Modeling tissue-specific signaling and organ function in three dimensions

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Summary

In order to translate the findings from basic cellular research into clinical applications, cell-based models need to recapitulate both the 3D organization and multicellular complexity of an organ but at the same time accommodate systematic experimental intervention. Here we describe a hierarchy of tractable 3D models that range in complexity from organotypic 3D cultures (both monotypic and multicellular) to animal-based recombinations in vivo. Implementation of these physiologically relevant models, illustrated here in the context of human epithelial tissues, has enabled the study of intrinsic cell regulation pathways and also has provided compelling evidence for the role of the stromal compartment in directing epithelial cell function and dysfunction. Furthermore the experimental accessibility afforded by these tissue-specific 3D models has implications for the design and development of cancer therapies.

Key words: Human epithelial cells, Three dimensional, Organotypic models, Tissue-specific signaling

Introduction

Qualitative evaluation of the cellular complexity and structural integrity of organ biopsies has been used by pathologists for decades to gain insight into human diseases. The well-accepted correlation between tissue structure and health or disease conveys important lessons for the development of experimental models for the study of normal human biology and associated disease progression. Optimally, model design should recapitulate both the 3D organization and the differentiated function of any given organ but at the same time allow experimental intervention. By doing so, cell-based models facilitate systematic analyses that address, at the molecular level, how normal organ structure and function are maintained or how the balance is lost in cancer.

Because of the ethical, technical and financial constraints inherent in research on human cells and tissues, the demand for models that faithfully parallel human form and function considerably outweighs the supply. We and others asserted more than two decades ago that development of physiologically relevant models of both rodent and human origin should recognize that organs and tissues function in a 3D environment (Elsdale and Bard, 1972; Hay and Dodson, 1973; Emerman and Pitelka, 1977; Bissell, 1981; Ingber and Folkman, 1989). Further, that in the final analysis, the organ itself is the unit of function (Bissell and Hall, 1987). We now know that exposure of cells to the spatial constraints imposed by a 3D milieu determines how cells perceive and interpret biochemical cues from the surrounding microenvironment [e.g. the extracellular matrix, growth factors and neighboring cells (for reviews, see Roskelley et al., 1995; Bissell et al., 2002; Cunha et al., 2002; Ingber, 2002; Radisky et al., 2002)]. Furthermore, it is in this biophysical and biochemical context that cells display bona fide tissue and organ specificity.

Here, we describe studies of epithelial-cell-based systems that demonstrate the importance of developing and utilizing 3D human organotypic models to understand the molecular and cellular signaling events underlying human organ biology (Fig. 1). In their most simplistic form, these models comprise homogeneous epithelial cell populations that are cultured within 3D basement-membrane-like matrices. These relatively simple ‘monotypic’ cell models have progressively evolved into 3D co-culture models containing multiple cell types, which approximate organ structure and function in vitro and enable systematic analyses of the molecular contributions of multiple cell types. Finally, we go on to explore how human 3D culture models are being coupled to existing technologies in the mouse to generate models in vivo that could elucidate the fundamental influence of stromal-epithelial interactions in normal organ function as well as those that perturb organ homeostasis and lead to disease. As a result of these advancements, we are equipped with a hierarchy of related models that appreciate the importance of 3D environments but vary with respect to cellular complexity. By using this collection of experimental models interchangeably, we can test molecular markers and targets in models of defined at the molecular level with increasing physiological relevance (culminating in vivo); conversely, results in vivo can be translated into less complex models that are more suitable for diagnostic and therapeutic development.

Monotypic 3D cell culture assays

For several decades, we and others have taken a relatively simple approach of developing monotypic 3D culture models that are composed of single cell types but nonetheless recapitulate the minimum unit of the differentiated tissue (Bissell and Radisky, 2001; Cukierman et al., 2002). In addition to cell source, the composition of two basic

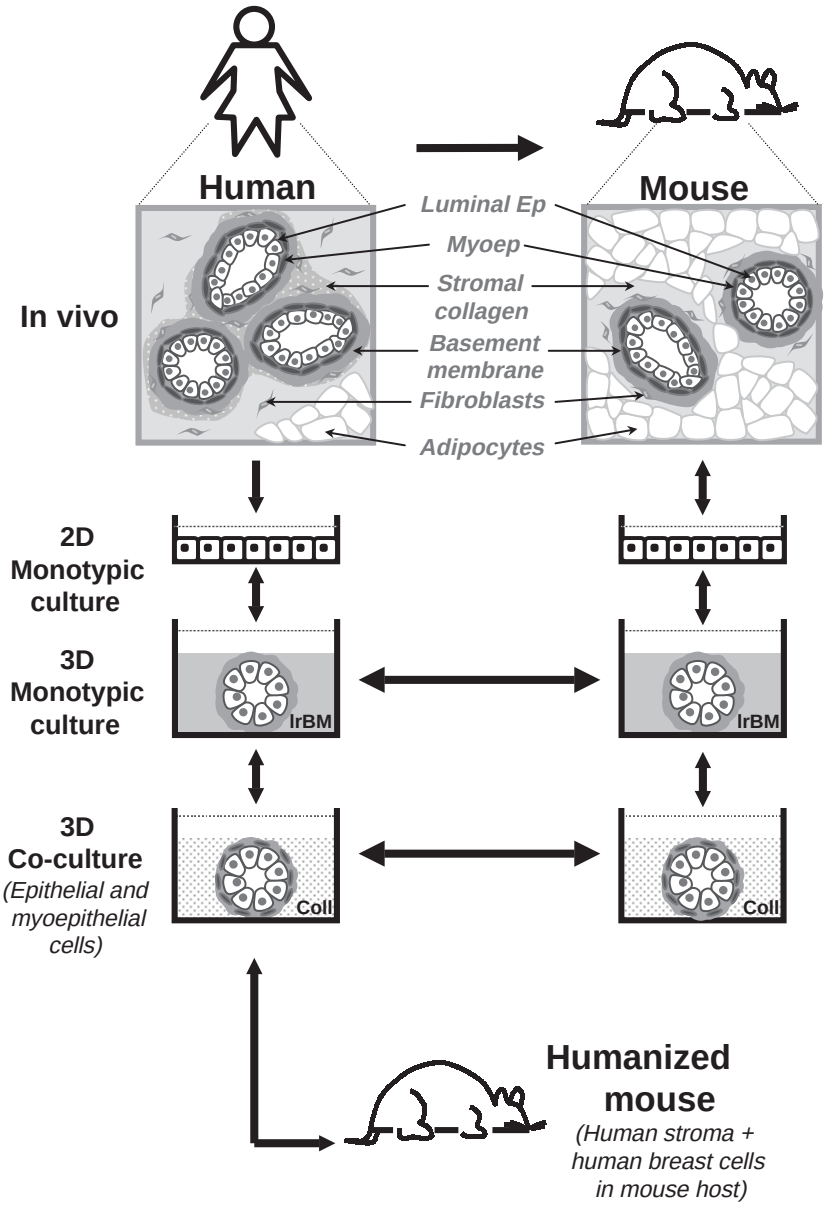

Fig. 1. Hierarchical modeling of human breast function. Similarities between the organization of human and mouse mammary glands have enabled observations in one tissue to be transferred to the other. This dynamic exchange of information has led to the gradual development of mammary gland models that now represent a continuum of organotypic systems ranging in complexity from monotypic 3D cultures to multicellular co-cultures to in vivo xenograft models. Each of the 3D models depicted here represents a physiologically relevant assay in its own right. However, when engineered with common cellular components and used in series, these models become invaluable tools for the identification and verification of disease-related molecules as well as for the design and translation of effective drug therapies. Future in vivo models that are more faithful to the human mammary microenvironment may be achieved in a 'humanized' mouse model in which mammary glands are entirely repopulated by breast cell types of human origin. Ep, epithelial cell; Myoep, myoepithelial cell. Adapted from previous publications (Ronnov-Jessen et al., 1996; Schmeichel et al., 1998).

components must be considered when one establishes physiologically relevant 3D culture models: the extracellular matrix (ECM) and the medium. Cell sources, both primary or immortalized, are most useful when they are capable of supporting tissue-specific differentiated function(s) in response to appropriate stimuli. Likewise, both the composition and delivery of the ECM and its molecules should reflect the physiology of the tissue in question. For example, laminin-rich Matrigel (Kleinman et al., 1986) is a reasonable substratum for use in 3D cultures of epithelia since epithelial cells are embedded in laminin-rich basement membrane (lrBM) in vivo. (In the discussion below, Matrigel will be generally referred to as lrBM. It should be noted, however, that whereas Matrigel is currently a cost-effective and biochemically reasonable substitute for BM, it is in fact a rather crude and ill-defined mixture of extracellular matrix proteins derived from a mouse tumor.) ECM presentation is also a critical parameter since cells cultured in (or on) thick and malleable matrices become differentiated, whereas cells grown on ECM delivered as a thin, planar coating on plastic do not (Roskelley et al., 1994; Cukierman et al., 2002). Finally, unlike chemically defined growth media, media containing serum or other complex supplements often interfere with differentiation.

A number of cell models have been coupled with appropriate 3D matrices and show fruitful results in recapitulating tissue functions in 3D. Extensive studies have been reported for liver, salivary gland, vasculature, bone, lung, skin, intestine, kidney and mammary and thyroid glands (see Table 1 and references therein). Other cells, such as MDCK (O'Brien et al., 2001; Troxell et al., 2001) and fibroblasts (Harkin and Hay, 1996; Cukierman et al., 2001), have also been monitored in 3D contexts and have provided valuable insight into the basic molecular mechanisms of polarity, branching morphogenesis, adhesion and cell migration (reviewed in Cukierman et al., 2002; O'Brien et al., 2002; Walpita and Hay, 2002).

In some cases, however, the relationship between these models in culture and a counterpart in vivo is unclear. We contend that, in order to be useful as a translational tool for the study of human disease progression, 3D organotypic models must be developed that are true to human form and function. In the following sections, we will describe the development of 3D culture models in human breast (and, briefly, in skin) that demonstrate how appropriate choice of cell source and ECM substrata can enable the establishment of physiologically relevant assay systems. By virtue of their resemblance to organ structure and function in vivo, these models facilitate meaningful dissection of the molecular mechanisms involved in the regulation of tissue specificity.

Table 1. Examples of 3D environments used in monotypic 3D cultures

Matrix	Cell type	Reference
Collagen I	Liver	Michalopoulos and Pitot, 1975; Bader et al., 1996
	Mammary gland	Emerman et al., 1977; Emerman and Pitelka, 1977; Howlett et al., 1995; Gudjonsson et al., 2002a
	Skin	Asselineau et al., 1985; Kopan et al., 1987
Amniotic basement membrane	Pancreas	Ingber et al., 1986
	Lung	Sakamoto et al., 2001
Reconstituted basement membrane	Liver	Bissell, D. M. et al., 1987; Ben-Ze'ev et al., 1988
	Mammary gland	Li et al., 1987; Barcellos-Hoff et al., 1989
	Pancreas	Oliver et al., 1987; Gittes et al., 1996
	Endothelia	Kubota et al., 1988; Grant et al., 1989
	Prostate	Bello-DeOcampo et al., 2001
	Lung	Schuger et al., 1990
	Salivary gland	Hoffman et al., 1996
	Thyroid gland	Mauchamp et al., 1998
	Kidney	Sakurai et al., 1997
	Intestine	Sanderson et al., 1996
	Bone	Vukicevic et al., 1990
Rotary cultures	Mammary gland	Runswick et al., 2001
	Prostate	Clejan et al., 2001

Monotypic 3D cultures for modeling mammary gland and epithelial signaling

A large body of work performed in mammary epithelial cells from mice demonstrates the central importance of 3D cell-microenvironment interactions in promoting a differentiated cellular response (Lin and Bissell, 1993; Roskelley et al., 1995; Boudreau and Bissell, 1998). Mammary cells embedded in lrBM adopt a spherical, polarized structure that resembles the normal mammary alveolus (or acinus) and that is capable of mammary-gland-specific function (e.g., producing milk in response to lactogenic hormones) (Barcellos-Hoff et al., 1989) (reviewed in Stoker et al., 1990). Human luminal epithelial cells, both primary and immortalized, respond to ECM in much the same way as their mouse counterparts by forming acini in 3D (Fig. 2) (Petersen et al., 1992; Howlett et al., 1995; Weaver et al., 1997). [The same cells grown in an interstitial ECM, such as collagen I, show altered integrins and abnormal polarity and organization (Howlett et al., 1995; Gudjonsson et al., 2002a; Weaver et al., 2002), thereby underscoring the importance of matching cell types with appropriate substrata.] Human breast tumor cells fail to show a differentiated phenotype in 3D lrBM, but instead form cellular masses that are disorganized and apolar (Petersen et al., 1992). Collectively, these studies demonstrate that human mammary epithelia respond to structural and biochemical cues provided by the ECM and that these cell-ECM interactions are sufficient to reveal innate cellular phenotypes.

3D organotypic cultures are amenable to a variety of experimental manipulations and have been effectively used to re-examine molecular pathways previously characterized by conventional culture methodologies as well as to elucidate novel signaling pathways. Recent examples are described below and are summarized in Fig. 2.

Coupled signaling mechanisms and reversion

When used in conjunction with well-defined human mammary cell cultures, monotypic 3D assays have proven useful for understanding how altered cell-ECM communication regulates breast tumor progression. One such cell source is the HMT-3522 human breast tumor progression series, which comprises a continuum of cell populations that arise from a common precursor but range in phenotype from non-malignant (S1) to tumorigenic (T4-2) (Briand et al., 1987; Nielsen et al., 1994; Briand et al., 1996). Dissection of the molecular differences between these cells revealed that surface expression of the ECM receptor β1 integrin is dramatically upregulated in T4-2 tumor cells in comparison with their non-malignant counterparts. When treated in 3D cultures with antibodies that block β1 integrin function, T4-2 cells dramatically reorganize: cell colonies become phenotypically reverted, assuming a polarized and growth-arrested status comparable to that observed of non-malignant S1 cells (Fig. 2) (Weaver et al., 1997). Reduced β1 integrin signaling results in downregulation of endogenous β1 integrins as well as in reduced signaling and levels of the epidermal growth factor receptor (EGFR) (Wang et al., 1998). In a reciprocal fashion, neutralizing the activity of EGFR effectively normalizes β1 integrin signaling and levels (Fig. 2). Such β1 integrin and EGFR reciprocal cross-modulation is apparently dependent upon a 3D context as neutralizing antibodies do not elicit reciprocal cross-modulation in cells cultured as 2D monolayers (Wang et al., 1998).

To determine the extent to which adhesion and growth factor receptor signaling is coupled in more aggressive tumors, Wang et al. recently assayed a series of metastatic human breast cancer cell lines for sensitivity to β1 integrin and EGFR cross-talk inhibitors in 3D lrBM cultures (Wang et al., 2002). Unlike results from the tumorigenic, but non-metastatic, T4-2 cells, single inhibitors induce only partial phenotypic reversion in aggressive carcinoma cells (Fig. 2). Instead, specific pairs of inhibitors, applied in tandem, are required to revert the malignant phenotype or cause apoptosis. Therefore, as signaling pathways become increasingly disconnected, they require intervention at multiple sites to elicit reversion and/or apoptosis.

Collectively, these studies show that recapitulation of phenotypically normal tissue in 3D lrBM assays correlates with

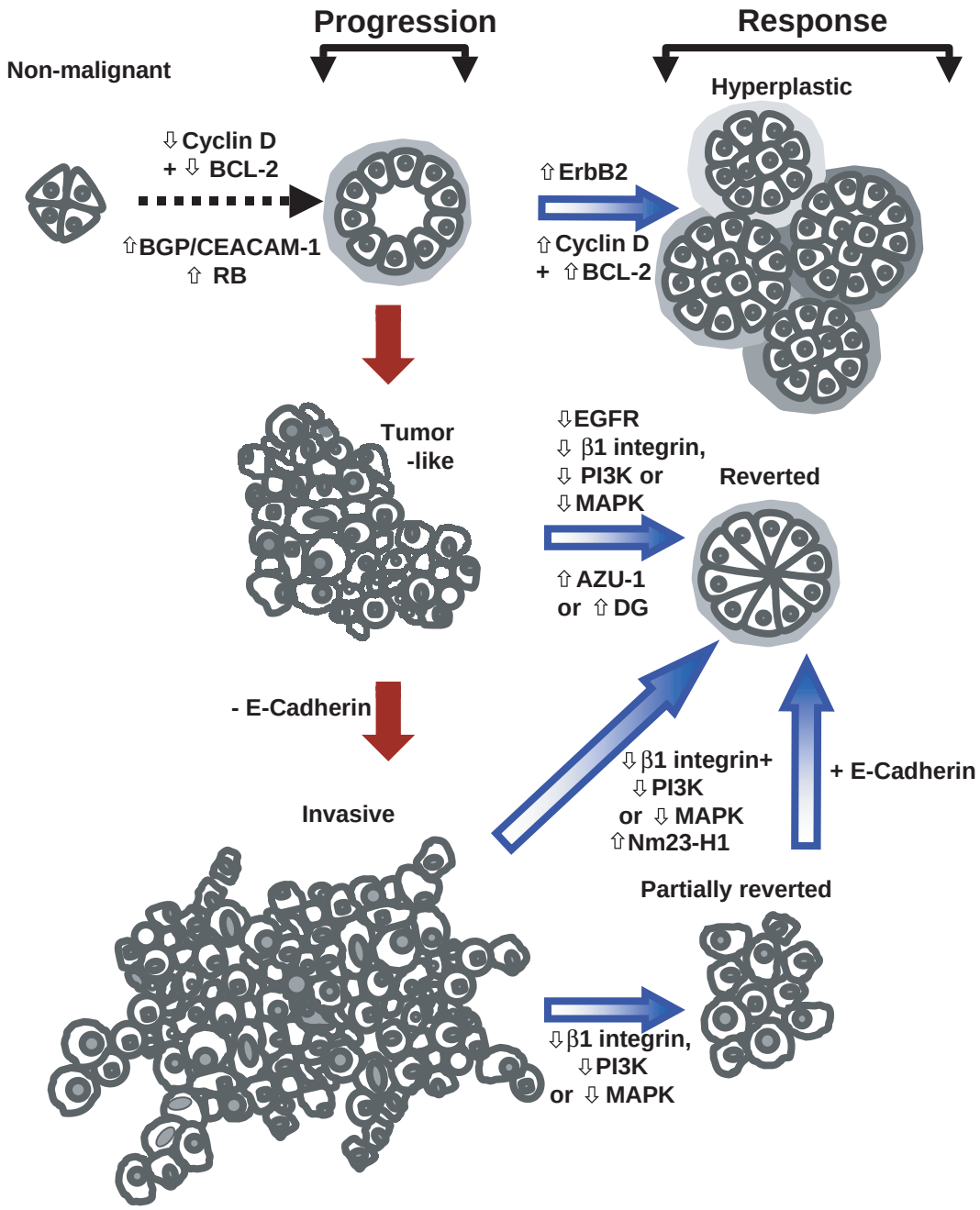

Fig. 2. Signaling mechanisms studied in monotypic 3D cultures of human mammary epithelial cell lines. The phenotypes of human mammary epithelial cells can be readily distinguished in the context of 3D lrBM assays. After 10 days in culture, non-malignant cells form growth-arrested, polarized acini with central lumens, whereas malignant cells form apolar colonies of continually growing cells that vary in size and shape depending on the degree of tumorigenicity. A number of studies, some of which are depicted here, have utilized this assay to explore the molecular regulation of normal breast (e.g., lumen formation) as well as aberrant signaling during tumor progression and/or reversion. Individual studies are referenced in the text.

the ability of adhesion and growth factor receptor signaling pathways to engage in reciprocal cross-modulation. Moreover, the intracellular signaling pathways directing cell polarity and proliferation in human mammary epithelial tissues are orchestrated in profoundly different ways, depending on whether cells are cultured in a 2D or 3D context. These studies also constitute proof that 3D assays of phenotypic reversion can be exploited further to characterize potential modulators of the malignant phenotype in breast.

Tumor suppressors and oncogenes

3D lrBM assays can be used to search for oncogenes and tumor suppressors as well as to understand their mechanisms of action. For example, Howlett et al. examined the extent to which restoration of Nm23-H1, a metastasis-suppressor gene (Leone et al., 1993), could restore ‘normal’ cell morphology (Howlett et al., 1994). They transfected a metastatic breast carcinoma cell line MDA-MB-435 with an Nm23-H1 transgene and assayed the resulting transgenic cells 3D lrBM cultures. They found that cells overexpressing Nm23-H1 formed organized acinus-like spheres with appropriately polarized basal and apical surfaces (Fig. 2) and thus provided evidence that suppressive effects of Nm23-H1 might be due to its role in growth inhibition and differentiation in response to cues from the ECM. Spancake et al. examined the effects of downmodulating the retinoblastoma (RB) tumor-suppressor pathway in human mammary epithelial cells. Whereas loss of

RB function did not affect polarity of these epithelial cells in 3D (Spancake et al., 1999), the 3D acini were not growth arrested and failed to display several markers of differentiation found in primary cells, thereby demonstrating that RB function plays a role in mammary cell differentiation.

AZU-1 is a gene product that was isolated by comparative gene expression analysis of premalignant and malignant cells of the HMT-3522 series (Chen et al., 2000). *AZU-1* [also called *TACC2* (Still et al., 1999)] mRNA is significantly downregulated in a variety of human breast tumor cells, which is consistent with it having a tumor suppressor role in breast tissue. Normalizing the expression of AZU-1 in T4-2 tumor cells causes phenotypic reversion of the cells, as revealed in 3D lrBM assays. These findings, in combination with in vivo tumorigenicity assays, provides experimental evidence that AZU-1 is a novel breast tumor suppressor. Interestingly, AZU-1 levels, which are very low in T4-2 cells, become normalized in tumor cells reverted by EGFR or β1 integrin inhibition, which suggests that AZU-1 expression is also sensitive to cues from the microenvironment (Chen et al., 2000).

More recently, the non-integrin cell surface ECM receptor dystroglycan (DG) was also shown to display a tumor-suppressive function in T4-2 cells (Muschler et al., 2002). Re-expression of DG in tumor cells lacking α-DG expression but expressing E-cadherin produced profound repolarization of cells in the 3D lrBM assay. This finding suggests that, at the cell surface, normal cellular function might be a result of a competitive balance that is achieved by signaling through integrins, growth factors and dystroglycan.

Novel aspects of the function of the oncogene ErbB2 in tumor progression have been revealed in 3D human mammary epithelial cell cultures as well. ErbB2 is particularly interesting as a potential oncogene in the breast because its overexpression correlates with a poor clinical prognosis (reviewed in Eccles, 2001; Yarden, 2001). Human MCF10A mammary epithelial cells were recently engineered to express conditionally activated ErbB2 and analyzed in monotypic 3D cultures (Fig. 2) (Muthuswamy et al., 2001). When ErbB2 receptor, but not ErbB1, is activated in mature 3D acini, the MCF10A cells lose their polarized organization and develop structures consisting of multiple acinar-like units with filled lumina. These structures do not display any invasive properties and thus represent a reasonable model for ductal carcinoma in situ (DCIS). This finding also raised the possibility that excessive signaling through ErbB2 in 3D cultures is sufficient to induce growth and to protect cells from apoptosis within the luminal space (Huang et al., 1999; Muthuswamy et al., 2001) (see also discussion below).

Sensitivity and resistance to cell death

More than two decades ago, Hall et al. showed that, when murine mammary cells are sandwiched between two layers of ECM, they form a lumen (Hall et al., 1982). Since then, 3D monotypic cultures have been used to explore the basic developmental pathways of the mammary gland, including lumen formation. The work of Frisch and colleagues indicated that adhesion to any ECM molecule is a survival cue and that loss of adhesion hastens a cell's demise (anoikis) (Frisch and Francis, 1994). Boudreau et al. showed that adhesion to an inappropriate ECM ligand, at least in the case of epithelial cells, only delays cell death temporarily (Boudreau et al., 1995) and that adhesion to relevant substrata, such as lrBM or laminin, is necessary to maintain long-term survival. Once the cells lose contact with BM, caspases are induced and the cells apoptose. Coucouvanis and Martin showed subsequently in developing mouse embryos that, whereas contact with BM protects the outer cell layer, the inner cell mass cavity is carved by apoptosis of cells that had no contact with BM (Coucouvanis and Martin, 1995). Others have since shown that cavitation of the lumen of the mammary gland is also mediated by induction of apoptosis (Blatchford et al., 1999) (see also below).

Several recent reports using 3D human mammary epithelial cell models have provided important insights into the molecules and pathways involved in the apoptotic events leading to lumen formation. For example, Huang et al. showed that biliary glycoprotein (BGP, also known as CEACAM1 or CD66a), a transmembrane protein expressed on the luminal surface of mammary epithelia, is required for lumen formation in MCF10A-derived acini cultured in 3D lrBM (Huang et al., 1999). Re-expression of a short isoform of BGP in MCF7 breast carcinoma cells (that is, cells that lack BGP and fail to form lumena in 3D lrBM assays) results in cells that form morphologically normal mammary acini with properly formed central lumena in 3D cultures (Kirshner et al., 2003). This model revealed that BGP adopts an apical localization during morphogenesis and influences lumen formation by initiation of apoptotic pathways.

Two recent studies by Muthuswamy et al. and Debnath and colleagues show that lumen formation in the MCF10A model is not mediated solely by the action of pro-apoptotic signals (Muthuswamy et al., 2001; Debnath et al., 2002). Rather growth control signals and increased apoptotic signaling cooperate to direct lumen formation in this 3D monotypic model. Because chronic activation of ErbB2 is sufficient to cause accumulation of colonies with cell-filled lumina, ErbB2 oncogenic signaling probably exerts multiple biological effects, coordinating signals that affect both proliferation and apoptosis during cavitation (Debnath et al., 2002). These studies provide a compelling example of how the study of developmental processes (such as lumen formation) can benefit from the experimental accessibility of these simple tissue-specific models to yield physiologically relevant information at the molecular level.

The HMT-3522 culture model was recently employed to address mechanisms of resistance to chemotherapeutic-agent-induced apoptosis in vivo (Weaver et al., 2002; see also Yamada and Clark, 2002). Formation of 3D polarized structures confers protection against apoptosis in both non-malignant and malignant mammary epithelial cells. Destabilizing the polarity of these structures by disrupting β4 integrin ligation, and thus perturbing hemidesmosome organization, allows induction of apoptosis. Loss of β4 integrin results in the inactivation of NFκB, a known positive modulator of expression and stability of apoptosis regulators. This topic is of critical relevance to considerations of apoptotic drug resistance and tumor dormancy. Metastasized tumor cells, either as single cells or clusters, may resist death induced by chemotherapeutic agents when the microenvironment and spatial information at the secondary site is conducive to the establishment of cell polarity and survival.

The functional significance of nuclear structure

The status of nuclear organization is an important indicator of tissue homeostasis and differentiation in vivo (Lelievre et al., 2000; Nickerson, 2001). In 3D cultures, the nuclear structure of HMT-3522-S1 cells differs radically from that of the same cells cultured in a monolayer (Lelievre et al., 1998). Furthermore, a series of structural changes are evident within the nucleus throughout acinar morphogenesis in 3D. The observed structural modifications are apparently coupled to S1 cell function, as targeted disruption of the nuclear structure in fully differentiated S1 cells causes upregulation of matrix metalloproteinase activity, an event that ultimately alters the quality of the underlying basement membrane (Lelievre et al., 1998). Collectively these studies point to a dynamic and reciprocal functional connection between nuclear structure and cell function that is dependent upon the presence of an appropriate 3D context. Much remains to be understood about how communication between the BM, the 3D structure and the nucleus is established. However, the idea that understanding these connections requires 3D models is now being more widely championed. Commenting on recent papers by Debnath et al. and Weaver et al., Jacks and Weinberg conclude: "Suddenly, the study of cancer cells in two dimensions seems quaint, if not archaic" (Debnath et al., 2002; Weaver et al., 2002; Jacks and Weinberg, 2002).

Modeling organs in vitro: organotypic co-cultures

Whereas the monotypic 3D culture models described above effectively approximate the biochemical and spatial contributions required for tissue-specific function, organs comprise numerous cell types, matrices and other environmental factors. By reproducing a total organ environment in culture models, the significance of autocrine and paracrine interactions, long appreciated for their roles in organ biogenesis and development (for a review, see Cunha, 1994), can be examined in molecular and cellular terms. This is particularly important given the growing interest in the broader role of the stroma (loosely defined as other cell types plus the ECM) in regulating normal epithelial cell function (see Bissell and Radisky, 2001; Liotta and Kohn, 2001; Silberstein, 2001; Tlsty and Hein, 2001; Cunha et al., 2002; Fuchs and Raghavan, 2002; Mueller and Fusenig, 2002). Studies in human skin provide extensive examples of how 3D co-culture methodologies can be used to recapitulate organ function and allow systematic analysis of underlying molecular pathways.

The co-culture paradigm: human skin

3D organotypic co-culturing methodologies have been particularly successful in epidermal biology. A cultured version of a 'skin equivalent' has been achieved through culturing of keratinocytes either on de-epidermized dermis (Regnier et al., 1981; Watt, 1988; Fartasch and Ponec, 1994) or on collagen gels embedded with dermal fibroblasts (Bell et al., 1981; Regnier et al., 1981; Asselineau and Prunieras, 1984; Asselineau et al., 1985; McCance et al., 1988; Watt, 1988; Coulomb et al., 1989; Fartasch and Ponec, 1994). Such co-cultures give rise to stratified epithelium that displays many of the morphological and functional features of an epidermis in vivo (Bell et al., 1981; Kopan et al., 1987; Watt, 1988; Kopan and Fuchs, 1989; Hertle et al., 1991; Parenteau et al., 1991; Fusenig, 1994; Smola et al., 1998).

Differentiated human skin equivalents are produced in co-cultures containing fibroblasts of either human or mouse origin (Choi and Fuchs, 1990; Turksen et al., 1991; Kaur and Carter, 1992). Given this inherent compatibility, which probably reflects a similarity in the synthesis of paracrine factors between species, one can supplement skin co-cultures with fibroblasts derived from genetically engineered mice. An elegant example of such a substituted culture allowed examination of the role of AP-1 transcription factor subunits c-jun and junB in skin homeostasis (Fig. 3) (Szabowski et al., 2000; Angel and Szabowski, 2002). Unlike wild-type murine fibroblasts, the presence of c-jun-deficient or junB-deficient fibroblasts had dramatic and distinct effects on keratinocyte proliferation and differentiation when included in skin co-cultures. These studies elucidated a double paracrine mechanism in which keratinocytes produce Il-1, which, in turn, induces the expression of keratinocyte growth factor (KGF) and granulocyte macrophage colony stimulating factor (GM-CSF) in dermal fibroblasts through AP-1 activation (Maas-Szabowski et al., 2000; Szabowski et al., 2000; Maas-Szabowski et al., 2001).

When used in combination with skin tumor progression models, 3D co-cultures also effectively distinguish between non-malignant and malignant phenotypes. Such strategies were used to show that MMP1, a matrix metalloproteinase implicated in tumor induction and progression, is upregulated not only in more aggressive tumor types but also in the co-cultured fibroblasts themselves (Fusenig and Boukamp, 1998). Other studies have established that embryonic stem (ES) cells from normal animals can produce well-differentiated epidermis in 3D co-cultures (Bagutti et al., 1996). Taking advantage of this compatibility, Bagutti et al. recently demonstrated that β1-integrin-null stem cells respond and differentiate in epidermal skin cultures only in the presence of excess stromal factors and that loss of β1 integrin decreases the sensitivity of ES cells to soluble factors that induce differentiation (Bagutti et al., 2001).

Given the development of these tractable models of human skin that display an unquestionable resemblance to skin in vivo, it is not surprising that these models are being productively utilized in pharmacotoxicological studies (Gay et al., 1992) and in skin grafting procedures (Boyce and Warden, 2002). Furthermore, they provide critical guidance for modeling the complexities of other human organ systems, such as the breast, in culture.

Modeling breast complexity in culture

The unit morphology of a mammary duct is a double-layered structure in which a continuous sheet of polarized epithelium is surrounded by a layer of myoepithelial cells. Because of their contractile nature, myoepithelial cells have generally been recognized for their function in the extrusion of secreted milk from mammary gland during lactation. However, accumulating evidence indicates that myoepithelial-luminal epithelial cell interactions contribute to homeostasis within the mammary gland and that disruption of this interaction might be an

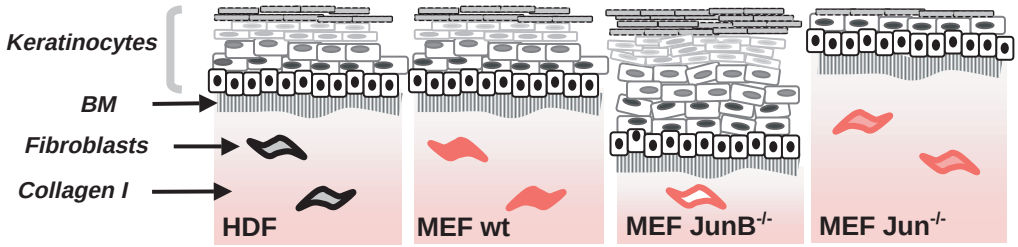

Fig. 3. Using genetically engineered fibroblasts to elucidate stromal-epithelial interactions in organotypic skin co-cultures. Primary human keratinocytes maintain their stratified and differentiated morphology in 3D organotypic co-cultures regardless of whether dermal fibroblasts included are of human (HDF, A) or mouse (MEF, mouse embryonic fibroblasts, B) origin. Substitution of wild-type mouse fibroblasts with genetically engineered fibroblasts from transgenic animals allows for a detailed analysis of the molecular underpinnings of epithelial stromal interactions. Here, c-jun$^{-/-}$ (C) and junB$^{-/-}$ (D) fibroblasts are shown to have hypo- or hyperproliferative effects, respectively, on the morphology of human skin. This study demonstrates the utility of 3D co-culture methodologies in dissecting the molecular determinants of paracrine signaling networks. This figure is summarized from a previously published figure (Szabowski et al., 2000).

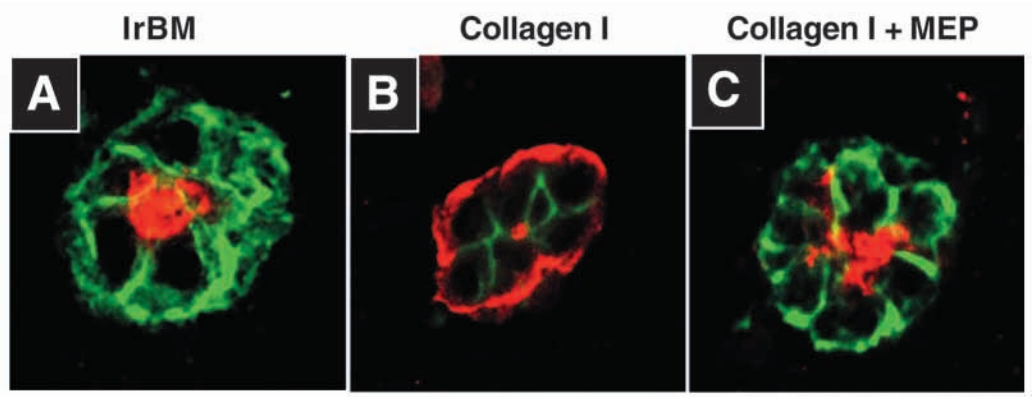

Fig. 4. Modeling mammary acinar structure in 3D organotypic co-cultures. Purified primary human luminal epithelial cells were embedded and cultured in lrBM (A) or collagen I gels (B,C) in the absence (A,B) or presence (C) of purified myoepithelial cells (MEP). Cultures were double stained for the lumenal marker, sialomucin (red) and the basolateral marker, epithelial-specific antigen (ESA; green). Luminal epithelial cells form polarized organotypic spheres in lrBM but adopt inverse polarity in collagen I gels. Addition of purified myoepithelial cells to luminal epithelial cells in collagen I corrects acinar polarity (C) and results in formation of a bilayered organotypic structure. Reproduced with permission from Gudjonsson et al. (Gudjonsson et al., 2002a).

important step in tumor progression (Zou et al., 1994; Sternlicht et al., 1997; Man, 2002).

Recently, several studies performed in 3D organotypic co-culture models have examined the role of myoepithelia in organizing and maintaining normal gland structure and function. Runswick and colleagues mixed purified human luminal epithelial cells and myoepithelial cells and incubated them in a 3D rotary culture environment (Runswick et al., 2001). Under these conditions, double-layered structures formed, containing a central core of polarized luminal epithelial cells surrounded by a layer of myoepithelial cells. Perturbation of myoepithelium-specific desmosomal cadherins disrupted basal positioning of myoepithelial cells (Runswick et al., 2001), thereby demonstrating that physical associations between myoepithelial and luminal epithelial cells are important for the establishment of higher-order organ structure in the mammary gland.

The interdependence of luminal and myoepithelial cells has also been analyzed in 3D ECM cultures in which purified primary luminal epithelial cells were combined within 3D collagen I gels in the presence or absence of purified myoepithelial cells (Gudjonsson et al., 2002a). As expected from previous studies of human mammary epithelial cells in 3D collagen I cultures (Howlett et al., 1995; Lelievre et al., 1998), the primary luminal epithelial cells alone fail to show appropriate polarity in 3D collagen I. However, collagen-based co-cultures containing both luminal epithelial and myoepithelial cells show polarized, bilayered organization (Fig. 4). Normal myoepithelial cells direct luminal epithelial cell polarity by synthesizing laminin 1; tumor-derived myoepithelial cells, expressing no or low levels of laminin 1, fail to yield double-layered organotypic structures in 3D collagen co-cultures (Gudjonsson et al., 2002a). Thus, the basal positioning of myoepithelial cells is not only well-suited for contractile events that occur during lactation (a known function of myoepithelial cells) but also provides important spatially restricted biochemical cues that drive cell polarity and normal function in the breast (see Bissell and Bilder, 2003).

Indeed, it is now conceivable that the human breast could be reasonably recapitulated in vitro by systematic pairing of different cell types in 3D culture. In a recent report, Hass and Kratz reported the results of co-culturing primary human mammary epithelial cells and adipocytes derived from the same patient (Huss and Kratz, 2001). In these cultures, differentiated epithelial structures are embedded in clusters of adipocytes in patterns reminiscent of the human breast in vivo. Whether these structures reflect the ability of adipocytes to contribute to a BM and whether there are other functions provided by the fat cells remain to be determined. Additional modeling work, perhaps incorporating stem cell populations as described above for skin or immortalized progenitor cells of the breast (Gudjonsson et al., 2002b), will be required if we

are to achieve the full complement of mammary gland components in 3D culture.

Stromal-epithelial interactions in 3D in vivo

Mouse xenograft models

The question of stromal control of organ function has been effectively addressed in whole animal studies in which human epithelial cells are transplanted as 3D xenografts along with normal or aberrant stroma and monitored over time. Overall, the findings parallel observations made in 3D organotypic cultures and indicate that progression of the epithelial cell tumor is not cell autonomous. Rather, the chromosomal instability that eventually leads to cancer is promoted, and probably sometimes induced, by the anomalies in the surrounding stroma and microenvironment (Moinfar et al., 2000; reviewed in Petersen et al., 2001).

One compelling demonstration of the stromal control of epithelial behavior comes from Olumi and colleagues, who developed a model in which non-malignant prostatic epithelial cells (normal or SV40-immortalized) were mixed in collagen gels with prostate-derived fibroblasts from normal epithelial organoids or cultures of carcinoma-associated cells. These 3D cultures were then transplanted beneath the renal capsule of athymic mice, and epithelial outgrowth was monitored (Olumi et al., 1999). Neither epithelial cells nor fibroblasts, alone, promoted tumor formation on their own. However, xenografts that included carcinoma-associated fibroblasts (CAFs) along with SV-40-immortalized epithelial cells shows a dramatic tumorigenic response. These findings demonstrate in an in vivo setting that the altered signaling capacity of the CAFs is sufficient to catalyze tumor progression in a cell type that displays a mildly altered genotype.

Parmar et al. have recently used a similar renal grafting approach to address the role of mammary stromal fibroblasts in mammary gland function and development (Parmar et al., 2002). They mixed normal human mammary epithelial cells, prepared as organoids, with mammary fibroblasts and grafted them into renal capsules of nude mice. These transplants show a robust elaboration of a mammary structure, which does not occur in the absence of mammary stroma and appears to be responsive to hormonal stimulation from estrogen and progesterone (e.g., they can be induced to produce milk when analyzed in pregnant animals) (Parmar et al., 2002).

Human xenograft and tissue transplant models are thus powerful tools for analyzing the complexities of organ function, especially when results can be reciprocally tested and scrutinized in simpler 3D culture models. However, opinions differ with respect to the optimum site of tissue transplantation within the animal and its preparation. Does the outgrowth of human mammary epithelium in the kidney, for example, truly represent normal mammary events or does the mouse fat pad provide a more relevant environment for outgrowth? Indeed, it has long been known that mouse mammary epithelial cells display differential developmental responses depending upon their site of delivery (see Miller et al., 1981; Neville et al., 1998). A recent study revealed that human mammary epithelial cells harboring three cancer-predisposing genetic alterations have differential tumorigenic responses in nude mice, the most extreme response being associated with transplantation into cleared mammary fat pad (Elenbaas et al., 2001). Considering the range of responses from both ‘normal’ and tumorigenic cells within a given animal, perhaps we should also ask whether the rodent fat pad is of sufficient relevance to reconstitute a human cell behavior that is true to the human form or whether we should be striving to humanized mouse mammary models?

Developing a ‘humanized’ mammary gland in the mouse fat pad

In very general terms, the mouse and human mammary glands share a reasonable level of similarity but also some differences (Fig. 1) (Ronnov-Jessen et al., 1996). Because the mouse mammary gland has high levels of adipose stroma, it is reasonable to suggest that the environment of the mouse mammary fat pad in mice is not entirely equivalent to human breast (Neville et al., 1998). Cleared fat pads, commonly used in transplant studies, also include several other cell types, such as fibroblasts, endothelial cells and cells of the immune system, all of which could influence mammogenesis. Moreover, techniques routinely used to prepare orthotopic sites, such as irradiation, may in fact induce profound and lasting stromal effects by themselves (Barcellos-Hoff and Ravani, 2000; Barcellos-Hoff, 2001). Furthermore, human mammary epithelial cells injected into cleared fat pads do not elaborate ductal structures (Sheffield, 1988), which emphasizes the apparent incompatibility between human and mouse mammary organs.

To study breast homeostasis with ultimate physiological relevance, we must model not just tissues but entire organs in vivo (Bissell and Hall, 1987; Bissell and Radisky, 2001). One future goal would be to develop ‘humanized’ mammary glands in rodents by replacing mouse mammary gland components with their human counterparts and reconstructing an organ that is comparable to the human gland in its architecture and organization (Fig. 1). As daunting as this task seems, progress is currently being made to craft such a ‘humanized’ mammary gland model in mouse. Human stromal fibroblasts grafted into the fat pads of nude mice support elaboration of transplanted human mammary epithelial cells into an infiltrating ductal tree that is highly differentiated and responsive to lactogenic cues (C. Kuperwasser and R. A. Weinberg, personal communication). Establishment of such a ‘humanized’ animal paves the way for the systematic inclusion of genetically modified epithelial or stromal cells and thereby provides a model for controlled analysis of specific molecules and pathways in an in vivo context. Furthermore, the ‘humanized’ mouse could also be important in the development of strategies for reconstructing human breast after surgical intervention (Huss and Kratz, 2001).

Future versions of ‘humanized’ mice could incorporate human mammary progenitor cells in this type of transplant model. The existence of mammary epithelial stem cells has been the subject of much debate, but recent evidence demonstrates the existence of precursor cells within the luminal epithelial pool (Smith, 1996; Stingl et al., 1998; Smalley et al., 1999). Recently, Gudjonnson et al. have isolated and immortalized a human mammary progenitor cell that gives rise to structures that resemble terminal ductal lobular units (TDLU) when implanted either in Matrigel or orthotopically into nude mice (Gudjonsson et al., 2002b). Thus it seems

plausible that introduction of human progenitor cells in the mouse mammary gland may be a useful strategy for generating chimeric animals with extensively humanized mammary organs.

Concluding remarks

We have described a series of experimental models that range in complexity from monotypic 3D cultures to multicellular 'humanized' organs in vivo. Because of the physiologically relevant structural and functional features they display, each model is a powerful tool in and of itself. However, when these models are linked together in progressive fashion, their potential for defining the molecular determinants of normal organ function and for elucidating pathways compromised during disease progression is amplified. Already, by using a combination of 3D culture and animal xenograft strategies, researchers have demonstrated a role for the stroma as an important regulator of epithelial function and carcinoma progression. Streamlining our approaches to utilize the same cell type at each incremental step will clarify the underlying molecular details of these phenomena. Moreover, effective utilization of a hierarchy of 3D models has an enormous potential for improving disease target identification and drug design. If the behavior of candidate regulators can be verified in models of increasing physiological relevance, this would provide a more convincing rationale for the design of clinical trials in human subjects. Moreover, implementation of high-throughput screening in the context of simpler 3D organotypic cultures will probably waste less time during drug development and have the potential to yield molecules that will sustain efficacy in clinical trials (Balis, 2002; Bhadriraju and Chen, 2002; Weaver et al., 2002). The progress made thus far towards these goals is already reason for excitement.

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