Cell

Modeling Tissue Morphogenesis and Cancer in 3D

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Three-dimensional (3D) in vitro models span the gap between two-dimensional cell cultures and whole-animal systems. By mimicking features of the in vivo environment and taking advantage of the same tools used to study cells in traditional cell culture, 3D models provide unique perspectives on the behavior of stem cells, developing tissues and organs, and tumors. These models may help to accelerate translational research in cancer biology and tissue engineering.

Tissues and organs are three dimensional (3D). However, our ability to understand their formation, function, and pathology has often depended on two-dimensional (2D) cell culture studies or on animal model systems. Studies in standard cell culture have produced many important conceptual advances. Nevertheless, cells grown on flat 2D tissue culture substrates can differ considerably in their morphology, cell-cell and cell-matrix interactions, and differentiation from those growing in more physiological 3D environments (Birgersdotter et al., 2005; Cukierman et al., 2002; Griffith and Swartz, 2006; Nelson and Bissell, 2006). At the other end of the experimental continuum, animal models frequently provide definitive tests of the importance of specific molecules and processes. However, there also can be puzzling discrepancies between conclusions from gene ablation studies and studies using chemical genetics approaches to interfere with the function of specific proteins (Knight and Shokat, 2007). In addition, animal models may not adequately reproduce features of, for example, human tumors, drug therapeutic responses, autoimmune diseases, and stem cell differentiation. In vitro 3D tissue models provide a third approach that bridges the gap between traditional cell culture and animal models (Griffith and Swartz, 2006; Rangarajan et al., 2004).

These in vitro 3D tissue models fulfill a need for reductionist approaches to understand in vivo molecular mechanisms. Moreover, the powerful tools of cell and molecular biology currently used in traditional cell cultures can often by applied to 3D tissue models. Increased use of 3D models that mimic specific tissues should promote advances in tissue engineering and could also facilitate the development and screening of new therapeutics. Our Review focuses on general principles, ideas, and caveats concerning the use of in vitro 3D model systems for studying tissue morphogenesis and tumorigenesis. We present recent examples chosen to illustrate key concepts. We also discuss exciting opportunities for further fundamental and translational research on cancer, stem cells, and tissue engineering.

Types of In Vitro 3D Models

One commonly used approach makes use of tissues harvested in vivo (microscopic embryonic organs or intact tissue slices), which are then explanted and cultured in vitro. They often retain their original 3D architecture in culture. This approach has been particularly effective in relatively short-term cultures for experimental analysis of numerous tissues including brain and embryonic glands (Gahwiler et al., 1997; Sakai et al., 2003). For most studies, the tissues must be thin enough to permit adequate oxygenation and nutrition of the tissue interior, e.g., less than ~0.3 mm thick. On the other hand, studies of tumor biology have benefited from systems that mimic the internal nutrient insufficiency of tumors to induce necrosis, which is important in studying tumor-host interactions such as the induction of tumor angiogenesis and resistance to chemotherapeutic drugs (Hicks et al., 2006).

Many 3D models have been established starting from isolated cells, e.g., from cell lines, dissociated tissues, or stem cells. A widely used strategy is to propagate cells in tissue culture and then implant them in a 3D matrix scaffold as either single cells or as tissue-like aggregates. 3D scaffolds have been generated from purified molecules such as collagen I, synthetic biomaterials, and even from native extracellular matrices from which living cells were previously extracted (Table S1). Another approach is to use more than one type of isolated cell or a tissue fragment in combination with another cell type. An example of this is a 3D tissue model of human skin that combines keratinocytes and fibroblasts with cancer cells to simulate human melanoma (Smalley et al., 2006).

Table 1. Key Strengths and Weaknesses of 3D Models

Advantages
• Cell morphology and signaling are often more physiological than routine 2D cell culture
• Permit rapid experimental manipulations and testing of hypotheses
• Permit much better real-time and/or fixed imaging by microscopy than in animals
Disadvantages
• Vary in their ability to mimic in vivo tissue conditions
• Currently lack vasculature and normal transport of small molecules, host immune responses, and other cell-cell interactions
• Generally mimic static or short-term conditions, whereas in vivo systems often progress

Table 1 lists major advantages and disadvantages of current 3D systems compared to regular cell culture and animal models. Comparisons of 2D and 3D models reveal that the latter are better, but not exact, models of in vivo tissues. Table 2 compares specific biological properties and their regulation in 2D and 3D systems, and details are presented in Tables S1 and S2. For example, glandular epithelial cell organization, signaling, and secretion are more similar to what occurs in vivo in 3D settings than comparable 2D approaches (Debnath and Brugge, 2005; Nelson and Bissell, 2006). The morphologies of fibroblasts, including cytoskeletal organization and types of cell adhesions, are also more similar to their in vivo behavior when the fibroblasts are grown in a 3D matrix than when grown in 2D (Figure 1). This is also true of their intracellular signaling characteristics (Cukierman et al., 2001, 2002; Grinnell, 2003; Walpita and Hay, 2002). Studies of gene expression and mRNA splicing patterns also reveal considerable differences when cells are cultured under 2D versus 3D conditions (Birgersdotter et al., 2005; Li et al., 2006).

Model Choice Affects Outcome

In any 3D model system, the specific cellular and matrix microenvironment provided to cells can substantially influence experimental outcome. For example, embedding tumor cells in a 3D collagen matrix as single cells, small aggregates, or larger aggregates can result, respectively, in individual cell migration and invasion, collective cell invasion, or mixtures of invasive and necrotic cells (Friedl, 2004; Mueller-Klieser, 1997). Because 3D in vitro model systems lack the complex vascular systems that perfuse tissues in vivo, oxygenation, nutrition, and waste removal occur by simple diffusion. Consequently, as the tissue thickness of a 3D model increases, transport limitations for these molecules will become increasingly important. Although nutrient restrictions may sometimes mimic in vivo tissue and tumor microenvironments better than the uniformly rich oxygenation and nutrition provided to monolayer cells in 2D cultures, they also introduce significant and potentially confounding variables to 3D models. This is because cells at different depths from the surface can be in different nutritional states (Keith and Simon, 2007; Levenberg, 2005).

In addition, both the composition and stiffness of the extracellular matrix surrounding the cells have major effects on cell signaling and behavior (Cukierman et al., 2002; Discher et al., 2005; Grinnell, 2003; Paszek et al., 2005). For example, collagen gels can mimic loose or dense connective tissue depending on the concentration of collagen; such gels have been used widely in studies of fibroblast and tumor cell migration and signaling (Grinnell, 2003). For growth and differentiation of epithelial cells, however, reconstituted gels of an extract containing basement membrane components and growth factors termed Matrigel (or EHS matrix or Cultrex) are much more effective (Kleinman and Martin, 2005). 3D matrices

Table 2. 3D-Dependent Cell Behavior and Signaling

Biological Function	2D versus 3D	Regulatory Mechanisms
Cell Shape	Loss of epithelial cell polarity and altered epithelial and fibroblast shape in 2D	Growth factor receptors and pathways; cell-adhesion signals associated with cell survival and matrix plasticity
Gene Expression	Cells in 2D versus 3D often have different patterns of gene expression	ECM, hormones, and adhesion molecules
Growth	3D matrix-dependent regulation of cell growth	Adhesion and growth factor-related pathways plus survival or apoptotic genes
Morphogenesis	3D matrix-induced vessel sprouting and gland branching	ECM, adhesion, growth factor-related pathways and apoptotic genes
Motility	Altered single and collective cell motility patterns in 3D matrices	ECM and its regulators; adhesions and growth factor-related pathways; phospholipids
Differentiation	3D matrix-induced cell differentiation	ECM and growth factors; motor molecules

See Table S2 for references and additional information.

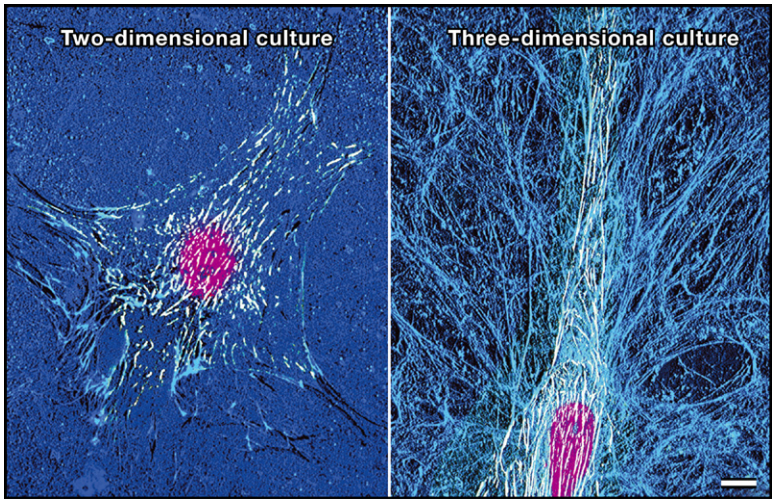

Figure 1. Human Fibroblasts in 2D versus 3D Microenvironments

Cells were cultured on planar fibronectin (left panel) or within a mesenchymal cell-derived three-dimensional (3D) matrix (right panel). Note the striking differences in overall morphology, fibronectin matrix organization (blue), and architecture of α_5 integrin-positive adhesion structures (white); nuclei are magenta. Scale bar = 10 μm.

generated by cells in vitro provide yet another class of matrix (Cukierman et al., 2001). Each type of matrix can also have experimental drawbacks. For example, collagen gels lack other components of connective tissue, and they differ in the extent of covalent crosslinking. Matrigel consists of basement membrane components, but it is a 3D cell culture material rather than a mimetic of the flat basement membranes underneath cells. Finally, cell-derived matrices can have lower amounts of collagen, larger internal spaces, and less depth than mature tissue matrices. Thus, an important point is that because each tissue in vivo has a characteristic matrix microenvironment, for a given study it is crucial to select an appropriately matched 3D in vitro matrix.

Matrix Stiffness

Although the molecular composition of the extracellular matrix is a well-known regulator of cellular responses, physical properties of the matrix in 3D models can also play surprisingly important roles. In particular, recent evidence points to direct roles for the stiffness (compliance) of the extracellular matrix in regulating multiple cellular functions (Discher et al., 2005; Paszek et al., 2005; Pelham and Wang, 1997). This property, also described as rigidity, elasticity, or pliability, is sensed by cells through bidirectional interaction between cells and the surrounding extracellular matrix. Cell surface integrin receptors and the contractile cytoskeleton pull against the extracellular matrix to sense the stiffness of the microenvironment (Ghosh et al., 2007; Ingber, 2006; Vogel and Sheetz, 2006). Biologically, cells need to sense and respond appropriately to their local microenvironment. The stiffness of microenvironments is variable; examples include loose versus dense connective tissue, soft versus hard tissues (such as bones and teeth), and early versus late stages of wound healing.

The stiffness of a matrix and its susceptibility to remodeling by cellular contractile processes, matrix secretion, and enzymatic degradation can affect the distribution of cell surface integrin receptors and the types of cell adhesions and cytoskeletal structures formed (Cukierman et al., 2001; Katz et al., 2000; Walpita and Hay, 2002). Matrix stiffness also alters intracellular signaling via Rho kinase and Rac (Pankov et al., 2005; Paszek et al., 2005; Wozniak et al., 2003). Stiffness can also enhance cell proliferation, in some cases promoting neoplasia (Paszek et al., 2005; Pelham and Wang, 1997).

Different in vitro 3D models provide a range of matrix stiffness that can mimic the range found in specific tissues in living organisms. Wide differences in stiffness exist between soft adipose tissue and the tightly woven basement membrane (such as encountered by mammary epithelial cells), as well as between loose matrices used by cells for migration during embryogenesis, dense connective tissue in skin, and precalcified osteoid versus rigid mature bone (Discher et al., 2005; Engler et al., 2006; Paszek et al., 2005). Pathological processes such as fibrosis or microenvironmental changes within and around developing tumors can also alter tissue stiffness and cellular responses (Engler et al., 2004). For example, dense, nonpliable desmoplastic tissue is associated with some carcinomas (Paszek et al., 2005) and sites predisposed for secondary metastases (Kaplan et al., 2005).

Cell and Tissue Polarity

Another critical in vivo property provided by 3D models is appropriate cell polarity. Polarity in vivo depends both on the cell type and the cellular microenvironment (Figure 2). Epithelial cells are often polarized, with apical and basal surfaces that are important for tissue organization and directional secretion of products. Their basal surfaces rest on thin, flat basement membranes comprised of collagen IV, laminin, and many other matrix proteins. In many tissues, particularly secretory organs, epithelial cells are organized into spherical 3D structures surrounding a lumen to function as acini of glands, alveoli (lung), or glomeruli (kidney). Tissue organization is lost when these cells are explanted onto flat 2D tissue culture substrates, and this organization and differentiated function can be restored or maintained when the cells are placed into 3D culture conditions (Griffith and Swartz, 2006; Nelson and Bissell, 2006).

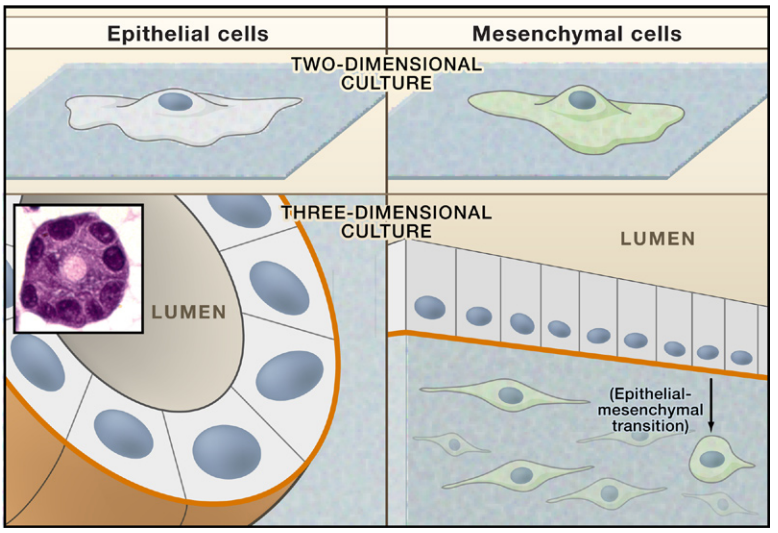

Figure 2. Cell and Tissue Organization in 2D versus 3D

Cells explanted into routine tissue cultures often flatten and lose differentiation markers. When placed back in appropriate 3D culture conditions, epithelial cells generally regain apical-basal polarity, and glandular cells form a lumen into which differentiated products are secreted. The inset image shows the morphology of human salivary gland cells reaggregated in vitro. In contrast, mesenchymal derivatives in 3D (lower right) regain a fibroblastic spindle shape and lose their artificial dorsal-ventral polarity. Epithelial cells often rest on a relatively thin 2D basement membrane facing a lumen. They can sometimes undergo an epithelial-mesenchymal transition to become migratory in a 3D stroma.

Because basement membranes are thin and basically 2D, epithelial cells in vivo resting on a flat basement membrane can be considered to be adhering to a 2D substrate (Figure 2). We predict, therefore, that it should eventually be possible to elicit normal, differentiated epithelial cell function on appropriately designed 2D surfaces in vitro, e.g., with a composition and stiffness mimicking basement membranes when combined with soluble stromal factors.

In direct contrast, cells such as fibroblasts lack this highly polar apical-basal organization in vivo. However, when placed onto 2D culture substrates, these cells acquire an upper (dorsal) and lower (ventral) surface, and prominent cell adhesions to the substrate form on the ventral surface. Fibroblasts lose this artificial dorsal-ventral polarity when placed back into a mesenchymal 3D matrix, and they regain their in vivo morphology (Amatangelo et al., 2005; Cukierman et al., 2001; Grinnell, 2003). Three-dimensionality per se—independent of matrix composition—can physiologically reprogram fibroblasts. There are striking differences in morphology, proliferation, and directionality of migration between cells cultured on a 3D matrix versus cells cultured on a matrix of identical biochemical composition that has been flattened to provide a 2D surface (Cukierman et al., 2001; Pankov et al., 2005; Zaman et al., 2006). One explanation for this may involve cellular detection of matrix contact with both ventral and dorsal surfaces. Simply bringing a collagenous substrate into contact with both cell surfaces restores more normal 3D morphology in fibroblasts (Beningo et al., 2004); however, whether other cell functions such as signaling and proliferation are similarly regulated remains to be examined.

Taken together, studies of epithelial cells and fibroblasts indicate that an important feature of 3D models is their ability to mimic normal tissue organization to induce appropriate polarity of each cell type (Figure 2). Nevertheless, polarity and phenotype are not always fixed; at specific stages of embryonic development and in some cancers, epithelial cells can undergo an epithelial-to-mesenchymal transition involving the loss of cell-cell adhesions and polarity, accompanied by activation of cell migration (Hay, 2005; Thiery and Sleeman, 2006).

3D Models in Developmental Biology

Morphogenesis—the development of form in the embryo—has recently been analyzed extensively in a variety of 3D model systems, particularly organ cultures and cell line models. A major goal in embryology has been to understand the mechanisms and regulation of branching morphogenesis (Figure 3), a process essential for the formation of glands and organs, including lungs, kidneys, salivary and mammary glands, prostate, and the vasculature (Affolter et al., 2003; Monte et al., 2007; Patel et al., 2006; Sternlicht et al., 2006). This complex process involves a daunting number of transcription factors, growth factors, and receptors (Monte et al., 2007; Patel et al., 2006).

In order to understand the key steps and physical mechanisms of branching morphogenesis, organ cultures of early glands have provided invaluable 3D models. Isolated epithelium stripped of its surrounding mesenchymal tissue can continue to undergo branching if provided with appropriate growth factors and extracellular matrix support. In fact, embryonic lung and salivary gland epithelia can be dissociated into single cells, which can reaggregate and regenerate branched organoids in an appropriate 3D microenvironment. Established epithelial cell lines such as Madin-Darby canine kidney (MDCK) cells have also been used (Mondrinos et al., 2006; Wei et al., 2007).

The steps in morphogenesis can then be analyzed in depth using tissue culture approaches that include antibody inhibition, regulation by exogenous proteins (e.g., growth factors and matrix proteins), cDNA overexpression, and gene knockdown by RNA interference. Recent findings that could not have been established without 3D models of embryonic acinus formation and branching morphogenesis include the importance of Cdc42,

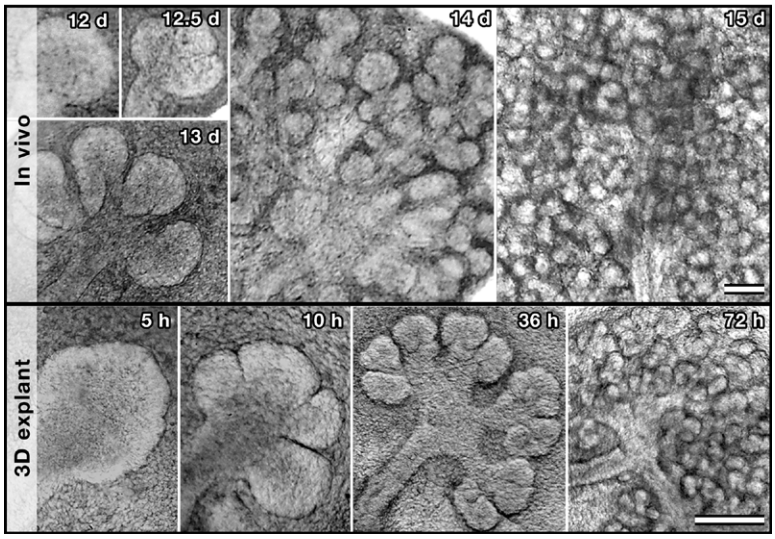

Figure 3. Branching Morphogenesis of Epithelial Organs

Branching of epithelial organs such as these salivary glands is essential for rapidly generating a large functional surface area, as shown here between embryonic days 12 and 15 of mouse development (upper panels). Branching morphogenesis of these and other glands can be studied readily in 3D organ culture, as shown in the lower panels spanning 72 hr in vitro (modified from Sakai et al., 2003). All images in the upper or lower panels are at the same magnification; scale bars = 100 μm.

PTEN, and differential phosphoinositide localization for determining apical-basal polarity of epithelial cells (Tables 2 and S2; Martin-Belmonte et al., 2007). Other intriguing findings coming from the use of 3D organ culture models include identification of mechanisms of growth factor signaling, the observation of vigorous random cell migration of epithelial cells during branching morphogenesis, and a previously unsuspected role for the matrix protein fibronectin in driving epithelial branching (Costantini, 2006; Larsen et al., 2006b; Monte et al., 2007; Patel et al., 2006; Sakai et al., 2003).

3D models are also making important contributions to our growing appreciation of mechanobiology, including the responses of bone, blood vessels, and connective tissue to mechanical forces. Numerous studies have analyzed the bidirectional mechanosensory responses of fibroblasts or myofibroblasts to collagen gels (Cukierman et al., 2002; Discher et al., 2005; Ghosh et al., 2007; Grinnell, 2003; Orr et al., 2006). For example, cellular signaling differs depending on whether the gel is under tension or free floating and compliant (Grinnell, 2003). Conversely, extracellular matrices are actively remodeled by fibroblastic cells by synthesis, degradation, and contraction, thereby altering their mechanical effects on cells (Grinnell, 2003; Larsen et al., 2006a). Experimental manipulation of cell interactions with 3D matrix models should provide new opportunities for evaluating the mechanisms of mechanochemical signaling, including the study of the interactions of integrin binding to the matrix and the regulation of the cytoskeleton, kinases, and stretch-sensitive ion channels (Griffith and Swartz, 2006; Orr et al., 2006).

Stem Cells in 3D

Determining the factors that regulate stem cell fate will provide tools for controlling their differentiation in potential clinical applications. In addition to responding to growth factors, retinoic acid, and other soluble regulators (Chen et al., 2003; Griffith and Swartz, 2006), stem cells can be regulated by three-dimensionality. Cultured stem cells self-assemble into embryoid bodies that mimic the inner cell mass of embryos. These 3D embryoid bodies have been used for numerous studies, providing insights into mechanisms underlying cell polarity (Yang et al., 2007) and revealing the roles of laminin and integrins in the formation of the basement membrane (Li et al., 2002).

Recent efforts have sought to identify optimal 3D matrices for stem cell maintenance or differentiation (Table S1). For example, in 3D collagen matrices embryonic stem cells can differentiate into epithelial and other lineages; coculture of embryonic stem cells with fibroblasts promotes a neural lineage, whereas coculture with keratinocytes promotes endothelial differentiation (Chen et al., 2003). Conversely, forcing human embryonic stem cells to grow as 2D cultures instead of embryoid bodies stimulates their subsequent differentiation, in one case into blood vessels (Wang et al., 2007).

As with other cells, stem cell fate and differentiation can also be altered by varying the stiffness of the substrate. The ability of stem cells to undergo neuronal, myoblastic, or osteogenic differentiation is strikingly altered by substrate stiffness (Engler et al., 2004, 2006), providing a potentially powerful physical means of altering stem cell fate for potential clinical application.

3D Models of Cancer Growth and Metastasis

The mechanisms of aberrant proliferation and invasion in cancer have been analyzed fruitfully in 3D model systems (Bissell and Labarge, 2005; Debnath and Brugge, 2005; Kim, 2005). As discussed below, systems have ranged from single cells to tumor spheroids and acinar models probed by genetic, chemical, and immunological approaches.

Tumor Proteases and Invasion

At the single-cell level, local cleavage of the surrounding matrix by transmembrane proteases of the membrane type matrix metalloproteinases (MT-MMP) family is required for cell proliferation in model tumors and integrin-mediated invasion into collagen gels (Hotary et al., 2006). Proteolysis of 3D crosslinked collagen, which surrounds

and imprisons the cells, facilitates their release. Similarly, MT1-MMP allows the expansion of normal endothelial cells to form new blood vessels and promotes the differentiation of preadipocytes into white adipocyte tissue (Chun et al., 2006). However, in seemingly conflicting findings, experimental blockade of all extracellular proteases, including MMPs, was reported to have no effect on cell invasion in another 3D collagen gel system; instead, tumor cells switched to an ameboid type of migration analogous to that used by T cells, which does not require integrins and uses different Rho-ROCK signaling (Friedl, 2004; Wyckoff et al., 2006). These conflicting conclusions concerning requirements for proteases in tumor cell invasion may be explained by the different 3D collagen gels used for assays, such as different extent of crosslinking or pore size. This discrepancy is important to resolve concerning potential therapeutic targeting of specific proteases.

Spheroid Models

In vitro aggregates of embryonic cells have been studied for decades to understand the principles of morphogenesis and tissue formation. These aggregates, called "spheroids," often consist of stem cells or tumor cells from malignant cell lines or fragments of human tumors (Kunz-Schughart et al., 2004; Mueller-Klieser, 1997; Wartenberg et al., 2001). Spheroids can be studied in suspension in hanging drops of medium, in bioreactors, or in 3D matrices, and they can be established from a single cell type or can be multicellular mixtures of tumor, stromal, and immune cells. These aggregates can mimic tumor behavior more effectively than regular 2D cell cultures because spheroids, much like tumors, usually contain both surface-exposed and deeply buried cells, proliferating and nonproliferating cells, and well-oxygenated and hypoxic cells (the latter secreting tumor cell cytokines; Frieboes et al., 2006). Homogeneous populations of spheroids may prove valuable for high-throughput drug screening (Kunz-Schughart et al., 2004).

Spheroids can also be used to study the adhesive properties of tumor cells (Winters et al., 2005), and their behavior can be analyzed by computational modeling (Frieboes et al., 2006). Interactions between different cell types can also be analyzed in spheroids; for example, tumor-induced angiogenic responses can be modeled using cultures in which tumor spheroids interact with vascular cells generated from embryonic stem cells (Wartenberg et al., 2001).

Tissue Architecture and Signaling in Cancer

In addition to oncogenic mutations, common features of neoplasia include aberrant tissue organization and signal transduction. Recent studies using 3D in vitro models have shown that the interdependent balance of activities of integrins, growth factor receptors, and intracellular signaling distinguishes malignant from normal tissue architecture. Human 3D in vitro epithelial models of mammary acini can mimic the increasingly abnormal tissue organization characteristic of breast carcinoma progression, where tumor cells suppress normal apoptotic mechanisms to invade the lumen (Debnath and Brugge, 2005; Wang et al., 1998). Polarity also plays a role because oncogene-induced disruption of acinar architecture by ErbB2 requires interaction with the Par polarity complex components Par6 and aPKC (atypical protein kinase C). This interaction between oncogene and polarity signaling induces acinar disruption while suppressing apoptosis independently from oncogene stimulation of proliferation (Aranda et al., 2006).

Remarkably, individually inhibiting the function of β_1 integrin, EGF receptor, MAP kinase, PI 3-kinase, or Par6 can restore acinar architecture to a state closer to normal, even though genomic abnormalities persist (Aranda et al., 2006; Liu et al., 2004; Wang et al., 1998). These findings reveal an interwoven system regulating tissue organization. Conversely, protease disruption of extracellular matrix organization can sometimes lead to carcinogenesis via a pathway involving the GTPase Rac1b and reactive oxygen species (ROS) leading to genomic instability (Radisky et al., 2005). These studies show crucial roles for cell-matrix interactions and tissue architecture in cancer. In addition, analyzing patterns of gene expression in 3D in vitro mammary acini may be useful for predicting breast cancer outcome (Bissell and Labarge, 2005; Fournier et al., 2006).

Microenvironment and Stroma

Tumorigenesis is akin to a developmental process in which the interaction between pluripotent stem cells within the tumor and the local microenvironment determines the success or suppression of tumorigenesis (Beacham and Cukierman, 2005; Bissell and Labarge, 2005; Mintz and Illmensee, 1975). Gene expression is selectively altered in the tumor microenvironment (Allinen et al., 2004), and specific stromal factors can promote tumor growth and angiogenesis (Bissell and Labarge, 2005; Orimo et al., 2005). In fact, secondary niches that are preconditioned by changes that make them similar to tumor stroma can serve as sites for homing of metastasizing cells. For example, myofibroblasts can deposit a fibronectin-rich matrix to create a favorable environment for incoming tumor cells (Kaplan et al., 2005). The tumor microenvironment can mimic aspects of wound healing, and tumor factors induce a "primed" stroma that can in turn promote tumor initiation or progression. Further stromal alteration via immune responses and other processes results in a characteristic "activated" stroma with high rates of proliferation and abnormal production of matrix and matrix proteases (Beacham and Cukierman, 2005; Birgersdotter et al., 2005). In 3D models, tumor cells respond to this matrix with Rho-dependent cytoskeletal contractility that promotes focal adhesions, hinders epithelial polarity and lumen formation, alters differentiation, and promotes growth via ERK, Rho, and Rho kinase signaling (Paszek et al., 2005; Wozniak et al., 2003).

Future Applications

We emphasize that in vitro 3D tissue models are experimental tools. Although some 3D models make use of intact tissues, embryonic organs, or reconstituted mimics of living tissues, they are not intact animals and for that reason do not reproduce all of the normal microenvironmental inputs depicted in Figure 4. For example, transport limitations of oxygen, other nutrients and pH can become

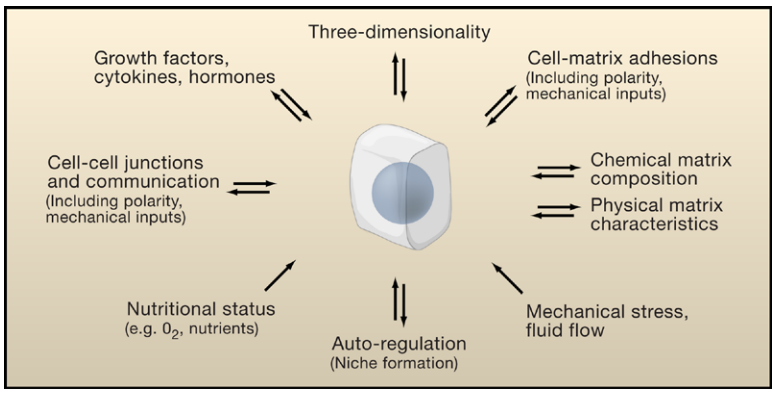

Figure 4. Microenvironmental Factors Affecting Cell Behavior

Numerous aspects of the microenvironment that change spatially and temporally may affect how accurately a 3D model reflects cellular behavior in vivo. Conversely, cells (center) can actively modify their local microenvironment.

important in these avascular models. As such, 3D systems must be considered models, and conclusions from their use may require further testing in vivo. Nevertheless, they provide potentially powerful tools for new applications represented by the following examples.

Prediction of In Vivo Outcomes

3D models provide systems for in-depth analyses of biological mechanisms. In addition to direct analysis and testing of hypotheses, they can provide an efficient way to probe the function of candidate genes and proteins before proceeding to laborious gene targeting approaches in animals. For example, projects involving analysis of gene expression commonly identify hundreds of candidate genes that might mediate a particular cellular process. 3D models can allow investigators to screen their functions in vitro to determine which genes are most interesting for further in vivo study.

In addition, 3D models based on human cells can potentially provide new systems for high-throughput chemical genomics and therapeutics screening (Khademhosseini et al., 2006; Kunz-Schughart et al., 2004; Langer and Tirrell, 2004). Because rodents or other animals can metabolize and respond to drugs differently than humans (Rangarajan et al., 2004), using human 3D tissue surrogates for "in vivo-like" drug testing might help reduce the high failure rate in the development of new drugs. It might also be possible to use 3D models for individualized therapy—that is, to predict the responsiveness of a particular patient's tumor to chemotherapy or radiotherapy. Human 3D tissue models can also provide systems for analyzing the pathogenesis of diseases, including cancer and various genetic disorders. Although iterative in vivo validation of hypotheses remains the "gold standard," 3D models can accelerate the process by quickly identifying the most promising targets and establishing mechanisms.

Multiparameter Quantitative Systems Analyses

Because 3D models are in vitro, experimenters can control and vary each of any number of parameters to generate quantitative data for systems analysis. For example, model tissues can be assayed for changes in gene and protein expression after systematically altering levels of growth or differentiation factors and/or nutrients in any combination and temporal sequence. Although many such studies have been performed on cells grown as monolayers, 3D models are likely to provide data with greater physiological relevance for mathematical models of growth, signaling, migration/invasion, and morphogenesis (Hicks et al., 2006; Nelson and Bissell, 2006; Zaman et al., 2006). They can also provide a bridge between cell culture and in vivo modeling of developmental pattern formation (Khademhosseini et al., 2006; Langer and Tirrell, 2004; Reeves et al., 2006). In addition to testing roles of diffusible regulatory factors, it should be possible to measure quantitatively the effects of changing the 3D matrix scaffold, altering cell adhesions, or introducing additional cell types such as immune cells. Such studies tracking multiple parameters under many conditions are impractical in vivo, and they can permit development of sophisticated models in quantitative biology.

Determining the Role of Stroma in Carcinogenesis

Now that it is clear that the stroma can play important roles in supporting or even inducing tumorigenesis, the specific roles of stroma in tumor development, progression, local invasion, and homing to specific secondary sites need elucidation. 3D systems should help dissect these processes in the tumor microenvironment that interact with, and are modified by, cancer stem cells. Although the primary focus of chemotherapeutic drug development has been on the tumor cells, new insight into the importance of the stroma in tumor progression argues for analyses in systems containing both tumor and tumor stroma. Interactions with the stroma may influence drug sensitivity and development of drug resistance, which can be tested in 3D models.

Tissue Engineering and Stem Cells

The rapidly expanding field of bioengineering of 3D tissues will be a particularly active interface between in vitro studies and the clinic (Griffith and Swartz, 2006; Langer and Tirrell, 2004; Lutolf and Hubbell, 2005). A major strategy involves developing artificial mimetics of matrix molecules, which could ideally provide structural support, appropriate integrin signals, and growth factor-binding functions of native extracellular matrix (Matsumoto and Mooney, 2006). An impressive range of 3D biomaterials, including synthetic polymers that self-assemble, are now available (Table S1). The ongoing empirical testing of these new scaffolds (both synthetic and containing reconstituted matrix molecules) for induction and maintenance of specific tissue phenotypes represents one area of rapid progress.

Because stem cell fate can be regulated by matrix stiffness (Engler et al., 2006), the manipulation of matrix stiffness and crosslinking to alter human stem cell dif-

ferentiation pathways is another area poised for future advances. These efforts may lead to additional insight into the factors that promote normal differentiation or into the processes by which stroma stimulate self-renewing cancer stem cells. A useful advance would be new methods to control stiffness without changing matrix density, i.e., without changing the concentration of sites bound by integrin adhesion receptors as occurs currently when the concentration of ligands (such as collagen) is increased to increase stiffness. Although stiffness can be controlled by crosslinking polyacrylamide gels (Pelham and Wang, 1997), alternative methods using biocompatible materials and natural reagents such as transglutaminase or chemical crosslinkers are needed to produce nontoxic implantable gels. However, because the range of changes in biomaterial stiffness is limited compared to the many possible stem cell phenotypes, this parameter will provide only one of multiple inputs to the cells (Figure 4). Nevertheless, advances in precisely controlling matrix stiffness will also advance fundamental research on mechanisms of matrix-mediated mechanotransduction and cytoskeletal regulation.

Organization into Tissues

Current research is focusing intensely on controlling stem cell differentiation, but another major challenge involves integrating the cells into functional tissue structures. One approach could be to intercalate stem cells into damaged tissue, but the local environment may not sustain normal biological function if it is pathological (for example, if it is fibrotic; Engler et al., 2004). A major challenge will be to establish the appropriate topological interactions and spatial organization of cells into acini of glands, islets, ducts, or other morphological patterns (Khademhosseini et al., 2006; Langer and Tirrell, 2004). An additional important in vivo feature that can be modeled in vitro is the effect of a second or third cell type on developmental fate and morphogenesis through cell-cell and cell-matrix interactions. One example of this is the role that extracellular matrix deposited by fibroblasts has in facilitating angiogenesis (Berthod et al., 2006).

Although a goal of tissue engineering is to develop well-defined biomaterials and cells for replacing defective tissues, an important potentially confounding element involves the secretory responses of cells to these materials and their microenvironment. Cells continually secrete complex mixtures of extracellular matrix proteins and other regulators of cell behavior, which may affect what happens when exogenous cells or materials are implanted. For example, embryonic stem cells may generate their own microenvironmental niches when cultured in collagen or matrices from extracted tissue (Postovit et al., 2006b). Cellular responses to local mechanical stresses and hypoxia need to be incorporated into existing 3D models to make them more realistic. 3D models also need to take dynamics within tissues into account, such as interstitial fluid flow and local chemotactic gradients (Griffith and Swartz, 2006). An important general function of the extracellular matrix is to serve as a reservoir for growth factors and cytokines. Matrix engineering can provide site-specific delivery of growth factors (Lutolf and Hubbell, 2005; Matsumoto and Mooney, 2006), though a challenge will be to ensure long-term function in vivo of these implanted materials.

Another focus of research will be on ways to manipulate cells prior to implantation, through partially differentiating or genetically engineering cells in 2D or 3D settings, especially for stem cells. Another major challenge involves linking vasculature to engineered tissues; further studies of angiogenesis in 3D models may provide approaches to this difficult problem (Jakobsson et al., 2007; Laschke et al., 2006).

Conclusions

The goals of researchers attempting to engineer functional tissues for implantation in patients, clinicians interested in developing human tissue models for testing drugs, and investigators probing the basic mechanisms of morphogenesis, differentiation, and cancer frequently intersect at 3D in vitro models. These models provide a bridge between simple cell cultures and animals, though many parameters need to be considered (Figure 4). Studies with 3D model systems have repeatedly identified complex interacting roles of matrix stiffness and composition, integrins, growth factor receptors, and signaling in development and cancer. These insights suggest that plasticity, regulation, and suppression of these processes can provide strategies and therapeutic targets for future cancer therapy and stem cell engineering (Bissell and Labarge, 2005; Postovit et al., 2006a). Besides providing model systems for testing ideas and potential therapeutic interventions, they may also permit high-throughput drug screening on human tissues in vitro. Insights from 3D in vitro systems into the mechanisms and regulation of morphogenesis, stem cell differentiation, and cancer should continue to crossfertilize these fields.

Supplemental Data

Supplemental Data include two tables and References and can be found with this article online at http://www.cell.com/cgi/content/full/130/4/601/DC1/.

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