Modeling Tumor Microenvironments In Vitro

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Tumor progression depends critically upon the interactions between the tumor cells and their microenvironment. The tumor microenvironment is heterogeneous and dynamic; it consists of extracellular matrix, stromal cells, immune cells, progenitor cells, and blood and lymphatic vessels. The emerging fields of tissue engineering and microtechnologies have opened up new possibilities for engineering physiologically relevant and spatially well-defined microenvironments. These in vitro models allow specific manipulation of biophysical and biochemical parameters, such as chemical gradients, biomatrix stiffness, metabolic stress, and fluid flows; thus providing a means to study their roles in certain aspects of tumor progression such as cell proliferation, invasion, and crosstalk with other cell types. Challenges and perspectives for deconvolving the complexity of tumor microenvironments will be discussed. Emphasis will be given to in vitro models of tumor cell migration and invasion. [DOI: 10.1115/1.4026447]

Keywords: mechanical stress, 3D culture, extracellular matrix, invasion, interstitial flow

Introduction

The tumor microenvironment plays critical roles in tumor progression as it controls the ease at which tumors can grow and invade, the chemical signaling between cells, and the host immune response to the tumor, among others. Evidence is mounting that the molecular gradients and mechanical stresses around tumor cells influence tumor progression in significant ways [1,2]; for example, chemokines abundantly expressed in chronic inflammatory conditions are implicated in tumor progression [3] and matrix stiffness promotes tumor malignant phenotypes [4]. Clinically, “occult” tumors (i.e., benign tumors that do not invade or become malignant) are far more common than cancer, and it has been argued that this is largely due to the microenvironment; e.g., normal tissue environments can suppress malignancy, while certain pathogenic tissue features may induce malignancy [5]. Furthermore, the tumor microenvironment can also suppress any host immune responses against the tumor, even those induced by immunotherapies [6–8]. Thus, understanding what defines malignancy-promoting tumor microenvironments, and teasing out the contributions and crosstalk between various cellular and molecular players, are critical for eventual development of therapeutic strategies that target the microenvironment.

The tumor microenvironment is known for its complexity, both in its content as well as its dynamic nature. It consists of various immune cells, stromal fibroblasts, extracellular matrix (ECM) components often associated with wound healing and inflammation, blood and lymph vessels, and various other cell types like endothelial progenitor cells [9]. Tumor cells interact and evolve with this complex community in multiple ways. Aside from its composition, two key biophysical parameters controlling tumor interactions with its microenvironment are molecular gradients and the mechanical stresses. *Molecular gradients* within the tumor microenvironment typically originate from cell–cell signaling (e.g., chemokines and growth factors), metabolic stress (e.g., pH, oxygen, glucose, and other nutrients) [10,11], and physical features of the ECM like composition, stiffness, hydraulic conductivity, and growth factor binding that affect transport properties [12,13]. *Mechanical stress* within a tumor microenvironment can arise from fluid shear and drag forces on the ECM and cells from interstitial flows or the mechanical tension generated by either the

expanding tumor on its surroundings or matrix contraction from the stromal fibroblasts [14–17]. Because mechanical stresses can cause pressure gradients that drive interstitial fluid flow, mechanical stresses are also inherently coupled to chemical gradients [11,18–22].

In vitro models have the potential to explore mechanistically the individual contributions of various components of the microenvironment on tumor cell behavior, and the ability to modulate the biophysical and biomechanical aspects. At the same time, in vitro models cannot recapitulate every aspect of the tumor microenvironment, and thus care must be taken to identify their limitations and assumptions. For example, while a three-dimensional (3D) culture of tumor cells in a collagen gel under interstitial flow might be used to explore direct effects of interstitial flow on tumor cell invasion [23–25], it ignores other factors present at an invasive edge of the tumor such as the architecture and composition of the ECM (which is highly heterogeneous), the diversity of cell types present in the tumor stroma and their physiological interactions, and gradients of oxygen, pH, metabolites, and nutrient that are formed from the tumor mass. As such, one can only infer from such studies the direct effects of interstitial flow on uniformly distributed tumor cells within a homogeneous collagen ECM (which itself is important), but not necessarily translate those findings directly to the tumor invasive edge, since one does not know the relative importance of interstitial flow versus the other factors that were excluded from the in vitro model. The field has often suffered because of erroneous interpretations drawn from oversimplified in vitro models, starting with the earliest two-dimensional (2D) monolayers; even as more complex models are built, the questions they can be used to address, and how data they generate can be interpreted, require careful consideration.

Several advances in tissue engineering have allowed more physiologically relevant in vitro tumor microenvironment models to be developed. These include better control of 3D matrices with defined compositions and mechanical properties, and improved microtechnology advances at the “mesoscale” ($\sim 10^2$ – 10^3 μm , which is needed for recapitulating most multicellular 3D microenvironments) for developing culture chambers that can be perfused, that can maintain well-defined and controlled chemical and mechanical environments for cells, and that allow real-time imaging [26–28]. Additionally, great advances in intravital imaging using animal models has revealed much of what we know about tumor microenvironments [29] and thus helped to guide development.

We describe in vitro modeling efforts of the tumor microenvironment, comparing the biological events during tumor progression

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Table 1 Engineering design considerations and models used for various steps in tumor initiation, progression, and metastasis

Biological event	Engineering considerations	Models used
Tumor initiation and growth	<ul style="list-style-type: none"> • ECM composition and stiffness • Compressive stress 	<ul style="list-style-type: none"> • 3D spheroid assay • Hanging drop assay
Blood and lymphangiogenesis	<ul style="list-style-type: none"> • Adhesion molecule density • Endothelial sprouting into 3D ECM • Growth factor gradients • Luminal and interstitial flow • Role of macrophages, stromal fibroblasts, and other cells 	<ul style="list-style-type: none"> • Bead assay • Tubulogenesis assays • Microfluidic devices • 3D flow chambers
Cell invasion and chemotaxis	<ul style="list-style-type: none"> • Cell contraction and motility • Interstitial flow and ECM stress • Co-culture with stromal cells • 3D matrix, stiffness, adhesion molecule density • Oxygen gradients/hypoxia • Chemokine gradients 	<ul style="list-style-type: none"> • Modified Boyden chamber • 3D cell tracking • Microfluidic devices • Vertical layered assay
Intravasation into blood or lymphatic vessels	<ul style="list-style-type: none"> • Interactions between tumor cells and endothelial cells • ECM composition; presence of basement membrane • Transendothelial flow (e.g., into lymphatic vessels) • Luminal flow on endothelial cells 	<ul style="list-style-type: none"> • Modified Boyden chamber • Microfluidic devices
Tumor cell circulation	<ul style="list-style-type: none"> • Luminal shear stresses • Complex geometry (e.g., branching, narrowing capillaries) • Platelets, blood cells, clotting factors 	<ul style="list-style-type: none"> • Microfluidic flow chamber
Adhesion and extravasation	<ul style="list-style-type: none"> • Co-culture with endothelial cells • Luminal flow • Chemokines and adhesion molecules 	<ul style="list-style-type: none"> • Parallel plate flow chamber
Growth in ectopic site	<ul style="list-style-type: none"> • Tissue-specific features of ectopic site (e.g., lymph node, liver, brain, bone) including chemokines and ECM components 	<ul style="list-style-type: none"> • 3D cultures

with the corresponding tissue engineering considerations (see Table 1).

Tumor Initiation and Growth

3D spheroid tumor cell cultures serve as powerful tools for dissecting the roles of various biochemical and biophysical cues in carcinoma initiation and progression [30]. Pioneering work from the Bissell lab [31] demonstrated that normal mammary epithelial cells and carcinoma cells display distinctly different phenotypes both in structure and function when cultured in a reconstituted basement membrane matrix (matrigel); e.g., normal mammary epithelial cells self-organize and form acinar structures with lumen, while carcinoma cells form cell-filled spheroids. This model has been used extensively to elucidate the roles of mechanical forces (both tensional and compressive stress), ECM components, and other factors in tumorigenesis [2,30,32,33].

Blood and Lymphangiogenesis

The size of prevascularized tumors is limited mainly by oxygen diffusion and consumption rates, such that tumors cannot grow beyond hundreds of microns before requiring new blood vessels [12,34,35]. Thus, angiogenesis is required for tumor growth beyond this size, and inhibitors of angiogenesis are used clinically to slow tumor progression [9]. Because of the ongoing development of therapeutic targets that block tumor angiogenesis, there is a need for physiologically relevant *in vitro* models of tumor angiogenesis. Furthermore, although much less studied, tumor lymphangiogenesis is thought to be important for metastasis of many solid tumors, and thus models of lymphangiogenesis are also included here.

Previous studies comparing the transport properties within tumor and normal tissues found that tumor tissues were more heterogeneous in their perfusion [36–39], which may need to be considered in model systems. Microfluidic models that incorporate on-chip blood vessels have been developed to provide well defined microenvironments for studies of blood and lymphatic angiogenesis, and their roles in tumor cell invasion [40–43]. For more detailed review on this subject, please refer to other review articles [17,34].

Tumor Cell Migration and Invasion

Tumor cell migration and invasion are important first steps in cancer metastasis [44,45]. During epithelial to mesenchymal transition, tumor cells downregulate cell–cell adhesions like E-cadherin, upregulate matrix metalloproteinases (MMPs), and invade the ECM. Often, tumor-associated macrophages or stromal fibroblasts also help guide tumor cells.

Modes of Cell Migration in 3D. Two types of cell migration behaviors within a 3D matrix have been described: amoeboid and mesenchymal [29]. Amoeboid migration, commonly seen in dendritic cells and macrophages, occurs primarily by squeezing through pores of the fibrous ECM network via a series of well-orchestrated front actin filament polymerization and back cell cortex contraction [46–48]. Since it is independent of firm adhesion to the ECM, amoeboid migration is fast—up $\sim 4 \mu\text{m}/\text{min}$ in animal models [29]—and has been observed in breast, prostate, and skin cancers. Mesenchymal migration; on the other hand, involves integrin-mediated cell adhesion [49] and tension generation on the ECM fibers [50,51]. Furthermore, it may also involve proteolytic degradation of the ECM via MMPs as cells invade [52,53], leading to much slower migration speeds (~ 0.1 to $1 \mu\text{m}/\text{min}$) than amoeboid. Zaman et al. observed DU-145 prostate carcinoma cells migrating in ECMs with varying stiffness and adhesion molecule density, and concluded that cell migration speed in this system was dependent on the balance between ECM stiffness and adhesion. Both types of migration have been observed in most tumor types, including breast, prostate, and skin cancers [1]; tumor cells are not committed to a specific migration phenotype, but rather switches between the two depending on microenvironmental factors [54,55]. For example, total MMP inhibition can transform tumor cells from mesenchymal into amoeboid phenotypes in both 3D culture and animal models [53]. Such plasticity of tumor cell invasive behavior can pose challenges for therapeutic strategies targeting invading tumor cells [56].

Mechanical Forces Involved in Cell Migration. Mechanical forces generated by cells are important during cell migration, as cells squeeze through the fibrillar ECM network and pull on ECM

fibers [see Fig. 1]. Matrix stiffness determines the extent to which cells can deform the ECM, and stiffness has been associated with the invasion of breast cancer [4] and glioma [57], among others. Compressive stresses have been found to promote phenotypic changes in cancer cells cultured in either 2D or 3D conditions toward more invasive phenotypes [58,59]. Microfabricated devices have been useful for measuring forces generated by single cells [60,61] as well as populations of cells [62]. Figure 1(b) illustrates one such example, where cells are cultured on a bed of flexible microposts that deflect as the cells pull on them; these

deflections can be measured optically to infer cellular traction forces [63]. A number of recent work estimated traction forces of single cells moving through 3D biomatrices by the movement of beads embedded within the gel [64,65]. A challenge here is the modeling of the natively derived biomatrices, which often are nonlinear in nature [66].

Chemokine and Growth Factor Gradients. Molecular gradients of oxygen and metabolites, growth factors, cytokines, and pH within a tumor microenvironment arise from numerous factors

Fig. 1 Tumor microenvironments and the corresponding in vitro models. (a) Single cell embedded in a 3D biomatrix. The cell is pulling on the collagen fiber. Dots represent adhesion molecules. (b) A microfabricated cell force sensor. The bending of the micropillars is used to report the cellular force. Graph is adapted from [60] and copyright of the Proceedings of National Academy of Sciences. (c) Molecular gradients via paracrine signaling. (d) A modified Boyden Chamber assay for tumor cell invasion and transendothelial migration under interstitial flow conditions. Graph is adapted from [22] with permission from American Association for Cancer Research. (e) Illustration of interstitial flow near a lymphatic vessel and tumor cell intravasation. (f) Microfluidic model for studies of tumor cell intravasation in the presence of a flow. (Graph is adapted from [41]. with permission from the Royal Society of Chemistry.)

including cell–cell signaling, cell and ECM heterogeneity, metabolic stress, abnormal vasculature and thus perfusion, and mechanical forces like interstitial flow [1,10,12,14,15,23,67–70] [see also Fig. 1(c)]. Gradients are particularly important in tumor invasion; for example, hypoxia in the tumor core due to rapid tumor growth, aberrant angiogenesis, and heterogeneous perfusion can drive tumor cell invasion up oxygen gradients in the tumor margin. Indeed, this may contribute to the increased metastasis that has been observed after antiangiogenic therapy [9,71]. Hypoxia can also drive the expression of growth factors and cytokines such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and interleukin-8 (IL-8) [34,72], contributing further to their heterogeneity.

Chemokine gradients can play multiple roles in shaping the tumor microenvironment, as they can attract various subsets of immune cells, drive angiogenesis, and matrix remodeling, and directly affect tumor cell survival and proliferation [3]. In the context of chemokine-mediated tumor cell invasion, specific chemokine-receptor pairs have associated with the invasion and metastasis of specific tumor cells. The chemokine (C-X-C motif) ligand 12 and its receptor CXCR4 are well documented to help drive metastasis of breast cancer, glioblastoma, and others, and CXCR4 expression has been associated with cancer stem cells [3,55,73]. The most widely used model system to study tumor cell response to chemokine gradients (i.e., chemotaxis) is the Boyden chamber [1,74], which is typically in the form of porous culture inserts with pore sizes that allow cell transmigration ($\sim 8 \mu\text{m}$) and a chamber beneath containing other cells or chemokines [see Fig. 1(d)]. Modifications to the Boyden chamber include 3D gels in the upper or lower chambers and the addition of transmembrane flow due to a pressure head [21,22], described below.

Chemokine gradients can also arise from co-cultured cells. Condeelis and colleagues used a vertically layered co-culture platform to explore how macrophages affect in tumor cell invasion [75,76]; using this setup, they identified a paracrine signaling loop between colony stimulating factor from tumor cells and epidermal growth factor from macrophages that promoted tumor cell invasion. In co-cultures of tumor cells and lymphatic endothelial cells (LECs), Issa and colleagues demonstrated that tumor-secreted factors caused LECs to secrete tumor-attracting chemokines in a positive feedback loop [77]. Fibroblasts are another important cell type in the tumor stroma that signal to tumor cells and have been studied in 3D co-culture [21,78,79].

Interstitial Flow. Interstitial flow exists in the interstitial space between the blood and lymphatic vessels [12] and is driven by hydrostatic pressure differences [Fig. 1(e)]. Interstitial flow is directed toward draining lymphatic vessels, which carry cells, fluid, and tumor-secreted factors to the sentinel or tumor-draining lymph node (LN). Since the sentinel LN is a first site of metastasis for many tumor types, the role of interstitial flow on tumor cell invasion has recently gained some research attention [17].

Using a modified Boyden chamber [Fig. 1(d)] with a pressure difference driving flow through an ECM containing various types of tumor cells, and with a layer of LECs lining the underside of the insert, the first reported effect of interstitial flow ($\sim 1 \mu\text{m/s}$) was to enhance tumor cell invasion and migration of CCR7⁺ CCL21-expressing mammary carcinoma cells [22]. The hypothesis tested was that cells with autocrine chemokine signaling loops—that is, both secreting a chemokine and expressing its receptor—could use such a system to sense fluid flow and migrate towards a draining lymphatic vessel. Using computational models [18], it was shown that pericellular gradients would form around the cell with higher concentrations in the direction of flow. In the modified Boyden chamber, flow-enhanced migration or “autologous chemotaxis” could be blocked by blocking the receptor CCR7 [22]. Such a mechanism was also demonstrated with CXCR4⁺CXCL12⁺ glioblastoma cells [20]. Recently, microfluidic devices allowing interstitial flow have been developed to

directly visualize autologous chemotaxis [23] and study tensional effects between the ECM and tumor cells [24,25] [see also Fig. 1(f)].

One challenge of 3D cell culture, particularly when using tension-generating or contractile cells such as fibroblasts, is maintaining counteracting tension in the 3D matrix to avoid overall gel contraction. To overcome this limitation, a radial flow chamber was developed in which a thin annulus of porous polyethylene membrane was sandwiched between two glass slides to host the cell-embedded collagen [80]. The porous polyethylene, with pores of $\sim 100 \mu\text{m}$, served to anchor the collagen that permeated it before gelation, while the glass was pretreated with glutaraldehyde for collagen attachment. Flow entered from a polyethylene rod in the center and flowed outward such that a tenfold velocity range existed from inlet to outlet. Using this model, Shieh et al. demonstrated interstitial flow can also drive TGF- β -dependent autologous chemotaxis of fibroblasts that guided tumor cells in the flow direction [21].

The advantage of the modified Boyden chamber for chemotaxis studies is its ease of use, commercial availability, and high throughput nature. However, it does not provide a well-defined chemical gradient, does not allow for live cell imaging, and generally gives only endpoint results, making it difficult to differentiate increased (random) motility from directed migration, for example. Microfluidic models have been developed to overcome these limitations and allow more quantitative analysis of tumor cell migration under well-defined molecular gradients, interstitial flows, and with various co-culture possibilities [26,81].

Tumor Cell Intravasation Across Endothelium

Once a tumor cell approaches a blood or lymphatic vessel, it must cross the endothelial barrier. Here, again, modifications to the Boyden chamber has been used most widely [Fig. 1(d)]. In this case, a monolayer of endothelial cells are plated on the underside of the insert, and transmigration of the tumor cells from 3D matrix, across the porous insert, and through the endothelial layer is assessed at an endpoint. Using this model, it was found that LECs enhance human mammary carcinoma cell transmigration [22]. In another modification of this model, human mammary carcinoma (MCF-7) spheroids were placed atop LEC monolayers plated on insert membranes. This resulted in highly reproducible formation of circular discontinuities, mediated by tumor-secreted lipoxigenase, in the endothelial monolayers that correlated with tumor invasiveness [82]. To directly observe tumor cell interactions and transmigration across endothelial monolayers, however, microfluidic models have been developed in various configurations allowing a perfused, endothelial-lined channel in contact with a 3D ECM embedded with tumor cells [43,83–88].

Circulating Tumor Cells

Circulating tumor cells (CTCs) are considered indicative of tumor dissemination to distant organs and represent an important diagnostic marker due to access to a patient’s blood. The challenge, however, is the relative rarity of CTCs in a blood sample (as few as one cell in 10^9 hematologic cells). Microfluidic devices provide unique opportunities for capturing rare tumor cells, particularly in that they can be easily designed and fabricated to contain geometric barriers for blood cell filtration, large surface-to-volume ratios, and modified surfaces for CTC capturing [89–91]. Briefly, the CTC chip contains a microfluidic channel with embedded microposts for enhancing the surface-to-volume ratio, and the posts are coated with antibodies that recognize tumor cells. Recent work from the Toner lab using an antibody against EpCAM demonstrated that a CTC chip can capture spiked rare tumor cells with a 99% confidence level. Furthermore, they could successfully identify CTCs in the peripheral blood of patients with metastatic lung, prostate, pancreatic, breast, and colon cancer in 115 of 116 samples. The Kirby lab used an antibody against

prostate-specific membrane antigen to coat the microposts and demonstrated capture of CTCs from peripheral whole blood samples of castration-resistant prostate cancer patients [91].

Tumor Cell Adhesion, Extravasation, and Growth in Ectopic Sites

For blood-borne CTCs to form successful metastases in a secondary site, adhesion to the endothelium followed by extravasation, in a manner similar to that of leukocytes, is thought to be required [92]. Adhesion molecules that are common to leukocyte-endothelial interactions [93], such as selectins, have been implicated in cancer metastasis [94–99]. The most widely used in vitro models that mimic this process are standard parallel-plate flow chambers in which tumor cells introduced into the medium flowing over a monolayer of endothelial cells and imaged [100,101]. For example, using this model, E-, P-, and L-selectins have been found to mediate adhesion events [94,101,102]. However, numerous clinical observations have demonstrated that different tumor types have distinct organ-specific metastatic potential [44,103,104], and organ-specific chemokines are known to promote relevant tumor cell adhesion to endothelium and facilitate processes of extravasation into the target tissue [105,106]. To this end, the Fischbach lab developed a mineralized 3D tumor model to investigate the roles of mineral hydroxyapatite (HA) in breast tumor cell progression and found that tumor cell adhesion, proliferation, and secretion of pro-osteoclastic IL-8 was increased in mineralized tumor models as compared to nonmineralized tumor models [107]. However, our understanding of how the physical and chemical microenvironments at the ectopic site impact the initiation and growth of a new tumor remain limited, and more physiologically relevant in vitro models are needed that incorporate target tissue cells underneath the endothelial cells.

Perspectives and Future Development

Looking ahead, a major challenge is to develop the types of aforementioned in vitro tumor models into devices that are easy to use, high-throughput, and highly reproducible so that they can be used more readily for biological experiments and thus have more impact in cancer biology. Furthermore, they must contain enough complexity to faithfully recapitulate the problem at hand, while maintaining straightforward analysis. As discussed above, simpler devices like the Boyden chamber allow for more high-throughput experimentation, while the more recent microfluidic models typically require engineering knowledge to use, are lower throughput, and suffer from difficult reproducibility. Another major challenge is that developing and using such models requires interdisciplinary cooperation between biologists and engineers, although more bioengineers are being trained in tumor biology labs. A third challenge is to create a set of data analysis tools that can be used easily by biologists, clinicians, and engineers needed to understand large data sets generated by such models. The long-term hope is that human in vitro models will one day replace mouse models in recreating tumor microenvironments for the purpose of diagnostics, preclinical testing, and basic cancer biology research.

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