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Engineered Culture Models for Studies of Tumor-Microenvironment Interactions

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Abstract

Heterogeneous microenvironmental conditions play critical roles in cancer pathogenesis and therapy resistance and arise from changes in tissue dimensionality, cell-extracellular matrix (ECM) interactions, soluble factor signaling, oxygen as well as metabolic gradients, and exogeneous biomechanical cues. Traditional cell culture approaches are restricted in their ability to mimic this complexity with physiological relevance, offering only partial explanation as to why novel therapeutic compounds are frequently efficacious in vitro but disappoint in preclinical and clinical studies. In an effort to overcome these limitations, physical sciences–based strategies have been employed to model specific aspects of the cancer microenvironment. Although these strategies offer promise to reveal the contributions of microenvironmental parameters on tumor initiation, progression, and therapy resistance, they, too, frequently suffer from limitations. This review highlights physicochemical and biological key features of the tumor microenvironment, critically discusses advantages and limitations of current engineering strategies, and provides a perspective on future opportunities for engineered tumor models.

Keywords

tissue engineering; cancer; 3D culture; biomaterials; drug delivery; microfabrication

1. INTRODUCTION

Over the past few decades, our understanding of cancer has shifted dramatically with the recurrent discovery that aberrant cell-microenvironment interactions are as pivotal to tumorigenicity as oncogenic mutations are. In 1889, Stephen Paget's (1) seed-and-soil hypothesis already predicted the importance of a fertile ground for the maturation of disseminated cancer. Nevertheless, research into the influence of the tissue microenvironment on tumorigenicity gained significant momentum only in the early 1980s following two independent studies that showed that embryonic environments can prevent tumor formation by malignantly transformed cells (2, 3). From these, a new field of

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research has emerged that focuses on identifying the molecular-, cellular-, and tissue-level mechanisms by which the microenvironment modulates cancer initiation, progression, and therapy response.

Cell behaviors within tissues are largely predicated upon three-dimensional (3D) interactions with extracellular matrix (ECM) and other cells as well as with various mechanical stimuli, and it is clear that traditional two-dimensional (2D) culture approaches are insufficient mimics of these conditions. For example, merely switching culture dimensionality from 2D to 3D radically affects protein expression (4, 5), cell proliferation (6), differentiation (7), and metabolism (8), which may partially explain commonplace discrepancies between benchtop and clinical efficacy of new therapies (9). Alternatively, animal studies inherently provide tissue context yet are limited in their ability to resolve independent aspects of the human tissue microenvironment that contribute to disease progression and metastasis. Xenograft models that transplant human cancer cells into mice, in particular, not only introduce species-dependent discrepancies in cell signaling (10) but also are restricted to immunocompromised animals, necessarily excluding the participation of the immune response critical to cancer progression (11).

The application of tissue engineering, drug delivery, and microfabrication offers promise to overcome some of the aforementioned limitations of conventional in vitro and in vivo models. However, despite significant progress over the past decade, current approaches need further refinement to better represent the heterogeneity and complexity of tumor microenvironmental conditions, while allowing dependable high-throughput analysis. This review presents an overview of bioinspired physical sciences strategies suitable to address the inherent challenges of modeling and studying tumor-microenvironment interactions. We first define biological and physicochemical constituents of the normal and the tumor microenvironments and next describe engineering approaches to mimic these phenomena in a physiologically relevant manner. We also highlight the strengths and limitations of such models and present a future outlook for the field of tumor engineering.

2. BIOLOGICAL AND PHYSICOCHEMICAL CHARACTERISTICS OF THE TUMOR MICROENVIRONMENT

Carcinomas represent the most common type of cancer and are fundamentally a disease of unmitigated epithelial tissue growth (12). The typical architecture of epithelial tissues is defined by tightly interacting cells situated on top of a basement membrane—for example, a sheet of ECM rich in collagen type IV and laminin that tethers the epithelium to the underlying, or in the case of glands, surrounding, connective tissue (also termed stroma) (Figure 1a) (13). Although malignantly transformed epithelia are common in adults, most of these never manifest disease, as normal tissue architecture and homeostasis ensure their dormancy. Aberrant microenvironmental conditions may shift this balance, however, and not only promote unrestrained cell proliferation (14) but also facilitate tumor initiation (15) and direct metastasis (16). Historically, cancer biologists have defined the microenvironment in biological terms; however, emerging evidence indicates that physicochemical variations must be considered. In particular, changes in tissue dimensionality, altered ECM mechanical

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properties, gradients of oxygen and morphogens, and exogenous physical stimuli (e.g., shear stress, compression) may all participate in disease initiation and progression (Figure 1).

Altered tissue dimensionality, ECM composition, soluble factor signaling, and mechanical properties are instrumental to primary tumor development and progression. In benign neoplasms, excessive epithelial cell proliferation undermines tissue polarity and provides dimensionality as a 3D mass with an intact basement membrane (Figure 1a). Upon further malignant transformation, disruption of the basement membrane concurrent with altered expression of cell-cell adhesion receptors enables the migratory and invasive phenotype characteristic of advanced cancers. This epithelial-to-mesenchymal transition (EMT) involves downregulation of epithelial cell adhesion receptors (E-cadherins) and concomitant upregulation of mesenchymal markers (e.g., N-cadherins, vimentin) by tumor cells, thereby facilitating cell-cell detachment and permitting new association of the tumor cells with their neighboring stroma (17). Additionally, tumor cells drive differentiation of fibroblasts (18) or mesenchymal stem cells (13, 19) into highly contractile and matrix-depositing myofibroblasts, thereby promoting enhanced ECM assembly, alignment, and unfolding, and cross-linking of collagen type I– and fibronectin-rich matrices (20, 21). The resultant stiffness alters the stress and strain fields throughout the ECM, and deformational changes therefore occur in adjacent cells, regulating behavior such as cell adhesion and locomotion (22). Although 2D studies suggest that these changes can stimulate tumor cell proliferation and further activation of stromal cells, recent evidence from 3D experiments suggests that cells respond differentially to matrix stiffness in 2D and 3D, owing in part to variations in cell confinement (23). As such, future studies should embrace intelligent 3D model systems that permit independent variation of ECM stiffness, porosity, and molecular conformation to resolve their individual and integrated roles in disease progression.

Coupled with excessive cell proliferation, decreased oxygen availability, and increased interstitial acidosis, changes in ECM properties dynamically affect the spatiotemporal distribution of critical signaling molecules. By extending past distances at which effective diffusion can occur, nascent tumor cell proliferation rapidly depletes oxygen supplied by the local vasculature. Hence, regions of reduced oxygen tension (termed hypoxia) develop and activate hypoxia-inducible transcription factors (e.g., HIF) that regulate expression of many protumorigenic morphogens including proangiogenic factors [e.g., vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)] and chemotactic factors (e.g., CXCL12) (24, 25). The sum of these effects compromises endothelial barrier functions and leads to elevated interstitial fluid pressure (IFP) (26). Elevated IFP, in turn, generates flow-induced fluid shear and transcellular gradients of growth factors and cytokines (27), altogether stimulating further cell proliferation (28, 29). Although 2D studies have shown that parameters such as gradient profile and steepness may independently regulate cell behavior, appropriate 3D culture models for studies of gradient-related variability in signaling are still missing (30). The implications of this varied transport on tumor progression—or conversely, drug bioavailability—may be profound but, to date, are underappreciated. Additionally, exposure to hypoxia may permanently shunt cellular metabolism (Warburg effect) (31), leading to glycolysis-driven acidification of the tumor interstitium (32, 33), which may, in turn, affect morphogen presentation to cells by regulating their transcription, stability (34, 35), and matrix-binding characteristics (36).

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Nevertheless, no studies currently exist that evaluate the combined effects of hypoxia and acidosis on growth factor expression and/or delivery and the resulting effects on cell behavior.

Following invasion, tumor cells travel to distant sites, which requires adaptation to additional physicochemical constraints. To metastasize, tumor cells detach from the primary context and, via mesenchymal or amoeboid locomotion, travel toward adjacent blood vessels, which are established through hypoxia-driven angiogenesis (37). Once intravasated into the vasculature, tumor cells travel as single cells or aggregates throughout the body (i.e., circulating tumor cells, or CTCs) and are subjected to a loss of substrate adhesion and increased shear stress imposed by fluid flow (38). Pending survival in the systemic circulation, some CTCs lodge in capillary beds of secondary (metastatic) sites, via adhesion to endothelial cells or exposed basement membrane proteins, and exit the vessel (i.e., extravasate) (39). The physicochemical properties of these distant sites lead to the death of most cells; however, some cells can acclimate to this new environment, escape dormancy, and initiate secondary tumors. Metastasis to bone and lungs (i.e., frequent sites of metastasis) is extremely inefficient, but whether or not this is due to differential ECM characteristics and exogenous physical stimuli relative to the primary site is unclear. For instance, within bone, tumor cells are exposed to inorganic components and substantially increased ECM stiffness (due to the presence of hydroxyapatite) as well as to mechanical loads generated during physical activity. These parameters are widely explored for bone regeneration applications, but only initial steps have been undertaken to assess their importance for metastasis (40-42). Furthermore, within lungs, low matrix stiffness may inhibit metastasis, which favors a cross-linked and, therefore, more rigid ECM (43). Nevertheless, the role of respiratory mechanics on the establishment of lung metastasis remains largely elusive. Adapting technologies conventionally applied to regenerative approaches may thus provide access to multivariate analysis of tumor metastasis as a function of bone and lung physicochemical parameters.

3. ENGINEERING TOOLS TO MIMIC TUMOR-MICROENVIRONMENT INTERACTIONS

3.1. Tissue Dimensionality

As tumors develop, a shift in tissue dimensionality with functional consequences on juxtracrine signaling due to altered cell-cell contact significantly contributes to disease progression and warrants consideration in the design of engineered culture models. In fact, this dimensional change influences tumor growth (44-46), migration (47), signaling (48), and drug response (49), independent of other phenotypic changes. However, culture models used to dissect the individual contributions of varied juxtacrine signaling and tissue dimensionality in a relevant context are still limited.

Advances in microscale culture technologies may be invaluable for deciphering the consequences of direct cell-cell contact on cancer progression. In the simplest permutation, polydimethylsiloxane (PDMS)-based microcontact printing of ECM proteins such as fibronectin or laminin onto otherwise nonadhesive substrates has been used. This approach

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regulates interactions between defined cell numbers by merely varying pattern size (50). Despite its straightforward use and ready applicability by nonengineers, the extension of microcontact printing to studies of cancer is still in its infancy. This may be related to technical limitations of patterning large, uniform cell arrays—that is, at the scale necessary for comprehensive analysis of the proteomic or transcriptional changes typically applied by cancer biologists. Such scaling is challenged by sagging and swelling of PDMS and frequently involves desiccation of the protein-inked PDMS stamps, which compromises protein bioactivity. Parylene template–based patterning can overcome these limitations by allowing uniform and reproducible patterning of adhesion islands (and hence cell clusters) over large surface areas under aqueous conditions (51). However, a common limitation of both PDMS and parylene patterning is that individually patterned cells may exhibit a different morphology than cells patterned in clusters, which confounds the interpretations of results, for example, by varying signaling in response to soluble factors (52). In this case, utilization of more complex patterning approaches that permit controlling cell-cell contact without affecting cell morphology is indicated (53).

Another consideration when developing model systems of juxtacrine signaling is that changes in cell-cell interactions are not isolated and are directly tethered to other microenvironmental conditions. Specifically, tissue spreading is controlled via the coordinated interplay between cell-cell and cell-ECM interactions. If cell-cell cohesivity is greater than cell-substrate adhesivity, 3D cell aggregate formation is favored and tissue spreading inhibited, whereas the opposite holds true when cell substrate adhesivity is enhanced (54). These observations have important implications for tumors, as decreased cell-cell cohesivity due to EMT is directly linked to tumor invasion (55). However, the switch in tumor dimensionality may not be fully explained with this model: Studies with endothelial cells suggest that tumor-mediated changes in ECM stiffness, which can promote cell-cell adhesion through the generation of greater contractile forces, may also be involved (56). Patterning elastomeric microneedles (56) or inverse approaches in which the protein of interest is first patterned on a glass cover slip and then transferred to an otherwise inert hydrogel of varying stiffness [e.g., polyacrylamide (PA)] may be used to study the underlying mechanisms (57).

The approaches discussed thus far have the potential to provide valuable new insights into the effect of juxtacrine signaling on cell behavior, yet they lack mimicry of 3D tissue context (i.e., the ultimate outcome of varied cell-cell interactions). Although a variety of innovative 3D patterning techniques have already been developed, most have not been extended to cancer study. For example, 3D cell patterning may be accomplished via dielectrophoretic (DEP) forces, whereby cells exposed to an electric field become polarized and migrate toward specific locations dictated by a micropatterned electrical insulator placed between two conducting glass slides (Figure 2a) (58). This approach affords quantitative control of 3D microorganization by adjusting initial seeding density, and stabilization of the respective multicellular conformations can be achieved via subsequent gelation of the matrix (e.g., via photopolymerization). An inherent advantage of this approach is that multilayered coculture models can be fabricated, thereby permitting studies of the effects of stromal cells on 3D cell-cell interactions of tumor cells, for instance. However, potential artifacts, including altered DNA damage responses and hence increased mutation rates, arising

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from cell exposure to the fabrication process (i.e., electrical fields, UV irradiation for photopolymerization) need to be considered (59-61).

A critical shortcoming of the models discussed thus far is their inability to resolve cellular events mediated solely by cell-cell contact from parallel signaling mechanisms. Cellular responses to juxtacrine versus soluble stimuli may be distinguished micromechanically through the use of silicon culture substrates composed of interchangeable parts. These parts can be introduced or removed throughout the experiment, which allows exquisite control over cellular composition and organization and duration of juxtacrine interactions (62). However, this approach again lacks 3D tissue context, introduces nonphysiologic substrate rigidity, and manipulates cell-cell contact only in a select cell population. Tethering of intercellular adhesion proteins (e.g., cadherins) onto the surface of 3D hydrogel-incorporated microwells of controlled cell-scale dimensions can overcome these challenges and isolate the effect of cell-cell interactions from other confounding variables (63). Combined with varied hydrogel rigidity, this approach permits correlation of cadherin-mediated cell-cell contact with cell stiffness that decreases with malignancy (64). Regardless, the effect of cadherin lateral mobility that may occur concomitantly (e.g., owing to changes in membrane fluidity) is still overlooked. Modifying PDMS microwells with a supported phospholipid bilayer to which soluble E-cadherin extracellular domains can be coupled would be beneficial here (65). Interestingly, observations utilizing this system indicate that enhanced cadherin lateral mobility inhibits actin stress fiber formation (65). These findings are provocative because they suggest that increased cancer cell compliance modulates tissue invasion not only through cellular migration mode (66) but also by decreasing adherens junction–dependent cell-cell contact. Despite the innovation of these culture models, their interpretation is again confounded by nonphysiological microwell shape and hence cell morphology; adjusting microwell geometry may address this shortcoming (67).

Clearly, a variety of as-yet-unidentified parameters can be evaluated with the aforementioned 3D culture approaches. However, it is critical to retain focus on how dimensionality is defined in these studies and whether or not 3D culture is warranted for a particular cellular context. For example, from a molecular perspective, matrices deposited by cells in conventional tissue culture are routinely described as 3D networks; however, at the cellular level, global 2D morphology is assumed when cells are seeded on top of these scaffolds (68, 69). This may direct cell behavior, a conclusion supported by migration studies performed in 2D, 2.5D, and 3D culture systems with constant chemistry (47). Similarly, tumor cells may actually encounter pseudo2D surfaces during metastatic dissemination. For instance, cellular interactions with the blood vessel wall (i.e., prior to extravasation) or the surface of trabecular bone (i.e., during bone metastasis) may inherently be 2D in nature, although the unique topography of these sites could participate critically (70). Finally, interpretation of results obtained with microfabricated 3D culture systems must be approached cautiously, as common methodologies to regulate the physicochemical properties of these models can introduce other parameters including cellular confinement and steric hindrance. These parameters may independently modulate cell behavior by, for example, decoupling cellular stiffness responses or impacting the diffusion of secreted signaling molecules, respectively (23, 71).

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3.2. Biomaterial Mimics of the Extracellular Matrix

3.2.1. Natural materials.—Historically, the roles of matrix composition, structure, and mechanical properties in cancer pathogenesis have been studied primarily with natural polymers. In particular, Matrigel®, a basement membrane preparation derived from murine sarcoma, and ECM components from animal tissues (e.g., collagen I, laminin, fibronectin) were embraced owing to their inherent cytocompatibility, presentation of cell adhesion sites, and ability to tailor matrix porosity, fiber structure, and stiffness via gelling conditions (e.g., temperature, concentration, gel thickness, pH, and media composition) (72, 73). Undoubtedly, these materials have provided important insights into tumorigenesis (74); however, their inherent batch-to-batch variability and complex molecular composition complicate study reproducibility and hence the mechanistic conclusions that can be drawn from them. Furthermore, the range of physical variability with these substrates is relatively narrow. For example, the elastic modulus of reconstituted Matrigel is approximately 170 Pa and therefore suitable to mimic the mechanical properties of normal tissues; adjusting collagen concentration may yield matrices of a few kPa that permit recapitulation of the mechanical properties of certain tumors (75). However, the ECM rigidity that tumor cells encounter following metastasis to bone extends orders of magnitude beyond the stiffness limitations of natural hydrogels, despite the clear importance of this parameter in guiding the progression of metastatic lesions (76). Another important caveat is that the mechanical strength of natural polymers is frequently adjusted by varying protein concentration of the hydrogel (77), which simultaneously alters adhesion peptide density and thus cell behavior, independent of altered rigidity (78). Finally, another often overlooked consideration when using natural polymers is the integrity of their physicochemical properties relative to those of in vivo counterparts. For example, derivation of Matrigel destroys the covalent cross-links between collagen type IV molecules that are implicit to the barrier properties of the natural basement membrane (79); hence, studies of epithelial migration through soluble Matrigel may only partially recapitulate the cellular and molecular mechanisms underlying tumor progression and invasion. In the case of collagen, fiber formation—a critical determinant of cell migration (80)—differs between pepsin- and acid-solubilized collagen in that fibers formed from acid-soluble collagen are structurally faithful to collagen in vivo (81) but contain telopeptides that may compromise biocompatibility (82). As different isolation techniques are employed, studies using collagen matrices should be interpreted in this specific context.

3.2.2. Semisynthetic materials.—To overcome limitations of natural biomaterials while taking advantage of their inherent cell affinity, increasing effort has been directed to developing semisynthetic hydrogels. This approach entails selective variation of matrix mechanical properties, degradation, and perhaps growth factor binding sites via synthetic side chains (83, 84), yet it maintains the backbone and hence physiological nature of the natural polymer. Examples include modified collagen and hyaluronic acid, which are particularly relevant because both components are increased in cancerous tissues and correlate with malignancy (20, 85). Specifically, glycation strategies have been developed to modulate the mechanical stiffness of collagen independent of adhesion peptide density (86, 87) and, in doing so, have demonstrated tumor stiffness–mediated changes in tumor (20) and stromal cell behavior (88, 89). It warrants mention that glycation may simultaneously

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alter matrix hydrophilicity, which, in turn, could influence subsequent matrix remodeling by changing the deposition and degradation of other ECM components. In particular, increased hydrophilicity elevates matrix metalloproteinase (MMP) expression and functional responses and causes weak fibronectin binding and consequential deposition of larger fibrils, relative to hydrophobic substrates (90). This is significant in the cancer context, as fibronectin fibril characteristics in tumors are distinct from those in normal tissue (90).

Alternatively, acrylation strategies are often used to alter the mechanical properties of hyaluronic acid. This approach affords the simultaneous introduction of cell adhesion sites and proteolytically sensitive cross-links that degrade as cells invade and secrete MMPs (83, 91). However, the introduction of short MMP-responsive peptide sequences to otherwise sugar-based polymers models only a fraction of MMP functions. Specifically, MMPs modulate tumor progression not only by providing space for cell migration but also by exposing cryptic binding sites in a variety of signaling molecules and releasing ECM-bound morphogens formerly sequestered in an inactive form (92-94). This latter scenario could be investigated through use of designer matrices in which growth factor release is made dependent upon the degradation of MMP-sensitive molecular links (95).

3.2.3. Synthetic materials.—Fully synthetic matrices offer the greatest level of experimental control for modeling the ECM. These can be produced in large, highly reproducible quantities and permit selective tuning of the physical and biochemical composition of cell culture matrices over a much wider range than natural ECMs. Currently, PA- (75, 96) and poly(ethylene glycol) (PEG)-based matrices (97, 98) are the most widely utilized synthetic hydrogels for studies of cancer-associated cell behavior. PA gels, in particular, have elucidated mechanoregulatory mechanisms of tumors by providing a platform that independently controls elastic moduli and ECM ligand presentation. For example, PA systems have shown that matrix stiffness enhances tumorigenesis via perturbed epithelial morphogenesis (75) and that increased cellular traction stresses in response to stiffness may be an index of enhanced malignant potential (99). However, because PA gels are immune to cell remodeling, translation to 3D culture formats is hindered. This is a critical shortcoming because cellular response to rigidity varies significantly between 2D and 3D culture, owing in part to altered cell confinement that may result from changes in porosity (23). Recent findings further suggest that stiffness-dependent changes in PA gel porosity may independently govern cell behavior via altered conformation of covalently attached cell adhesion proteins (100). Cell seeding on arrays of elastomeric microposts that allow adjusting substrate rigidity via the height of the microposts (i.e., independent of changes in porosity) could be used in control experiments to verify individual contributions of ECM rigidity to a particular biological outcome (101).

PEG-based gels offer advantages over PA analogs in that, in addition to having biochemical and mechanical properties that can be tuned independently, they also can support cell-responsive degradation sites necessary for 3D culture conditions. Recent studies with Arg-Gly-Asp (RGD)-modified PEG hydrogels not only recapitulated 3D epithelial morphogenesis similarly to those using Matrigel cultures (Figure 2b) but also indicated that both adhesion peptide density and matrix mechanical properties are critical in this process. Interestingly, exposure to transforming growth factor β (TGFβ) activated EMT

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in these systems independent of matrix stiffness and adhesion peptide density, although previous studies indicated a functional link between these parameters (102, 103). As these were 2D culture studies, varied results could be due to changes in culture dimensionality; however, variations in integrin engagement may also be involved. In fact, most artificial ECMs are modified solely with RGD peptides, whereas in vivo cell-fibronectin interactions are mediated via balanced integrin binding to both RGD adhesion sequences and PHSRN synergy sequences, which result from partial fibronectin unfolding [e.g., due to stiffness-induced changes in cell contractility (104, 105)]. These differences may be critical because differential integrin usage caused by varied presentation of RGD and PHSRN may modulate EMT (106, 107). Modifying synthetic, otherwise nonadhesive, hydrogels with both RGD and PHSRN peptides may help to better define the individual and combined contributions of particular integrins to changes in cell behavior. Further complexity could be achieved through binding sequences of additional ECM molecules relevant to tumor progression (e.g., IKVAV for laminin) or by covalently immobilizing these peptides as gradients rather than distributed homogeneously throughout the bulk of the hydrogel (108).

To analyze the role of matrix structure, composition, and rigidity at metastatic sites such as bone, materials are required that recapitulate the mineral-containing nature of the bone ECM and have stiffnesses in the same MPa range. Synthetic polymers including poly(lactic) acid (PGA), poly(glycolic) acid (PGA), and their copolymer (PLGA) offer a foundation for such approaches, as they provide suitable substrates for mineralization and degrade under aqueous conditions into noncytotoxic by-products. Such scaffolds can be fabricated using a variety of approaches including gas foaming/particulate leaching (GF/PL) and electrospinning, whereby mineralization is achieved via incubation in simulated body fluid to yield a bone-like apatite layer (109) or by incorporation of synthetically derived hydroxyapatite nanoparticles during the scaffold fabrication process (110). Although interactions with mineral markedly affect tumor cell behavior in these matrices (110), it is possible that the presence of collagen type I influences bone mineral responses, but such responses are not frequently mimicked with most systems. To specifically recapitulate bone stiffness, scaffolds fabricated from poly(ester urethane) may also be suitable. These systems have been utilized to show that mechanical properties of the bone mineral matrix foster bone metastasis by inducing the osteolytic breast cancer phenotype (41).

3.3. Soluble Factor Signaling

3.3.1. Growth factors and cytokines.—To study the spatiotemporal complexity of soluble factor signaling in tumors, drug delivery approaches originally developed for therapeutic applications (111) may prove useful. Temporal control over soluble factor signaling may be achieved with delivery vehicles that mimic the growth factor binding and release kinetics of the ECM. For example, incorporating signaling molecules into polymeric scaffolds (e.g., PLGA-based systems) permits sustained, long-term delivery whose rate of release can be readily modulated by adjusting the degradation kinetics of the factor-releasing vehicle (e.g., via altering polymer molecular weights or the molar ratios of lactic and glycolic acid monomers) (112) and/or by tailoring the scaffold fabrication procedure (e.g., preencapsulating bioactive molecules into PLGA particles used for subsequent scaffold fabrication) (113). Despite the obvious advantages of these approaches, persistent

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presentation of active signaling molecules does not necessarily represent tumor conditions in vivo where factors are stored within the ECM in an inactive form and released only through cellular processes, for example, via MMP-mediated matrix breakdown (72) or cell contractility–dependent growth factor activation (18). Indeed, sustained signaling molecule exposure may independently alter malignancy (114) and hence warrants consideration during interpretation of results.

To recapitulate the soluble factor spatial complexity, changes to scaffold fabrication, transport phenomena, and materials chemistry may be explored. In the simplest scenario, locally confined release of factors can be achieved by placing delivery vehicles at the desired location. To increase complexity, layered scaffolds may be applied where each layer contains a different growth factor concentration (115). Although originally designed to generate tissue-scale gradients of growth factors in vivo, this approach may not provide the level of control needed to study individual cell behavior in vitro. Localized and cell-demanded release of factors from proteolytically degradable matrices may be used here to form gradients whose spatial dimensions can be tailored by additional parameters such as interstitial flow (116). Finally, 3D gradients may be established by patterning proteins into otherwise nonadhesive hydrogels (e.g., agarose) by using multiphoton-exposed gradated thiols (117). With these systems, covalently linked growth factors not only lead to receptor activation but also may enhance signaling by precluding receptor internalization (118). Nevertheless, the technical sophistication and low-throughput nature of this approach may limit its utility for conventional biochemical analysis.

Although single factors can significantly impact cell behavior, tumorigenesis typically involves complex, multivariate spatiotemporal interactions. These factors signal simultaneously or sequentially, underscoring the critical importance of delivery techniques capable of mimicking this interplay. Mixing strategies can emulate simultaneous delivery, whereas sequential factor delivery requires more sophisticated approaches such as composite systems of multiple polymer phases yielding distinct release kinetics (e.g., by mixing one factor with PLG particles and encapsulating a separate factor in microspheres prior to PLGA scaffold fabrication) (113). Although most delivery approaches focus on the supply of stimulating factors, inhibitory cues are critical as well, yet commonly overlooked. For example, delivery of both pro- and antiangiogenic factors from spatially restricted zones of a synthetic scaffold promotes temporally stable and spatially restricted angiogenesis (119). Consequently, it is likely that modification of individual signaling pathways in an effort to mimic tumor conditions may induce aberrant interactions between inducing and inhibitory factors.

A common challenge to the above-described approaches is their inability to mimic fluctuating or transient increases/decreases of ligands, which may be mechanistic to a specific cellular response. Stimuli-responsive delivery vehicles releasing factors in response to cyclic mechanical, electrical, or chemical stimulation may be invaluable here (120, 121); however, all of these stimuli concurrently modulate cell behavior, thereby conflating the precise contribution of fluctuating soluble factor signaling. Moreover, the spatial distribution of signaling molecules with such approaches would be compromised. Culture systems incorporating microfluidic conduits within engineered ECMs can ameliorate

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these shortcomings and enable soluble factor delivery with well-defined spatial and temporal resolution (122) (Figure 2c). Depending on microchannel arrangement, homo- or heterogeneous soluble environments can be formed: A single network of multiple channels that are diffusively coupled yields homogeneous morphogen concentrations throughout the scaffold (122), whereas a hydrogel-based, three-path device with a source, a sink, and a center channel can generate sustained linear gradients of chemicals across the center path (123).

Another key consideration is that most delivery vehicles do not appropriately mimic the biochemical, structural, and mechanical properties of the ECM. For example, a caveat of PLGA-based drug delivery vehicles is their high intrinsic rigidity, which, apart from directly altering tumorigenesis (124), can undermine signaling of the delivered factors by integrin-mediated activation of their receptor downstream cascades (125) and cell morphology (52). Delivery from compliant ECM mimics could serve as an alternative strategy, whereby matrix rigidity is adjusted through cross-linking density of RGD-modified alginate (126, 127) and release rates separately modified by heparin-binding interactions of many growth factors and cytokines (128). Furthermore, these materials are attractive because they can mimic enhanced signal transduction efficiency in the presence of proteoglycans (72). Fibrin-based matrices bearing recombinant fibronectin fragments with both integrin and growth factor binding sites represent another innovative approach (129), in which synergistic interactions between integrins and growth factor receptors enhance signal transduction. Although these systems better represent the growth factor microenvironment than most other systems do, they could be further refined. For example, fibronectin and heparin interactions modulate fibronectin conformation, which also influences growth factor (un)binding and consequently cell signaling (130). Finally, the spatial organization of ECM fibers and dimensionality is pivotal to guiding cellular responses to growth factor gradients (131) (C. Fischbach, unpublished data); however, this organization is largely ignored in study interpretations.

3.3.2. Hypoxia and acidosis.—The contributions of hypoxia to cell behavior and tumor progression are classically approached by exposing monolayer cultures to environments with reduced oxygen levels (typically $< 5\%$) (4). This setup has contributed to a better understanding of oxygen-limited cell signaling or gene expression, yet it poorly recapitulates the spatiotemporal oxygen variations characteristic of the tumor microenvironment or of in vivo conditions in general. In fact, ambient oxygen is commonly employed in cell culture to mimic normoxic conditions, but physiological tissue oxygen levels are significantly lower (132, 133). These limitations have been partially addressed through engineering approaches, which effectively stratify oxygen availability to the cells being studied. For example, incorporation of PDMS inserts, which throttle oxygen diffusion at defined distances from the cell monolayer, represents one approach to gradate oxygen distribution (134); however, this approach utilizes 2D systems that ignore the considerable impact of 3D microenvironmental context on the hypoxic-cell response (135).

A variety of 3D culture models may be applied to study hypoxia-induced cell signaling. For example, simple stacking of cell monolayers cultured on gas-permeable, ECM-impregnated paper sheets imparts declining oxygen availability to cells of a particular layer and mimics dimensionality and cell-cell and cell-ECM interactions characteristic of tissues in vivo (136).

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Similarly, dynamic culture of tumor cells within thick (i.e., >250 μm) 3D polymeric models may generate tumor mimicry of central hypoxia, simply owing to convective-diffusion-reaction processes (4). Although the aforementioned systems can assess global effects of oxygen-related signaling under 3D conditions, they are not suitable for isolating cell behavior under a particular hypoxic or normoxic condition in light of multiple other microenvironmental conditions impacted by varied oxygen levels. In this regard, 3D culture systems that generate homogeneous normoxic or hypoxic cell populations are invaluable. Such models can be designed by adjusting the thickness of the utilized polymeric matrix (e.g., alginate) to balance oxygen supply with cellular consumption (135).

Although these approaches effectively mimic oxygen profiles, it is essential to note that hypoxia simultaneously modulates a variety of other microenvironmental signaling parameters, which confound experimental interpretation. In particular, Warburg's observation over half a century ago (31) that transient hypoxia promotes a sustained metabolic shift toward glycolysis in cancer cells is frequently overlooked in conventional hypoxia model systems. However, the associated enhanced production of acidic metabolites (i.e., lactic acid) decreases pH in the tumor interstitium, a condition that is further exacerbated by elevated activity of carbonic anhydrase (137-139). These changes in pH can impart alterations to the ECM, affect morphogen presentation or the interaction of these with their cognate receptors, and further stimulate signaling cascades, culminating in vast outcomes ranging from angiogenesis to DNA damage to tumor cell migration (34, 35, 140, 141). In particular, decreased pH changes the binding affinity of certain growth factors (e.g., VEGF) not only to cells (142) but also to certain ECM components. Specifically, VEGF binds to fibronectin with higher affinity at acidic pH, and these interactions are further enhanced by heparin (143). Another commonly disregarded aspect is that hypoxia can indirectly affect cell behavior by increasing ECM stiffness through upregulation of the collagen cross-linking enzyme lysyl oxidase (LOX) (144). These changes in ECM mechanical properties not only can promote malignancy at the primary site (20); they also contribute to the formation of a premetastatic niche that enhances tumor cell seeding in distant organs (145).

Microfluidic models offer a means to maintain precise control over oxygen/nutrient delivery and waste removal in 3D culture context and may provide an opportunity to study long-term cell behavior in response to recurrent or increasing hypoxia. Integrating perfusion channels within the culture matrix directly enables the re-creation of spatiotemporal variations in oxygen and metabolic activities via diffusive coupling of neighboring channels, as described above (122). Alternatively, microscale 3D cultures can be loosely packed within the microfluidic conduits to yield tissues permeated with a pore network mimicking the vasculature (146). It is conceivable that flow of culture medium of varying oxygen concentrations through these culture models affords an opportunity to assess the resulting effects on normal tissue formation. Additionally, continuous removal of waste products or lack thereof by adjusting perfusion rates could potentially control acidification of the tumor interstitium. These applications could also be extended to even more complex studies evaluating not only the tumor but also its physicochemical interactions with the surrounding host tissue. For example, endothelialization of microfluidic channels embedded within tumor cell–seeded hydrogels (147) could be perfused with hypoxic culture medium. This would

allow study of both isolated and combined effects of oxygen deprivation, tumor-derived paracrine signals, and shear stress on vessel sprouting (148).

3.4. Mechanical Stimulation

The likely roles of stretch, compression, and shear-dependent mechanical stimuli on cancer progression are increasingly recognized, although they remain essentially uncharacterized. This includes the application of stresses and strains either to manipulate cell behavior (mechanobiology) or to reflect/determine a particular in vivo mechanical environment (biomechanics) (149).

3.4.1. Mechanical stretch.—Mechanical stretch commonly occurs in relatively compliant tissues that are distended, such as the lung or bladder; it can be applied to in vitro cultures grown on a deformable substrate (e.g., silicone, rubber) as well as to tissues. A variety of physiologically relevant strain fields (e.g. in plane, out of plane, uniaxial) can be achieved by altering substrate geometry (e.g., rectangular versus circular) or stretch application (pulling via grips, vacuum, etc.). In particular, the Flexcell® line of loading devices is frequently utilized to study strain-mediated cell behavior. This platform generally stretches a thin, deformable membrane and has revealed, for example, that stretch increased Lewis lung carcinoma cell proliferation (150) and may promote benign ovarian disease by increasing secretion of cancer biomarkers from both peritoneal macrophages (151, 152). However, these systems are cost restrictive and limited to 2D studies, although tissue dimensionality clearly affects mechanosensing and alters cytoskeletal architecture (153). For added dimensionality, tissue explants grown in vitro can also be stretched longitudinally, but this requires gripping the tissue and substrate, thereby inducing end effects (154). The strain distribution in these stretch devices is heterogeneous and thus difficult to correlate with observed cell behavior. Furthermore, the devices typically have low throughput and therefore are not suitable for screening applications. Microfabricated arrays capable of simultaneously applying cyclic equibiaxial substrate strains to small populations of cells or microtissues may help to overcome these limitations and could be used for cancer studies (155).

3.4.2. Fluid flow.—Fluid flow occurs throughout tumor-associated blood and lymphatic vessels as well as the interstitium. Indeed, in many primary tumor sites, interstitial fluid flow away from the center of the tumor invokes shear stress on cells, with direct and indirect impacts on tumor progression such as mediation of myofibroblast formation (156) and creation of soluble factor gradients, respectively (157). Historically, the effects of fluid flow were explored by flowing fluid over cell monolayers using devices such as parallel-plate chambers. Given the inherent stiffness and 2D nature of these chambers, alternatives are needed and could include microfluidic devices, which allow modulation of a wide array of signaling cues, including applied mechanical forces (both fluid and solid deformation), for cancer focus. For example, highly structured architectural features can be achieved, and the scaffold material can be embedded with cells, growth factors or cytokines, or material properties tuned to impart specific mechanical forces (both fluid and solid strain) (158, 159). Another area of interest focuses on resolving how fluid flow may influence CTC extravasation and metastasis. In particular, hemodynamic fluid flow alters the collision frequency between CTCs and endothelial cells or platelets as well as the formation of

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the resulting adhesive bonds (160, 161); yet, the specific role of the flow characteristics underlying this remains unclear. Furthermore, these parameters may vary as a function of fluid flow–dependent changes in stromal cells. For instance, inflamed endothelium may respond differentially to fluid flow, which could, in turn, modulate interactions with CTCs. Interestingly, previous studies suggest that endothelial cell behavior differs by organ type as well as in the tumorous context (162, 163), but whether or not this is due to differential response to fluid flow and modified CTC interactions is unclear. Studies of these issues would provide invaluable new insights as to why certain organs (e.g., bone, lung) are prone to metastasis, whereas others (e.g., heart, skeletal muscle) are not.

3.4.3. Mechanical compression.—In vitro compression is readily achieved via direct application of a moving platen to a fixed tissue explant or a 3D scaffold containing cells, which has led to important new insights such as elevated myofibroblast differentiation and matrix remodeling with dynamic compression (164). As with stretching, edge effects at surfaces in contact with platens limit the volume from which conclusions should be drawn; however, if the construct is large enough, areas sufficiently distanced from these surfaces warrant analysis (St. Venant's principle). Another complication of compression is that when applied to porous substrates, fluid flow necessarily arises within the pores, owing to changes in cross-sectional area and generation of pressure gradients. However, this may better reflect in vivo compression, which entails various other changes including fluid flow, pressure, and matrix deformation. Therefore, intentionally combining fluid flow and mechanical compression or stretch and applying them to 3D culture models may be most physiologically relevant. Perfusing 3D scaffolds (165) and engineering mechanically actuating microfluidic devices (166) are emerging technologies that pursue this route. For example, a model of a breathing lung has been engineered by coculturing alveolar epithelial cells and culture pulmonary capillary endothelial cells on opposite sides of a distensible membrane to which cyclic stretch is applied (Figure 2d). Cytokines or other molecular cues can be added to fluid flow for added relevance to cancer. A caveat, however, is that as the complexity of these models increases, so too does the difficulty in deciphering the isolated and/or combined effects of mechanical stimuli. Additionally, many of the current models are single-cell cultures that lack important cell populations. For example, osteocytes may magnify the level of stresses and strains imposed on the skeleton in vivo. Yet, they are mostly neglected in current in vitro loading models of bone, owing to fabrication or culture duration limitations. The absence of these cells may explain why changes in bone cell behavior require significantly higher load levels in vitro than in vivo (167, 168).

A variety of challenges exist that are inherent to all of the described models. Mechanically induced changes in biochemical signals are mediated on the molecular/cellular scale via integrin-dependent changes of the cytoskeleton (169, 170). This complicates interpretation because tumorigenesis influences integrin-mediated changes in cytoskeletal architecture independent of exogeneous stimuli. As stiff cells located within compliant tissues (as in normal tissues) deform less in response to mechanical loading than do soft cells in a stiffer matrix (as in tumors), loading responses should be accentuated in tumors relative to healthy tissues. Investigating this hypothesis will be critical to deciphering the role of mechanical stimuli in tumor progression and metastasis. Furthermore, all physiological

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mechanical environments are inherently complex; stresses and strains depend on material properties, architecture, anisotropy, temporal variations, etc., but because the variability of these parameters is not clear even for physiological situations, their mimicry in the context of cancer becomes tremendously complicated. Furthermore, the specific mechanical signals (e.g., stress versus strain, rate, frequency, duration) that best correlate with changes in cell behavior are controversial and unknown in the context of cancer. To study their impact in a meaningful manner, cells should be cultured in scaffold systems that recapitulate the architecture of the respective native tissue. However, generating matrices of physiologically relevant, complicated geometries (e.g., cancellous bone) is extremely difficult even when using 3D printing based on high-resolution images of the relevant tissue compartment. Finally, nonlinear, anisotropic, and heterogeneous material properties prohibit the use of any simple constitutive stress-strain relationship such as Hooke's Law, confounding data analysis and interpretation of findings from experiments.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

A multitude of tissue-engineered and physical sciences–based systems have been developed to mimic pathologically relevant aspects of cancer in vitro. By providing opportunities to decipher the complexity of this disease in a reductionist way, these culture models have led to important new insights. Nevertheless, a variety of additional prospects should be considered in moving forward. In particular, most current studies focus on investigating specific signaling events at a single scale (e.g., molecular, cellular, tissue, organ, or systems level), despite the fact that cancer is clearly a multiscale disease in which aberrations at one level automatically affect all other levels (Figure 3). For example, tumor cells secrete factors that, via systemic distribution, alter ECM assembly and remodeling at distant sites (e.g., lungs), eventually promoting metastasis (43, 171). Also, it is well known that systemic diseases such as obesity increase the risk for a variety of cancers including breast, prostate, and pancreatic cancers. However, the underlying systemic signaling is not well understood. Modular tumor-on-a-chip systems to model multiorgan interactions of a tumor with other metabolic compartments such as liver or kidney as well as common sites for metastasis would be invaluable for such investigations and could be based on previous technologies for studies of pharmacokinetics and dynamics (172).

Other considerations should include (*a*) the type and source of cells to be used in modeling the tumor microenvironment and (*b*) how cell behavior is analyzed. At present, most studies are performed with cells readily obtainable and maintainable in culture, including cancer cell lines and fibroblasts, as representative stromal cells. However, studies with other, perhaps more clinically relevant, cell types or with immune cell types are still lacking. In particular, there is an emerging emphasis on the importance of rare CTCs and tumor stem cells in tumor progression and metastasis (174, 175). Significant progress has been made in isolating these cells from patient samples and defining conditions for their subsequent growth/maintenance (176, 177). Still, routine applications are not possible, and focus should therefore be placed on identifying conditions that make these cells amenable for in vitro studies. This would allow the development of culture models based on patient-derived cells, which models could then be used for comprehensive analysis and predictive evaluation of treatments for personalized medicine. Finally, exposing tumor cells to varied

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microenvironmental conditions may result in the evolution of single-cell populations that further the development of tumor heterogeneity. However, as most current studies focus on assessing population averages and are performed over relatively short periods of time, these differences are often hidden (178). Performing long-term experiments, single-cell analysis, and/or utilizing appropriate data analysis tools may help to elucidate specific signaling events that foster the evolution of tumor complexity.

Given the importance of the ECM as a global modulator of cell signaling, more focus on understanding its role in modeling and guiding tumor progression would be beneficial. A number of grand challenges can be defined. For example, what are the dynamics of ECM remodeling in tumors, and how do the resulting temporal changes of the ECM contribute to tumorigenesis? In situ, photopolymerization and/or degradation strategies afford an opportunity to address these questions (179). Another open question is related to stiffness and how cells interpret such information. Cellular integrin engagement occurs at the fiber level; fiber stiffness, however, is multiple orders of magnitude higher than global ECM stiffness because deformation is distributed over a network of disordered and connected fibers that respond collectively to strain, rather than over single fibers (180). How do cells decode these differential mechanical properties into molecular information and changes in signal transduction? Furthermore, the way cells experience stiffness may be significantly altered by the glycocalyx (124), an ECM component that has been largely overlooked despite its significant upregulation in tumors and its impact on other, more conventionally studied, ECM components. Hence, focusing efforts on developing scaffolds that not only mimic individual ECM components but also aim to recapitulate the ECM's compositional complexity is highly desirable. Finally, not all tumors are stiffer; in fact, nonepithelial-derived tumors such as osteosarcoma are significantly softer than their tissue of origin. What are the underlying mechanisms, and how does integrin signaling in these tumors change in response to softening?

Translation of the developed technologies to biology labs should be a final consideration. Most of the culture models described in this review are relatively complex and require engineering tools and skills that are not readily available to cancer biology labs. However, the input of biologists is critically needed in order to perform appropriate biological analysis and interpretation of results. Hence, a future focus of the field should be on developing simple culture models that can be used by clinicians and other researchers without engineering backgrounds. Other limitations of most current technologies include their relatively low throughput, requirement of advanced 3D imaging tools, and difficulty with specimen isolation. In particular, photo-cross-linked materials present this challenge, as they cannot be simply dissolved or enzymatically digested like other ECM mimics (e.g., calcium-cross-linked alginate dissolved with EDTA, collagen digested via collagenase), and this may present difficulties during RNA and protein isolation for transcriptional or proteomic analysis. Addressing these shortcomings will significantly enhance the amount and quality of information that can be gained with engineered tumor models.

Looking forward, engineered tumor models may advance both basic and translational cancer research. To this end, close collaborations between engineers and cancer biologists are indispensable, as these interactions are critical for the definition of biologically relevant

design criteria that advance the development of novel tools and strategies. This approach may revolutionize how cancer is studied in culture and ultimately provide novel therapeutic opportunities that translate into better clinical management of the disease with improved patient survival.

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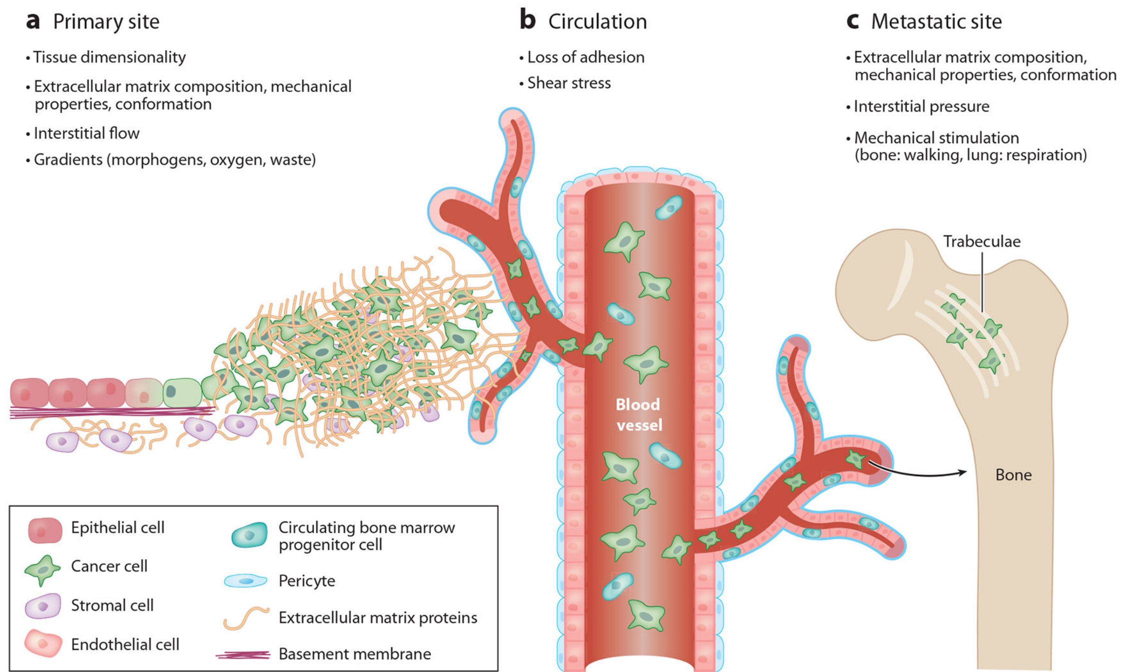

Figure 1.

Biological and physicochemical characteristics of the tumor microenvironment. (*a*) Tumor initiation perturbs 2D epithelial architecture and basement membrane organization. Tumor invasion and migration toward adjacent blood vessels are promoted by increased oxygen and nutrient demands of the growing 3D tumor as well as by epithelial-to-mesenchymal transition (EMT)- and stroma-mediated changes in extracellular matrix (ECM) composition, mechanical properties, and conformation. (*b*) Intravasation into blood vessels introduces tumor cells into the circulation where they lose substrate adhesion and are exposed to fluid flow–mediated shear stress. (*c*) Lodging of tumor cell(s) in capillary beds of secondary organs (e.g., bone) facilitates their extravasation via endothelial or basement membrane protein interactions and the formation of micrometastases. Pending survival and favorable microenvironmental conditions including appropriate ECM characteristics and mechanical stimuli, secondary tumor growth ensues.

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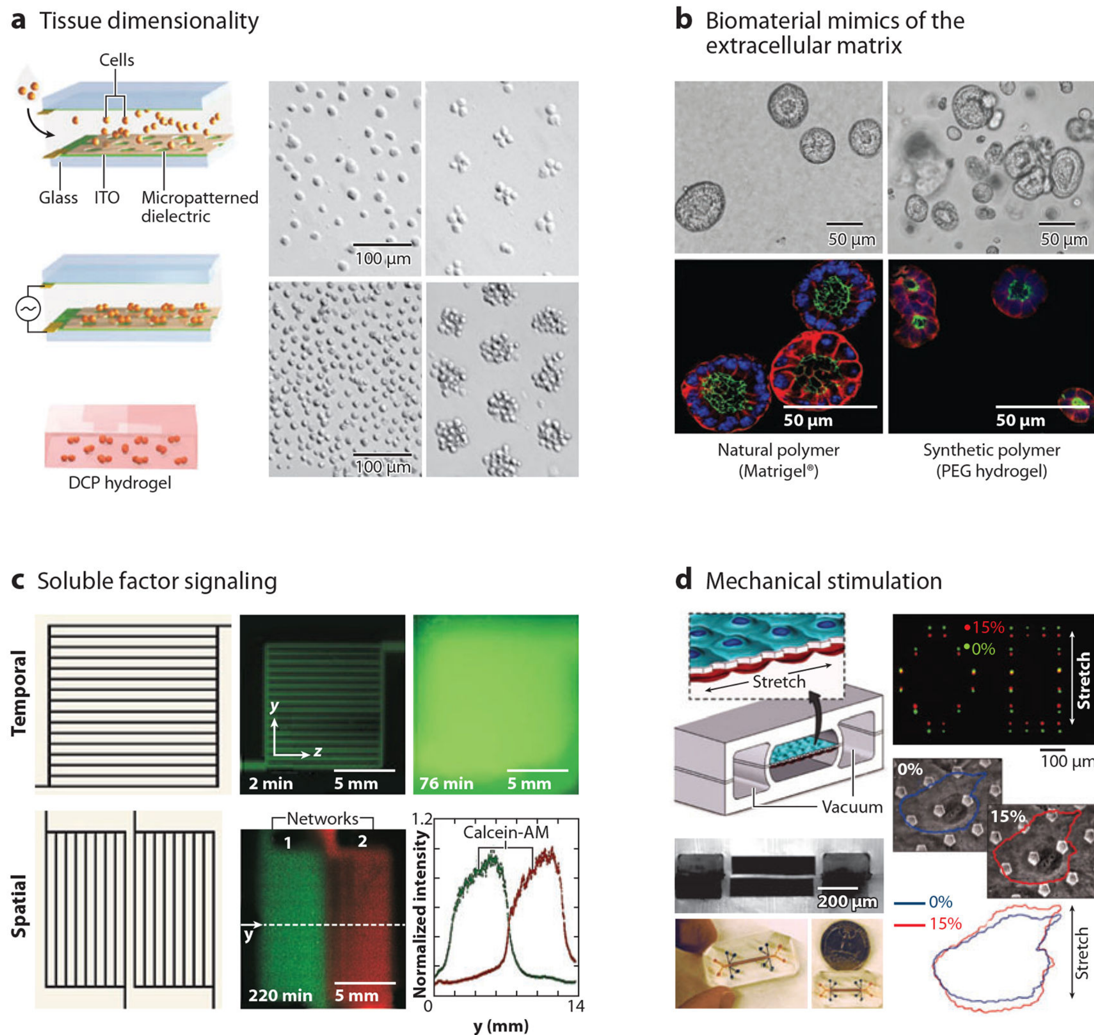

Figure 2.

Engineering approaches to mimicking tumor physicochemical and biological characteristics. (*a*) Tumor three-dimensionality and control over cell-cell interactions can be achieved through dielectrophoretic cell patterning; cells are exposed to an electric field and migrate toward specific locations of a micropatterned electrical insulator. Adjustment of seeding density controls 3D microorganization, and gelation of the migration medium stabilizes this configuration for subsequent culture (58). (*b*) Culture of lung cancer cells within PEG hydrogels covalently modified with RGD adhesion peptides and MMP-sensitive cross-linkers undergo morphogenetic and polarity changes similar to those that occur in Matrigel® (98). (*c*) Integrating microfluidic channels into hydrogels affords temporal and spatial control of soluble factor signaling by adjusting perfusion rate and modular network assembly, respectively (122). (*d*) Lung-on-a-chip microdevices mimic respiratory mechanics for possible future studies of their effects on lung-metastatic tumor cells (166). These devices recreate physiological breathing movements by applying vacuum to the side chambers, which causes mechanical stretching of a porous PDMS membrane seeded with lung epithelial cells on the top and endothelial cells on the bottom. Membrane

stretching creates cellular tension in the direction of the applied force. Abbreviations: DCP, dicalcium phosphate; ECM, extracellular matrix; ITO, indium tin oxide; MMP, matrix metalloproteinase; PDMS, polydimethylsiloxane; PEG, poly(ethylene glycol); RGD, Arg-Gly-Asp. (Images were modified and included with permission from Nature Publishing Group, American Association of Cancer Research, and American Association for the Advancement of Science.)

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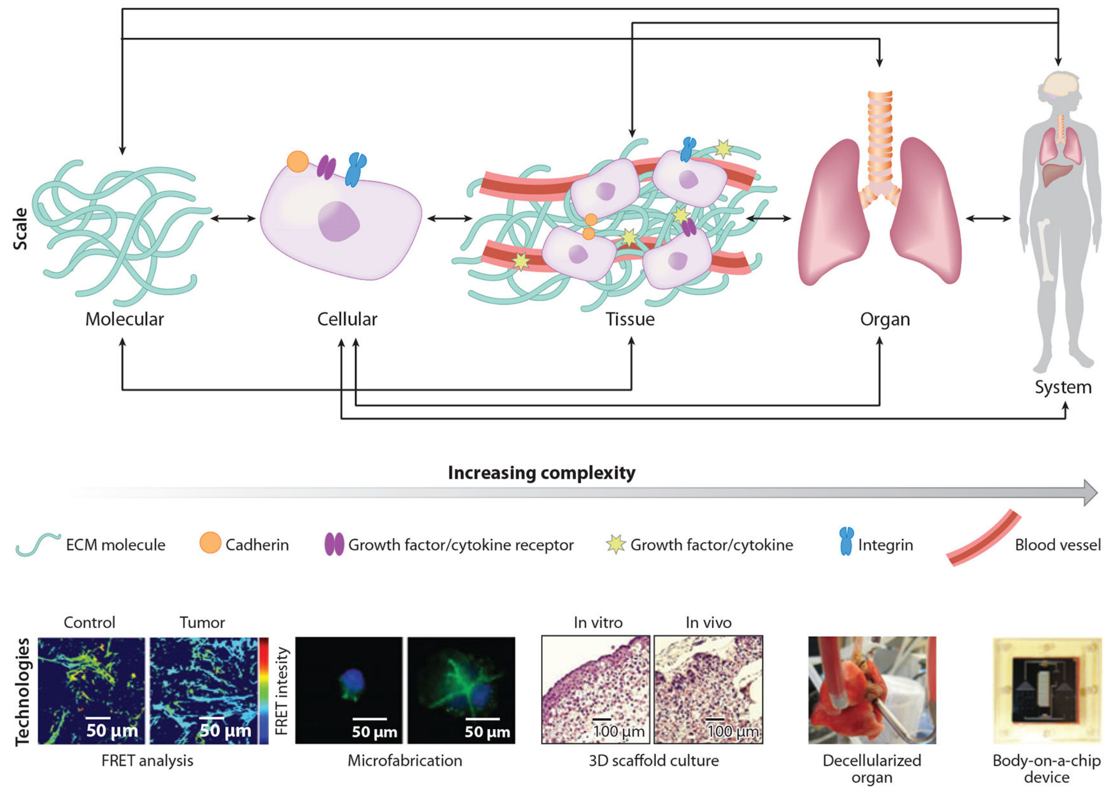

Figure 3.

Multiscale nature of cancer contributes to its complexity. Cancer represents a multiscale disease in which the integrated effects of molecular-, cellular-, tissue-, and organ-level signaling cause systemic disease that is characterized by tremendous complexity. Yet, most technologies focus on recapitulating signaling events at a single scale. Specific examples include (from left to right) analysis of fibronectin molecular conformation via FRET imaging to evaluate how tumors modulate ECM unfolding (21), cell patterning techniques to investigate signaling responses of individual cells and clusters of cells (51), 3D PLGA scaffold culture to recapitulate histological and functional characteristics of tumors in vivo (4), and combination of decellularized organs with bioreactor technologies such as artificial ventilation models to study cancer cell behavior within a fully functioning organ (e.g., the lung) (173). Integrating such single-scale models into more complex cell culture analogs that allow interconnecting various compartments similar to previously developed body-on-a-chip models (172) would be useful to mimic tumor complexity. Such systems would not only enable studies of cell metastasis to secondary locations or evaluate the toxicity of novel anticancer therapeutics but also test the effect of other conditions such as obesity or inflammation on tumorigenesis. Abbreviations: 3D, three dimensional; ECM, extracellular matrix; FRET, fluorescence resonance energy transfer; PLGA, poly(lactic-co-glycolic acid). (Photographs were included with permission from Nature Publishing Group, Royal Society of Chemistry, Annual Reviews, and Institute of Physics Publishing.)

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