important for the development of polarity in kidney [26]. The first ECM-(laminin) response element to be characterized was found in the promoter of the β-casein gene and was termed BCE-1 (bovine casein element 1) [27,28]. Transcription factor binding to BCE1 was necessary but not sufficient for signaling. There is an extensive body of work showing that these transcription factors are also necessary for milk-protein gene expression in response to hormonal and other signals *in vivo* (for reviews see [29,30]). The enhancer could be activated by BM and/or laminin, or by changes in histone acetylation, the latter even if the cells were on 2D substrata [31]. Functional differentiation depends upon the degree of complexity of the tissue architecture achieved in culture [32] (see Figure 3). Unlike β-casein, most other milk proteins were not synthesized under the above conditions, suggesting a need for cell–cell interactions and formation of polarized acini (reviewed in [32]). Thus, the level of function specified determines the unit of function. In studying mammary epithelial cell function in culture, it became evident that cell–cell interactions and closure of the acini around a lumen were equal partners in regulating the polarization aspect of functional differentiation. As the unit of tissue specificity was larger than the cell plus its ECM, the functional unit could be considered to be the organ itself [33].

The importance of laminin, polarity and myoepithelial cells

The importance of laminin 1, β1-integrin and other ECM receptors in mammary gland function has been amply demonstrated both in culture and *in vivo* in the last decade [24,34–37]. However, if mammary epithelial cells can form functional acini in the presence of a laminin-rich gel in culture, what then is the role of the myoepithelial cells which surround the luminal epithelial cells *in vivo* (see Figure 1)? Luminal cells embedded in 3D collagen-I express different surface integrins from those embedded in 3D BM [38]. The two assays were used to clarify the role of myoepithelial cells in functional integrity of the acinus. When purified primary luminal cells were

Figure 3



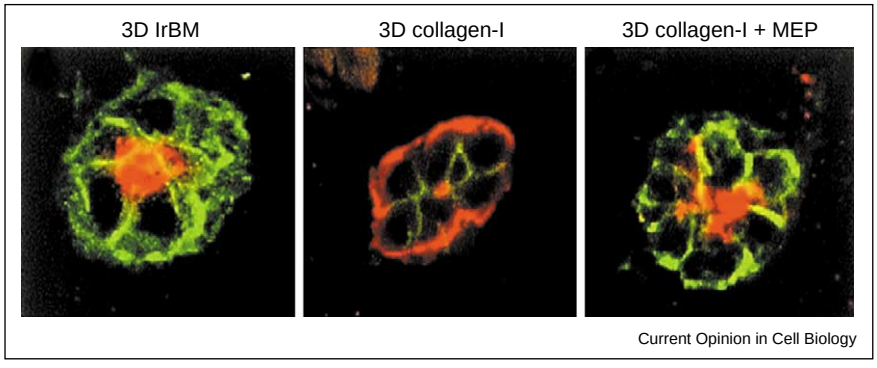
Milk protein production requires a hierarchical set of events including availability of lactogenic hormones, correct cytoskeletal organization, laminin-1, proper cell–cell interactions, formation of acini with apico-basal polarity, and cavitation and formation of lumina for secretion of milk [33].

embedded in collagen I gels, they formed an inside-out structure (i.e. they had reverse polarity). Incorporating purified myoepithelial cells, BM or laminin 1 (but not laminin 5 or 10/11) into these gels restored the polarity of the acinus [39•] (see Figure 4). Other structural entities such as desmosomes [40] and hemidesmosomes [41•] are also clearly needed to achieve and maintain acini polarity. These data support the previous findings regarding the importance of laminin in signaling to milk protein genes [25] and in tissue architecture and polarity in MDCK cells [42]. The mechanisms by which epithelial cells become polar and form junctions and the role of laminin I in this process has been reviewed extensively recently [42–47,48•]. It is important to remember, however, that although epithelial cells on tissue culture plastic can be considered 'polar' in that they have a distinct apical/basal polarity, this polarity is not functionally equivalent to that of the same cells in a 3D polar acinus. The discussion above on the production of milk proteins in culture makes it clear that functional differentiation in 2D and 3D are not equivalent; moreover, common signaling pathways also are regulated differently in 2D and 3D (see discussion below).

Normal and malignant breast cells can be distinguished in 3D BM

One characteristic of epithelial cells in tissue culture plastic is that, unlike with fibroblasts, it is not always

Figure 4



Myoepithelial cells contribute to correct polarity of luminal epithelial cell acini by providing laminin-1. Luminal epithelial cells make inside-out acini in collagen (middle) as shown by sialomucin (green) and ESA (red) staining. Addition of laminin-1 producing myoepithelial cells (MEP) to the 3D collagen cultures reverts the polarity (right) to resemble that of luminal epithelial cell acini in laminin-rich 3D BM (left) ([39•], reproduced with permission). Lr, laminin-rich.

easy to distinguish normal from malignant cells because they often grow at similar rates and are morphologically also similar. Together with Ole Petersen's laboratory, we developed a versatile assay to rapidly distinguish normal and malignant human breast cells in 3D BM in a defined medium [49] by modifying the rodent assay discussed above (see Figure 2); for recent detailed reviews on the use of this assay and some of its modifications, see [1,50–52]. The result was not only a tool for discriminating between normal and malignant cells, but also a system for investigating the phenotypic behavior of premalignant cells of a breast progression series [53,54]. These data indicate that breast cells lose architectural integrity before they become malignant. Furthermore, destruction of BM *in vivo* and in culture can lead to loss of mammary architecture [55], loss of functional differentiation [56], malignant behavior [57] and mammary tumors [58]. Given that aberration of the microenvironment and the tissue structure can lead to tumorigenicity, the question of whether the opposite can also be true is raised: can restoration of tissue structure restore normal behavior?

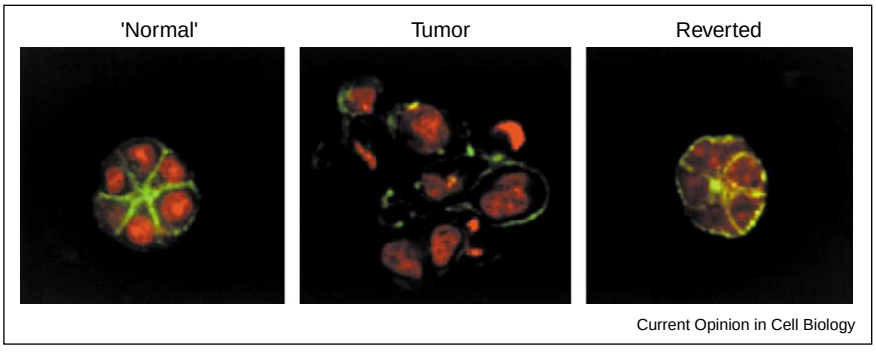
Restoration of tissue architecture can trump the malignant phenotype of breast cancer cells

Examination of surface receptors of the human breast cell progression series mentioned above (HMT3522) [53,54] indicated that several integrins and growth-factor-receptor pathways were in 'overdrive', leading to imbalanced signaling. Correcting β1 integrin and EGFR activities and/or inhibiting related signaling pathways (MAP kinase and PI3 kinase) could revert the malignant phenotype despite the malignant genotype (see Figure 5; [59–61]). Re-expression of several molecules that are altered or down-regulated in malignant cells, such as dystroglycan and a possible tumor suppressor molecule, AZU-1 (TACC1), could also restore the normal phenotype [62,63]. Surprisingly, even metastatic cells could be reverted (or killed) when treated with a combination of adhesion inhibitors and signaling molecule inhibitors [64].

The reversion assay gave a dramatic example of how the regulation of signaling pathways in 2D and 3D differs: when cells were reverted using β1-integrin or EGFR-inhibitory antibodies, signaling through these pathways was normalized. Surprisingly, the total protein levels of EGFR and β1-integrins were normalized as well. This feedback regulation did not occur in 2D cultures [60] (summarized in Table 1). More recent examples have provided additional evidence that several biological processes as well as adhesion complexes take different paths in 2D and 3D ([65,66]; for reviews see [67–70]; see also below).

The reversion assay can also be thought of as a screen that helps us to understand how to model the acini. A given malignant population (e.g. HMT3522-T4-2) can be reverted to a near-normal phenotype in multiple ways (Figure 6a), and other malignant cells may be similarly reverted to a morphologically normal form (Figure 6b). Gene expression arrays indicate that the different methods of reversion of T4-2 cells may modulate different genes, and yet produce a similar architectural and behavioral end point. For example, comparison of the number of genes the expression of which alters when T4-2 cells are reverted by blocking β1-integrin or EGFR shows that out of ~8000 genes tested there are only a handful of genes that are commonly altered in both methods of reversions, despite the fact that the expression of ~200–250 genes changes when T4-2 cells are reverted by either agents (Figure 6c). Interestingly, analysis of pathways in which the differentially expressed genes are involved and the biochemical data [59,60,63], however, indicate that several canonical signaling pathways are indeed intrinsically and reciprocally linked within the acini, irrespective of the reverting agent used. How the acini achieve this remarkable feat remains to be elucidated.

Figure 5



Non-malignant and tumorigenic breast epithelial cells can be distinguished from each other in the 3D BM assay. HMT3522–T4-2 (tumorigenic) cells (middle panel) can be reverted to a near-normal morphology, as discussed in the text. Organization of F-actin (green) that is lost in T4-2 (tumor) cells (nuclei shown in red) is restored in T4-2 cells reverted by down-modulation of EGFR signaling [60] or other means (see Figure 6).

Table 1

Cross-modulation of EGFR and β1-integrin

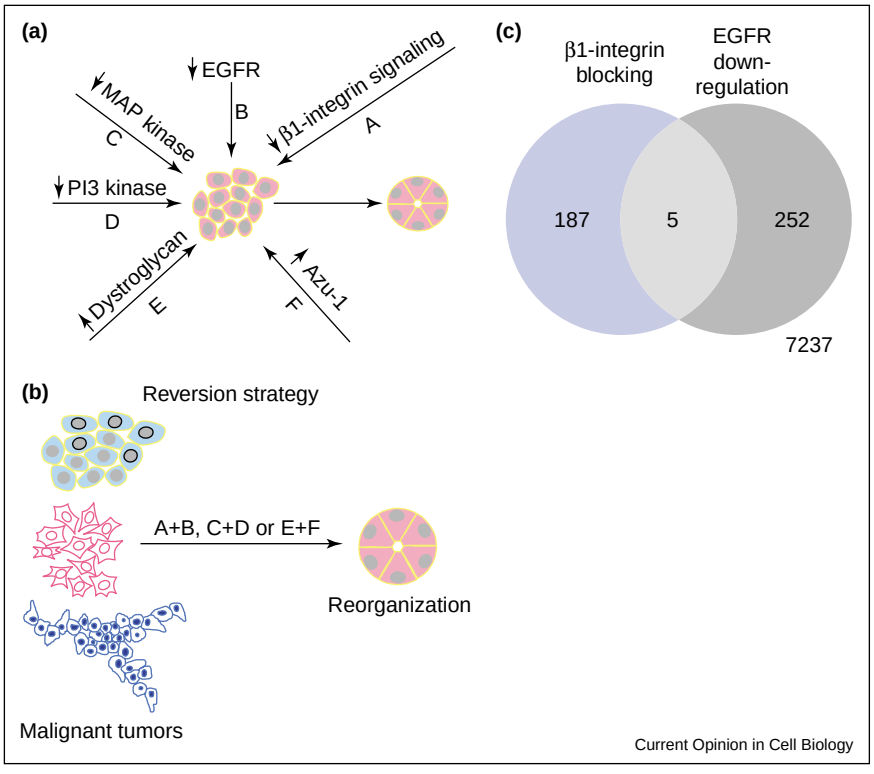
	3D				2D		
	S1	T4-2	T4-2		T4-2	T4-2 'treated'	
Inhibitor added	–	–	β1-integrin inhibitor	EGFR inhibitor	–	β1-integrin inhibitor	EGFR inhibitor
β1 integrin (total levels)	+	+++	+	+	+++	+++	+++
EGFR (total levels)	+	++++	+	+	++++	++++	++++
EGFR (activated)	+	++++	+	+	++++	++++	+

β1 integrin and EGFR protein levels and signal activation are coordinately modulated in HMT-3522 cells cultured in 3D lrBM but not in 2D. When 3D T4-2 cells are treated with functional inhibitors of either β1-integrin or EGFR (T4-2 treated) to revert the cells, endogenous β1-integrin and EGFR protein levels down-modulate coordinately. This phenomenon does not occur in 2D.

Restoration of form as a means of deciphering how form is maintained: modeling breast tissue architecture

In an effort to interpret our mammary-gland-specific results and to synthesize a conceptual framework for subsequent modeling, we have begun to seek inspiration from other disciplines with the expectation that any parallels that emerge can guide our future thinking. We briefly outline how a mechanism based on stochastic variation [3••] that is believed to be important in cell fate and guidance [71,72] may be relevant to the mammary gland acinus. In the literature, the nature of the relationship between PI3K, PIP3 and PTEN has emerged as a potential molecular mechanism underlying phenomena

Figure 6



Reversion strategies as a means of understanding signaling integration in acini. **(a)** Up- or down-regulation of many factors can cause the reverted phenotype of T4-2 cells in 3D BM. **(b)** Many different cell lines, including metastatic cells, can be reverted by combination of the treatments shown in (a). **(c)** Venn diagram summarizing gene expression analysis of ~8000 genes using cDNA arrays. Genes that showed differential expression between T4-2 and reverted T4-2 cells when reversion was achieved either by β1-integrin blocking (left) or by EGFR down-regulation (right) are shown. Differentially expressed genes are defined as genes that show a p-value of 0.05 or lower in four experiments.

such as spontaneous polarization, cell movement and differentiation, and morphogen-concentration-gradient-dependent chemotaxis. These phenomena are believed to arise from interplay between the coupled components of a cell-symmetry-breaking strategy, self-enhancing local activators that can amplify stochastic variation (noise) in a non-linear manner, and long-range inhibitors that promote competition for activation between different areas [71,72]. This scenario maintains a given state (positive feedback loops), represses undesired states (negative feedback loops), and prevents a change of state being triggered by small cues. The enzyme PI3K and the signaling phospholipid PIP3 form a positive feedback loop and can be equated with a local self-enhancer, whereas PTEN can be a candidate for a long-range inhibitor (it counteracts the activity of PI3K by dephosphorylating PIP3). In abstract, the local self-enhancer can be rewritten in terms of two components, A (PI3K) and B (PIP3), in which A affects B (A → B) and B affects A (B → A). The long range-inhibitor C (PTEN) affects A (C → A). In the discussion below, we expand the interpretation of A, B and C from molecular species to include the 'cellular modules' of Hartwell *et al.* [4]. At a scale more pertinent to the acinus and the mammary gland, A, B and C could represent tissue-level modules such as 'the ECM', 'polarity' and so on. It may be fruitful to consider that the organization and behavior of a tissue is characterized not only by its ability to maintain homeostasis (robustness to noise), but also by its capacity to make use of intrinsic uncertainty in a productive space- and time-dependent manner. Thus, the decision of cells in a tissue to proliferate, differentiate or apoptose may arise from intracellular and/or extracellular cues that bias stochastic variation to alter the balance between and/or select amongst pre-existing states. In our experimental organism, the mammary gland acinus, events such as BM signaling, receptor clustering, cross talk, cytoskeletal rearrangements and chromatin remodeling may allow inherent asymmetric amplification processes and events (local self-enhancement and long range inhibition) to be initiated at the correct site and appropriate time to allow reversion of the tumorigenic phenotype. Spatially and temporally enforced proximity, alignment and orientation would define regions able to exploit stochastic variation to increase the likelihood of activation of a target by an effector. An activation event would trigger a positive feedback loop that amplifies the signal, resulting in the accumulation of second messengers and additional downstream signals. Thus, specifying the position of a small initial event could yield a large localized signal able to set in motion cascade(s) that result in an observed phenotype. There are profound differences in the localization of a number of different molecules in 2D and 3D [59,60]. By causing the same event to occur in distinct cellular and tissue locations [73,74•], the 2D and 3D microenvironments may trigger different cascades and thus differences in eventual behavior. Although these ideas are purely speculative, we believe it is time to find experimental means of testing them, as has been done beautifully with bacteria [3••].

Conclusions

The efforts to model an acinus of the mammary gland was rooted in the early studies of cell and developmental biologists in the late 1960s and early 1970s, and yet it has taken a few decades to amass enough data for its utility to be recognized. It is now ready for broader use, and several laboratories in addition to ours are using the 3D BM model of breast acini to generate tissue-relevant data [66,75•,76•]. Models of skin, kidney, liver, and other tissues have also been attempted with varying degrees of success (briefly reviewed in [1]). The skin models, in particular, are sophisticated and robust (for reviews on this topic see [77–79]). Along with tissue-specific conditional knockout and transgenic mice, we believe it is imperative to develop functional 3D models of other tissues. The dearth of knowledge in areas such as pancreatic and other glandular epithelial cancers make them important candidates for a concerted effort to establish functional tissue models in culture.

This commentary has focused on efforts to model aspects of tissue architecture in culture, what has been learned from these studies, and thoughts on asymmetry-breaking mechanisms that might be relevant to cells in an acinus. An acinus is only one part of breast tissue. In general, tissues can be viewed as large, heterogeneous communities of cells that respond swiftly and dynamically to variations in their immediate microenvironment but nevertheless remain robust and essentially stable. Coordination, control and communication occur in a space- and time-dependent manner so that the demands on a tissue, for example the demand for milk in the breast, is handled appropriately. Such physiological processes take place on underlying anatomical structures. An emerging field of study is the 'anatomy' and 'physiology' of complex networks such as the World Wide Web, the Internet backbone and so on [5,6••]. Therefore, exploiting parallels between biological tissues and such systems may yield useful tools for modeling the human breast acinus discussed here. The challenge is to deconstruct a tissue into a hierarchy of functional units (nodes) and to reassemble them (i.e. to form connections between the nodes) in a manner that captures the key properties of the entire ensemble.

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