

Building Better Tumor Models: Organoid Systems to Investigate Angiogenesis

Venktesh S. Shirure, Mary Kathryn Sewell-Loftin, Sandra F. Lam, Tyson D. Todd, Priscilla Y. Hwang, and Steven C. George

Abstract Cancer remains a leading cause of death in the United States and other developed countries. In nearly all cases, the cause of death is related to complications associated with tumor metastasis to distant sites such as the brain, lung, liver, and bone. A central feature of tumor progression is the acquisition of a blood supply, which provides nutrients for the growing tumor as well as conduits for transport of cancer cells. Our understanding of how a tumor acquires and manipulates a blood supply has been gleaned largely from animal models, but more recent advances in tissue engineering and microfabrication have led to clever 3D in vitro models of tumors that include blood vessels. This chapter will first briefly review the process of blood vessel growth including our knowledge of blood vessels within the cancer microenvironment, and discuss the most recent advances to mimic blood vessel growth in the tumor microenvironment using 3D in vitro culture methods. Finally, we discuss several important factors that control blood vessel growth including hypoxia, cellular metabolism, and tissue mechanics, which provide rich opportunities for future investigation.

Keywords 3D models • Tissue engineering • Cancer microenvironment • Hypoxia • Mechanics • Metabolism • Metastasis

1 Introduction

Angiogenesis, the process of vessels sprouting from existing blood vessels, is a critical event in numerous pathophysiological processes, including embryogenesis, wound healing, inflammation, diabetes, and cancer. Early growth of a neoplastic tissue engenders a metabolic deficit (e.g., glucose and oxygen) that limits growth. Compensatory mechanisms that permit additional growth include changes in

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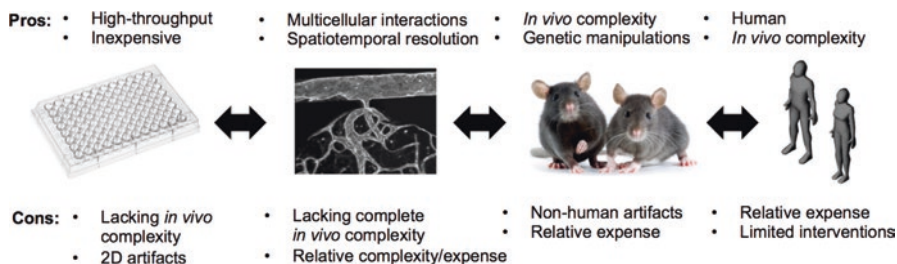


Fig. 1 Advantages and disadvantages of tumor model systems. Experimental model systems of the tumor microenvironment (TME) broadly include four categories: (a) traditional 2D monolayer cell culture, (b) 3D in vitro multicellular models, (c) animal models, and (d) human models. While simple and inexpensive, 2D monolayer culture cannot replicate the essential heterotypic cell-cell and cell-matrix interactions that have proven essential in the biology of the TME. Animal models can replicate the integrated response of the whole animal, but many times the immune system is compromised to allow the study of human cells and the temporal and spatial resolution of the TME is severely limited. While humans represent the “perfect” system, studies are limited to more advanced cancers, and most interventions, including genetic manipulation, are not possible. Advanced 3D in vitro multicellular models allow the step-by-step incorporation of key components in the TME, and high spatial and temporal resolution of dynamic events. While it may be difficult to fully recapitulate the TME in vitro, new advances in microfabrication and imaging provide opportunities to tease apart complex cell-cell and cell-matrix dynamics in the TME. The arrows linking the models are purposely two-way as observations made in one system can answer questions, but also generate new hypotheses that can be tested or confirmed in alternate systems. While all four approaches are important, a 3D in vitro model can provide unique opportunities to study angiogenesis in the tumor microenvironment

metabolism and the acquisition of a blood supply. The access to vasculature allows tumors to disseminate cancer cells to distant organ sites, leading to the formation of metastatic lesions. The hypothesis that angiogenesis is required for tumor growth was first proposed by Folkman in 1971 [1]. Thereafter, there has been reaffirmation of this concept by independent groups [2–5]. As a result, anti-angiogenic therapy is part of the treatment regimen for some types of solid cancers including breast, lung, and renal cancers.

The biology of angiogenesis within the tumor microenvironment has been largely studied in either mouse models or simple 2D monolayer culture systems (Fig. 1). Although these classical model systems have provided a wealth of understanding, they have limitations and there remains a need for novel model systems that can further improve our understanding of angiogenesis. Animal models offer a complex tissue environment compared to 2D cell cultures, but the biology of these animal models is simply not human. A more recent model used to study cancer biology is the patient-derived xenograft (PDX) in which a human tumor is implanted either subcutaneously or orthotopically in an immunocompromised mouse. The major limitation in the subcutaneous model is that it lacks important features of the original tumor microenvironment. In contrast, orthotopic tumor xenografts are implanted at the original tumor site and are considered more reliable for studying the biology or predicting drug response in humans. Nonetheless, in both cases the tumor xenografts are surrounded by non-human tissue stroma, developed in the absence of a

normal immune response, and take several months to grow [6, 7]. Furthermore, the animal models are expensive to maintain, are not high-throughput, provide barriers to some imaging technologies, and provide inherently limited spatial and temporal resolution. The 2D culture systems, on the other hand, are much easier to develop and utilize. However, these systems lack the fundamental complexity of the multicellular 3D tissue microenvironment. These limitations have led to the development of 3D culture systems, which are positioned between 2D and animal models in terms of advantages and limitations (Fig. 1).

The idea of mimicking 3D tissue function *in vitro* is not new in cancer research; in fact, tumor spheroids were first presented nearly four decades ago [8]. Since then, the model systems have evolved from 3D tumor spheroids comprised solely of cancerous cells to tumor spheroids composed of a mixture of cancer and stromal cells, vascularized tumors, and more recently perfused vascularized tumors [8–10]. Despite the recent progress that has been made in the field using conventional biological models, we still do not have a complete picture of the impact of microenvironmental factors that dictate the formation and maintenance of cancer-associated blood vessels. Herein we provide a limited discussion of the biological pathways of angiogenesis; unique features of vessels within the cancer microenvironment; the important roles of hypoxia, cellular metabolism, and mechanics on tumor angiogenesis; and tumor metastasis as a backdrop to understand how the creation of *in vitro* 3D tumor organoids can be used to further augment our understanding of tumor angiogenesis and its role in tumor progression.

2 Cell Signaling Pathways in Tumor Angiogenesis

The growth of blood vessels from existing blood vessels to meet the metabolic needs of a tumor defines tumor angiogenesis (Fig. 2a) [11]. The important pro-angiogenic factors involved in cancer are vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and angiopoietin-1 (Ang1) [12]. These pro-angiogenic factors activate otherwise quiescent vessels and “turn on” the so-called angiogenic switch. VEGF has been extensively studied for how it activates the angiogenic program [12]. Vessel exposure to proangiogenic factors leads to vasodilation and increased vascular permeability. The endothelial cells lining the vessel degrade the basement membrane by proteolytic activity of matrix metalloproteinases (MMP). The endothelial cells detach and organize into a new branch in which the endothelial cell leading the branch is called a tip cell and the endothelial cells following are called stalk cells (Fig. 2b). The tip cells sense gradients of morphogens and guide the direction of the new sprout. The tip cell restricts the stalk cells from transforming into other tip cells by secreting delta-like ligand 4 and signaling through the NOTCH-mediated pathway. The stalk cells, on the other hand, are responsible for proliferating and forming continuous extension branching from the original vessel. Eventually vessel junctions are reestablished, proteolytic activity is neutralized, the basement membrane is synthesized, and pericytes are recruited

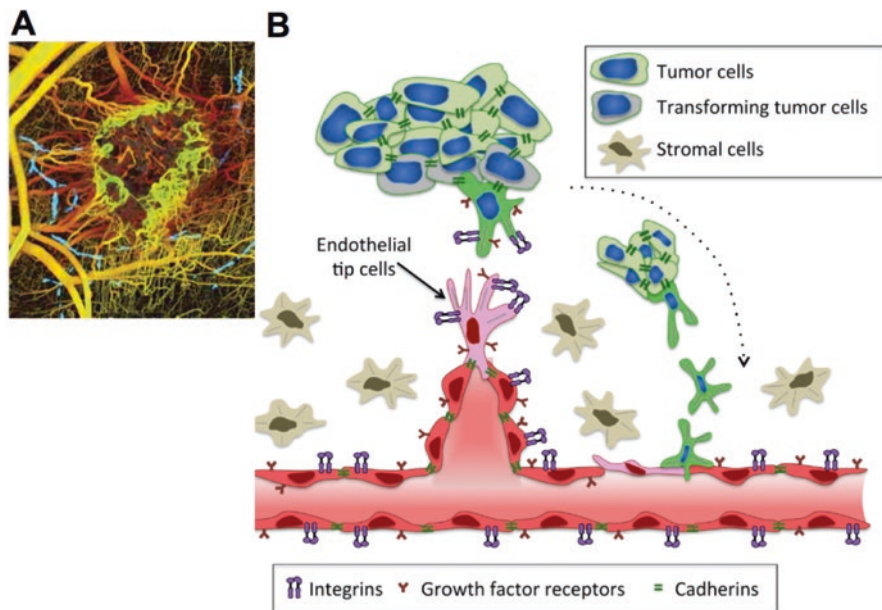


Fig. 2 Angiogenesis in the tumor microenvironment. (a) Angiogenesis is required to promote tumor growth. The angiogenesis in tumors of mice was captured using optical frequency domain imaging (OFDI) technique. This high resolution microscopy shows tumor associated vasculature is dense and unorganized compared to surrounding non-tumor tissue. The red and yellow indicate the depth of the tissue and blue indicates lymphatics. Scale bar = 500 μ m (reprinted with permission [11]). (b) Numerous signaling factors, including soluble growth factors and membrane bound receptors, integrins, and junction proteins, play a role in the development of vasculature during tumor progression. Such signaling is regulated both via tumor cells as well as vascular cells, including the tip cell of blood vessels undergoing angiogenesis

to stabilize the nascent vessels. The processes that stabilize the vessels are conducted by numerous molecular pathways mediated by platelet-derived growth factor B (PDGF-B), ANG-1, transforming growth factor- β (TGF- β), ephrin-B2 and NOTCH. The WNT signaling pathway also plays a role in tumor angiogenesis [13, 14]. Endothelial cells express an array of WNT ligands and their frizzled (FZD) receptors, some of which are essential for stimulating endothelial cell proliferation [14]. One of the regulators of WNT signaling is β -catenin. Activation of WNT/ β -catenin signaling can induce numerous tumor growth genes including Myc, Axin2, and Zeb1 in vivo. In contrast, abnormal β -catenin activation and signaling has been associated with solid stress from tumor masses [13, 14].

Another important cell signaling pathway that regulates vessel integrity is the angiopoietin (ANG) and TIE signaling system; this pathway stimulates basement membrane deposition to promote vessel tightness [15, 16]. However, dysregulation of ANG and TIE signaling in sprouting endothelial cells can lead to vascular permeability, inflammation, and defects that allow for tumor metastasis [17]. Finally, p21-activated kinases (PAKs) alter RhoGTPase signaling, causing irregular actomyosin

contractility and actin dynamics, and altered cell motility and permeability in endothelial cells [18, 19]. Furthermore, work by Ghosh et al. has shown that endothelial cells in the tumor microenvironment demonstrate aberrant Rho activity and fail to respond to mechanical strain in the same manner as normal endothelial cells [20]. In addition to altering cell motility and permeability, disruption of actin dynamics also changes contractile forces and tension in cells, resulting in changes to the transcriptional regulators YAP and TAZ [21–23]. Studies are beginning to investigate the exact mechanisms that YAP and TAZ have on endothelial cells in cancer-associated blood vessels, but some preliminary work reveals that translocation of YAP/TAZ to the nucleus can upregulate the expression of target genes such as connective tissue growth factor (CTGF) and can lead to an increase in monocyte adhesion to endothelial cells [21].

3 Organoid Systems to Model Angiogenesis

The early 3D systems were mainly devoted to create vessels to study activation of angiogenic programs. In these systems, either a single suspension of endothelial cells or endothelial cell coated microbeads were embedded into extracellular matrix (ECM) gels, such as collagen, fibrin, or Matrigel [24, 25]. These gels were placed in micro-well plates or similar assemblies to generate a 3D culture system. These approaches have successfully yielded capillary microvessels with lumens, but these systems have several limitations. The vasculature was not designed for perfusion of fluid through the vascular lumen, the vasculature formed was not stable over time, and creating controllable temporal and spatial concentration gradients around the vasculature is difficult.

The rapidly emerging “organ-on-a-chip” field utilizes tissue engineering and microfabrication to create in vitro microtissues in platforms that are optically clear, cost-effective, have high spatial and temporal resolution, can capture events in real time, and have the potential to be high-throughput. These systems are proving critical for uncovering novel aspects of angiogenesis. The general method to fabricate the organ-on-a-chip platform begins with the computer-aided design of the device. A mask is printed from these designs, and used to create a master mold. The master mold is fabricated using soft lithography techniques, in which a silicon vapor coated with photoresist, such as SU8, is covered with the mask and exposed to UV. The UV light polymerizes the exposed area of photoresist, which is the area of device design on the mask. The non-polymerized photoresist is etched, and the master mold is used to create numerous replicas of the device design using polymers such as polydimethylsiloxane (PDMS). The devices made of PDMS are ideal for many cell culture applications, including tumor organoids, as they are permeable to oxygen, transparent, and have a similar refractive index to glass to facilitate optical imaging.

We and others have developed several microfluidic platforms that capture features of the human microcirculation (Fig. 3) [26–32]. A critical feature of the

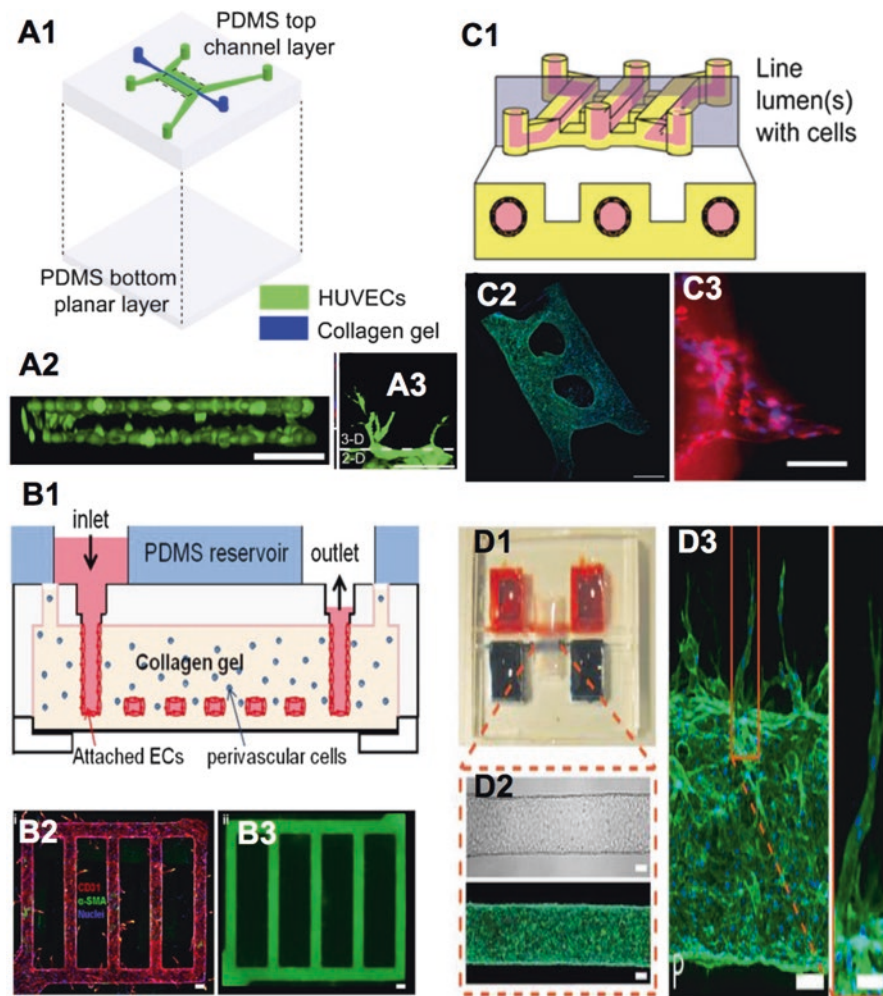


Fig. 3 In vitro blood vessels created by coating microfluidic channels with endothelial cells. **(a1)** Device design for coating endothelial cells on the surfaces of the PDMS and collagen gel. **(a2)** The endothelial cells (*green*) form monolayer in the device, and **(a3)** angiogenesis is observed from 2D coat into 3D collagen gel (reprinted with permission from [30]). **(b1)** Device design to create hollow tubes of circular cross section in collagen gel by using silicon master mold. The surface of the collagen tube are coated with endothelial cells to form a vessel. **(b2)** These vessels stained with endothelial specific CD31 (*red*) sprout and **(b3)** exhibit barrier function as they retain dextran in the lumen (*green*). Scale bar in **b2** and **b3** shows 100 μm (reprinted with permission from [27]). **(c1)** The channels in the hydrogel were developed by using viscous finger patterning and then **(c2)** endothelial cells (*green*) were coated on the hollow structures. **(c3)** These vessels show angiogenic response (*red*) to VEGF concentration gradients. Scale bar in **c2** and **c3** show 500 and 50 μm , respectively (reprinted with permission [32]). **(d1)** PDMS device in which a cylindrical tube is created in collagen gel using needle of 400 μm diameter. **(d2)** The gel cylinder was coated with endothelial cells (*green*). **(d3)** When the vessels exposed to angiogenic factors show angiogenesis. Scale bars in **d2** and **d3** are 100 μm . Scale bar in the insert of **d3** is 50 μm (reprinted with permission from [31])

microcirculation is the hollow capillary structure through which blood or a blood substitute can flow. The studies use mainly two approaches to create these structures. The first approach is lining a conduit with endothelial cells. Some studies first create a perforated rectangular shaped conduit in PDMS, and coat one side or all sides of the conduit with endothelial cells [30]. Others have used a more sophisticated approach of creating a cylindrical conduit in an ECM gel (i.e., collagen or fibrin) and then coat the lumen of the cylinder with endothelial cells [27, 31]. Once the endothelial cells adhere to the wall of the conduit and spread they form tight junctions with physiological permeability coefficients [27]. This method generally creates large diameter ($>100\ \mu\text{m}$) endothelial cell-lined tubes [27, 31, 32]. These endothelial-lined tubes can then be exposed to various levels of concentration gradients, which are created by using additional microfluidic lines. There are several creative designs to establish concentration gradients using microfluidics. These device designs initiate angiogenesis in response to physiological concentration levels of VEGF, bFGF, and several other proangiogenic factors or cocktails of factors [27, 31, 32]. As the endothelial cells are coated, a challenging task is to maintain the density of endothelial cells and shape of the tube to accurately mimic the *in vivo* vasculature.

The second approach to generate microvessels follows the developmental process of vasculogenesis, in which the endothelial cells are encouraged to self-assemble into capillaries (Fig. 4). In this method, the microvessels are produced from endothelial cells and stromal cells that are initially randomly distributed in an ECM. The stromal cells are a necessary component as they secrete factors necessary to support vessel formation, in particular tube formation and stabilization [33–35]. Our lab has shown that cord blood-derived endothelial cells and human lung fibroblasts in a fibrin gel generate dynamic, interconnected, and perfusable networks of microvessels [29]. When implanted in the mouse, the microvessels anastomose to mouse vasculature and become functional [36, 37]. This co-culture system provides a more physiologic alternative, compared to assays using only endothelial cells [27, 31, 32], to mimic *in vivo* angiogenesis. Moreover the physical dimensions, including diameter, of the microvasculature formed by our assay resembles that of the *in vivo* microvasculature ($<50\ \mu\text{m}$).

Vasculogenic vessel formation has also been shown to be facilitated by other types of stromal cells, such as bone marrow derived stromal cells [28]. In the context of tumor angiogenesis, such a system could be of interest, as bone marrow is one of the most frequent metastatic sites for multiple types of solid tumors, including breast, and colon cancers. Our lab has been working to develop microfluidic systems to place tumors in the immediate vicinity of perfused microvascular tissue developed by this vasculogenic process [38]. These systems are designed to recapitulate microenvironment of early and advanced tumors and study angiogenesis in response to the microenvironmental perturbations described in the following sections.

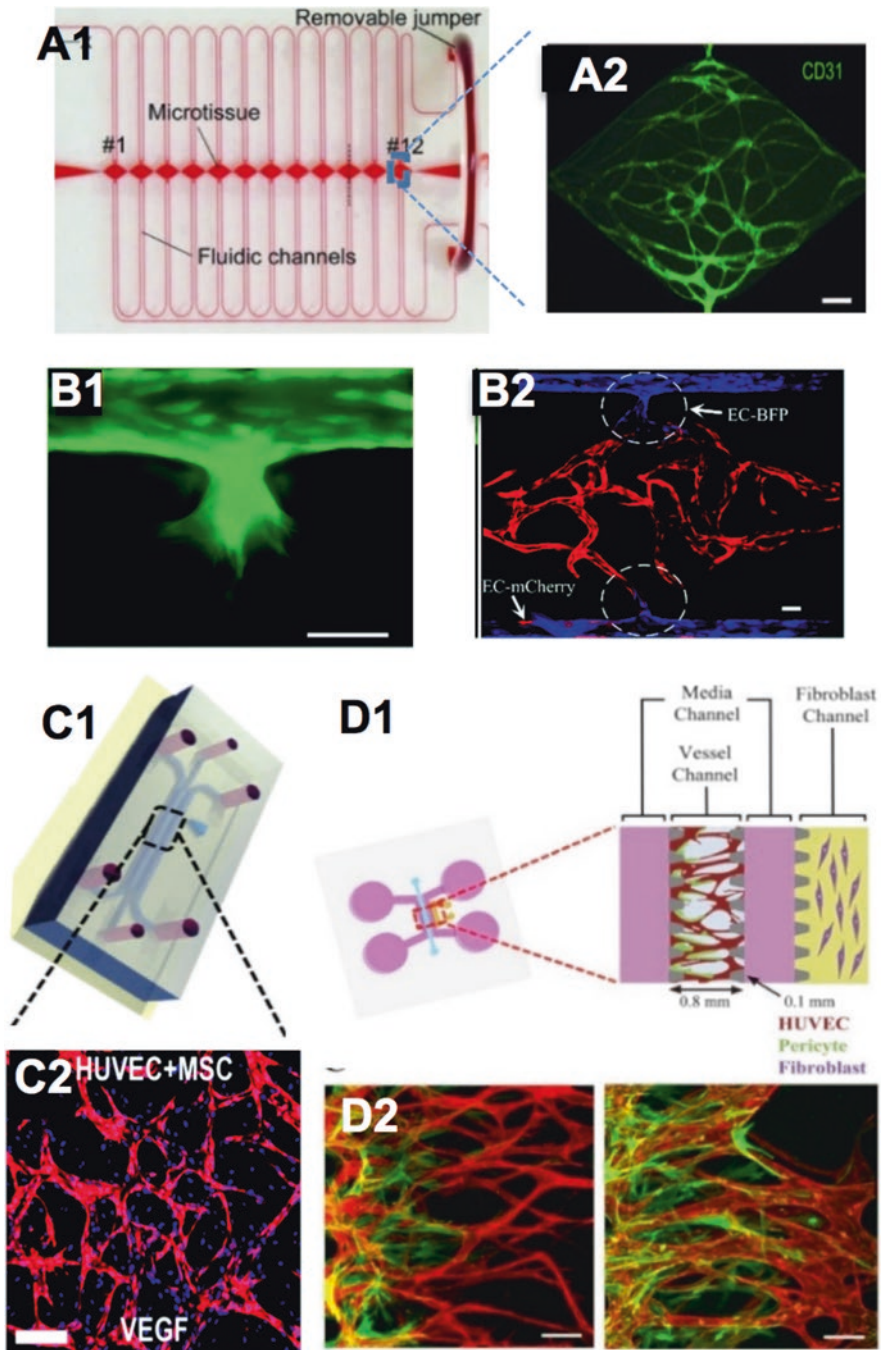


Fig. 4 Microfluidic systems to create in vitro vascular networks by vasculogenesis process. **(a1)** Device design for creating endothelial network from randomly distributed endothelial cells and normal lung fibroblasts in fibrin gel. **(a2)** The endothelial cells (*green*) form interconnected network of vessels (reprinted with permission from [29]). **(b1)** One approach to connect the vessels

4 Hypoxia and Tumor Angiogenesis

4.1 Overview

The deficiency of oxygen, an essential nutrient for cell proliferation and survival, is a critical stimulus for acquiring new blood vessels. Hypoxic tumors activate molecular programs that lead to secretion of proangiogenic factors by the tumor as well as tumor-associated stromal cells. Tumor hypoxia has been associated with poor patient prognosis, with clinical studies showing that advanced breast cancers have a median oxygen tension of 10 mmHg, compared with 65 mmHg in normal breast tissue [39–41]. Hypoxic cores exist in advanced stage tumors [42], and can also exist in tumors as small as 400 μm in diameter [43]. In hypoxic conditions, angiogenesis is primarily regulated by hypoxia inducible factors (HIFs). Of the highly conserved HIF family of transcription factors, HIF-1 has been the best studied [44–46]. It is known to be a heterodimer of α -subunit (HIF-1 α) and a β -subunit (HIF-1 β), where subunits are members of the basic helix-loop-helix (bHLH)-containing PER-ARNT-SIM (PAS) domain family of transcription factors. In addition to HIF-1 α and HIF-1 β , there are two additional oxygen regulated α -subunits (HIF-2 α and HIF-3 α) and two other constitutively expressed β -subunits (HIF-2 β , and HIF-3 β). Furthermore, the low oxygen environment stabilizes HIF-1 α in endothelial cells as well [47].

The HIF-1 activation follows a series of molecular events. Starting at oxygen concentrations below 6%, HIF-1 α stabilizes and translocates from the cytoplasm to the nucleus, where it dimerizes with HIF-1 β [48]. HIF-1 then binds to hypoxia responsive elements (HREs) within the promoters of HIF target genes leading to the increased expression of proangiogenic factors such as vascular endothelial growth factor (VEGF), VEGF-R2, angiopoietin 1/2, fibroblast growth factor, platelet-derived growth factor, and the decreased expression of anti-angiogenic factors such as thrombospondin-1 and carbonic anhydrase-9 [49]. In addition to angiogenesis, HIF-1 can activate more than a hundred genes that control important cellular processes such as epithelial-mesenchymal transition, stem-cell maintenance, and metabolism that impact tumor cell invasion, metastasis, metabolic reprogramming, and resistance to therapy [4].

Fig. 4 (continued) with the fluidic lines of PDMS device is to coat the fluidic lines with endothelial cells. **(b2)** The endothelial cells in the fluidic line connect with the vessel network in the gel forming a perfused network of vessels (reprinted with permission from [35]). **(c1)** Microfluidic platform design used to create vascular network from bone marrow derived mesenchymal stem cells and endothelial cells in fibrin gel. **(c2)** The micrograph shows endothelial network in this device (*red*). The scale bar is 200 μm (reprinted with permission [28]). **(d1)** Microfluidic platform used for co-culture of endothelial cells and pericytes in fibrin gel. **(d2)** The system supports formation of endothelial network (*red*) formation with pericyte coverage (*green*). The scale bar is 100 μm . The figure is reprinted from [26], and is covered under Creative Commons Attribution (CC BY) license

4.2 *Controlling and Measuring Oxygen In Vitro*

The traditional method to create hypoxic conditions utilizes cell culture incubators, where blending excess nitrogen with air lowers oxygen concentration. Alternatively, the exchange of oxygen from air can be controlled by an air tight glove box equipment, or hypoxic conditions can simply be generated due to consumption of oxygen by cell culture. Additionally, chemicals that consume oxygen, such as sodium nitrate, can also be used to manipulate oxygen tension [50]. Alternatively, cobalt chloride can stabilize HIF-1 α in the presence of normoxia, and allows for more flexible data collection. This “pseudo-hypoxic” condition can simulate the impact of HIF-1 α , but cannot fully recapitulate all features that hypoxia has on cell function [51].

Microfluidic devices have become attractive systems to study hypoxia due to their inherently small size, and thus small diffusion distances. A common technique to reduce oxygen in microfluidic devices is to use separate channels containing an oxygen scavenger such as sodium nitrate (Fig. 5). These channels are separated from the tissue chambers by a semipermeable material, such as PDMS, that allows diffusion of oxygen but not water [52–54]. By altering the concentration and flow of the scavenger, the oxygen tension within the device can be controlled with high spatial and temporal resolution. PDMS is ideally suited as a material of construction for these device as it is a highly permeable material with respect to oxygen compared to relatively impermeable materials such as cyclic olefin copolymer, polystyrene, polypropylene, poly(methacrylic acid), polyurethane, and poly(methyl pentene) [55]. By choosing an appropriate coating and/or using an oxygen scavenger, a wide range of oxygen concentrations can be controlled to study tumor hypoxia and its effects.

A major advantage of using in vitro systems is that real time oxygen measurements can be performed in a live tissue culture with minimal disruption of biological processes. The gold standard for the oxygen sensors is Clark-type electrodes which measure oxygen by detecting a current flow caused by the reduction of oxygen [56]. However, the method is operationally complex and less sensitive for oxygen measurement relative to other methods. Recently, more sensitive techniques have been developed that employs an oxygen sensitive luminophore. The luminescence of oxygen sensitive dyes is inversely proportional to the concentration of oxygen. When the dyes are excited by a laser in the presence of oxygen, the excited state energy of the phosphorescent indicator molecule is absorbed by oxygen instead of being emitted as a luminescent photon. In other words, oxygen quenches the phosphorescence, and reduces the lifetime of the phosphorescence decay. Generally, a shorter luminescence lifetime indicates a higher oxygen concentration. The lifetime of the phosphorescence, as opposed to the intensity, is a more robust method as it is insensitive to photobleaching and independent of the concentration of the dye. Detecting the luminescence lifetime generally requires a more complicated experimental setup because a pulsed laser needs to be used [57].

While many research groups have focused on controlling the oxygen environment around tumor spheroids, some groups, including ours, have begun to control

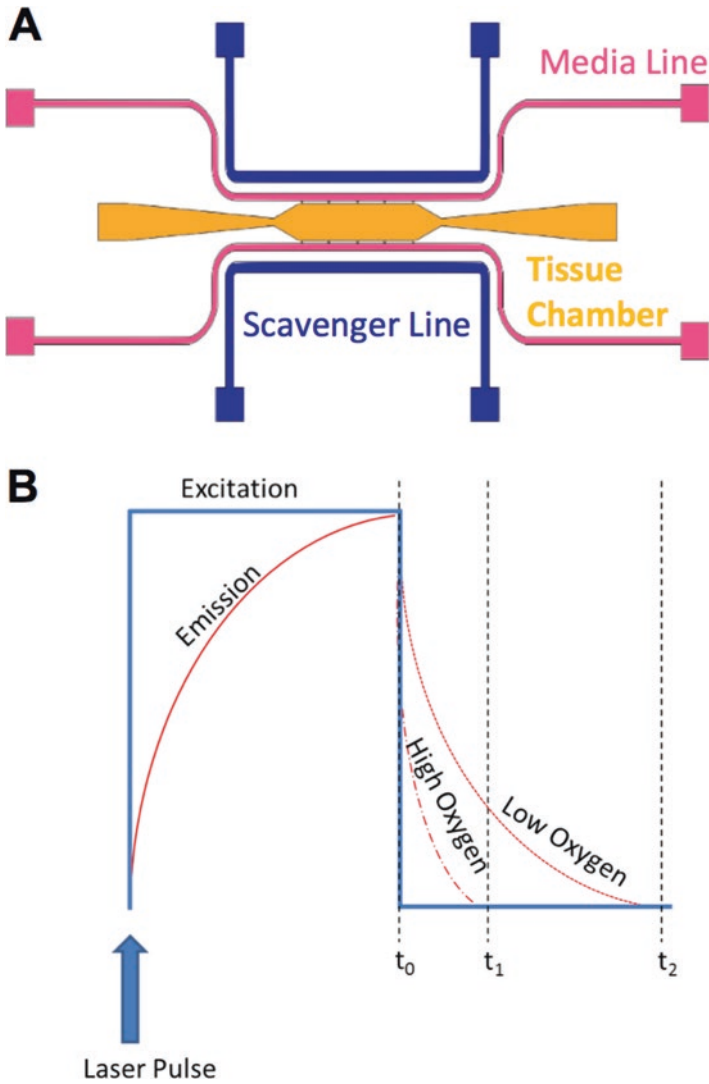


Fig. 5 Manipulating and measuring oxygen concentrations in vitro. (a) Oxygen scavenger lines can be designed into microfluidic platforms to generate hypoxic conditions inside tissue chambers. Typically these include materials such as sodium sulfite. (b) PhLM is a method used to measure oxygen concentrations in 3D culture systems. Using a pulsed laser to excite the oxygen sensitive dye and measuring the dye’s lifetime of decay, a longer phosphorescent lifetimes correspond to lower oxygen concentrations

oxygen tension in vascularized tumors [52, 53, 58]. Due to the role that the vascular network has in oxygen regulation and the interaction between the tumor and the vasculature during hypoxia, the inclusion of these components in the next generation of tumor organoid models is critical for a complete understanding of angiogenesis in the tumor microenvironment.

5 Cellular Metabolism and Angiogenesis in the Tumor Microenvironment

5.1 Overview

Endothelial cells act as a semi-permeable barrier between the circulating blood and various tissues. Being in direct contact with the blood, endothelial cells have the most ready access to the nutrients needed for healthy cell growth, including glucose, glutamine, and oxygen but are also responsible for delivering these nutrients to the surrounding tissue. Endothelial cells are able to balance their own metabolic needs and transport duties by executing a specific metabolic program that shares many similarities with cancer cell metabolism.

Endothelial cells are highly glycolytic and consume glucose at a high rate. Even during quiescence, endothelial cells generate more than 80% of their ATP through glycolysis alone [59, 60]. Glycolysis in endothelial cells tends to favor lactate as its end product, as less than 1% of pyruvate generated by glycolysis is oxidized in the tricarboxylic acid (TCA) cycle. By reducing the utilization of oxidative phosphorylation (OxPhos) and thus reducing the amount of consumed oxygen, they are able to more effectively deliver oxygen to the tissues.

When appropriate signals are received to form tip cells and induce angiogenesis, phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFK2 or PFKFB3) expression is upregulated (Fig. 6). PFK2 converts fructose-6-phosphate into fructose-2,6-bisphosphate (F2,6BP), a potent regulator of phosphofructokinase-1 (PFK1) which converts F6P into fructose-1,6-bisphosphate (F1,6BP), considered the first committed step in glycolysis. VEGF and fibroblast growth factor 2 (FGF2) have been shown to increase PFK2 expression and glycolysis. Kruppel-Like Factor 2 (KLF2), a transcription factor which responds to hemodynamic-induced stress on the EC glycocalyx, has been shown to reduce PFK2 expression in quiescent endothelial cells (De Bock et al.; Doddaballapur et al.; De Bock, Georgiadou, and Carmeliet). By upregulating PFK2 and increasing levels of F2,6BP, PFK1 activity and flux through glycolysis are both greatly increased. In many other cells, such as immune cells, this activation would lead to a 20- to 30-fold increase in glycolytic flux but only a two-fold increase occurs in endothelial cells. The end products, lactate, can later be used as a mitochondrial fuel by other stromal cells or regenerated through gluconeogenesis after reaching the liver.

Similar to endothelial cells, most tumor cells are highly glycolytic even in the presence of oxygen, known as Warburg Metabolism, and have reduced OxPhos [61]. In contrast to endothelial cells, tumor cells have a high rate of proliferation, and consume large quantities of glucose [61]. The rapid use of glucose and excretion of lactate in the tumor microenvironment stimulates angiogenesis. In low glucose conditions, endothelial cells can utilize Fatty Acid Oxidation (FAO) and amino acid metabolism, especially glutaminolysis, to supplement their energetic and macromolecular needs. FAO catabolizes circulating triglycerides to create acetyl-CoA, which can then be used for energy production in the TCA or for lipid synthesis and

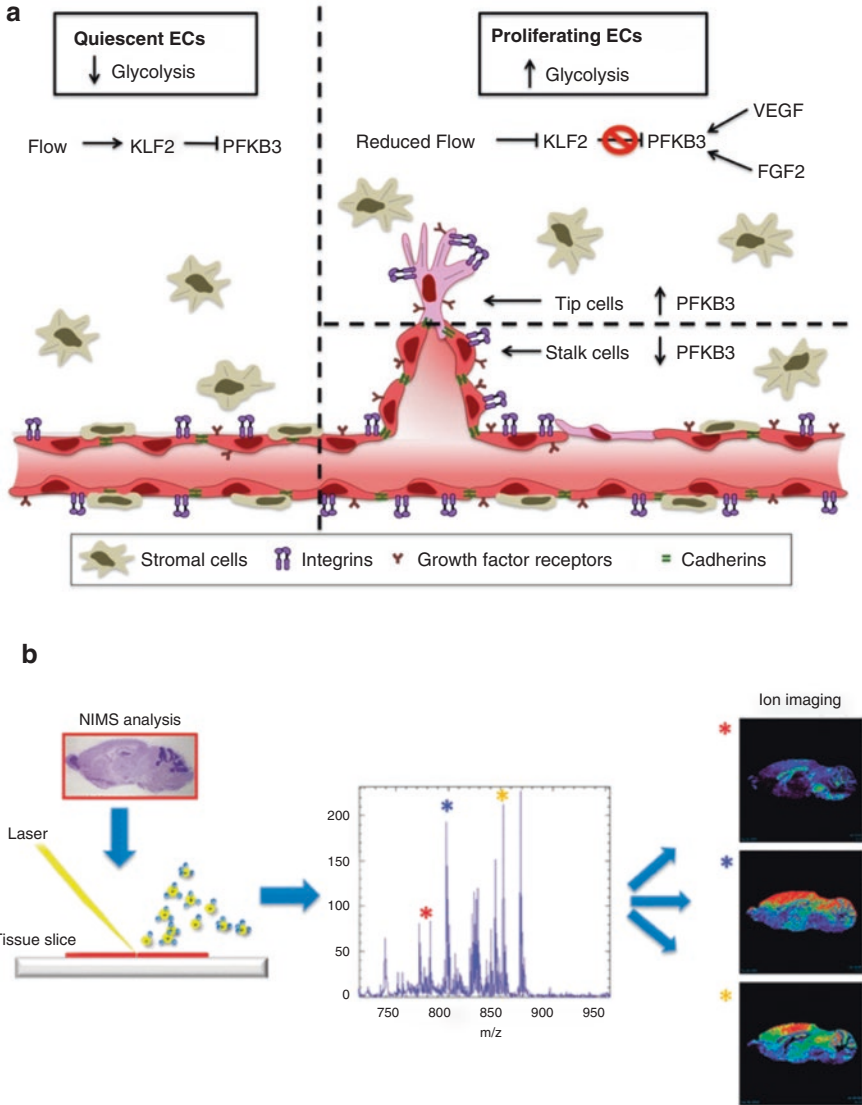


Fig. 6 Endothelial cell metabolism in cancer associated angiogenesis. **(a)** In normal tissues, ECs in blood vessels demonstrate a quiescent phenotype, with limited glycolysis for energy production due to hemodynamic stress-induced stimulation of Kruppel-Like Factor 2 (KLF2). In tip cells, glycolysis is upregulated by several growth factors and downregulation of KLF2 by reduced flow also serves to increase glycolytic flux as ECs become activated to proliferate and develop new vasculature via angiogenesis. **(b)** Nanostructure Imaging Mass Spectrometry of rat brain sections b. to resolve metabolic differences by brain region (reprinted with permission from [62]). An initiator is used, similar in function to the matrix from MALDI techniques, to desorb and ionize metabolites before analysis by mass spectrometry. Each metabolite can be visualized in its own ion image, as shown to the right, which allows for label-free spatial tracking of multiple metabolites in a tissue sample

also yields NADPH as a byproduct. Glutaminolysis catabolizes glutamine, whose concentration in tissues is typically 5 μM , yielding glutamate, which is then converted to α -ketoglutarate and can enter the TCA cycle. Glutamate can also act as a nitrogen source for amino acid or nucleotide synthesis and an NADH source. These alternative energy sources facilitate angiogenesis into the tumor microenvironment, which is typically hypoxic and low in nutrients necessary for proliferation.

5.2 *Methods to Characterize Cellular Metabolism In Vitro*

There are several techniques that have been utilized to study metabolism in 2D culture and are being adapted to study 3D tissues in vitro. Three promising options include the Seahorse Extracellular Flux (XF) Assay, Fluorescence Lifetime Imaging Microscopy (FLIM), and Mass Spectrometry (MS) based metabolomics analysis. Direct in vitro studies of metabolism in complex tissues, including organoids composed of multiple cell types, create many challenges compared to standard 2D metabolic analyses due to the heterogeneity of the system and the need to analyze multiple focal planes.

The SeaHorse XF assays are useful to characterize a broad metabolic phenotype (i.e., primarily glycolytic or OxPhos) using a microwell plate format. They employ a probe containing an embedded oxygen-sensitive fluorophore and an embedded proton-sensitive fluorophore to monitor minute changes in acidity and oxygen concentration which can then inform the oxygen consumption rate and the extracellular acidification rate. The former is characteristic of OxPhos whereas the latter is indicative of glycolysis with lactate as the terminal end product (Fan et al.). The XF assays were originally developed for 2D cell culture, but have since been adapted to analyze spheroids as well, allowing for the rapid profiling of organoids grown in spheroid plates [62]. This system has the advantage of being label free, high-throughput, customizable for reagent studies, and fully adapted to 3D assays. However, it measures only two of the tens of thousands of possible metabolites, and contains no spatial resolution.

Fluorescence Lifetime Imaging Microscopy (FLIM) utilizes confocal or multiphoton microscopy with rapidly pulsed lasers to detect the lifetime of endogenous fluorophores. FLIM can offer incredible temporal resolution, on the order of nanoseconds, of the chemical state of a system. In vivo and in vitro, NADPH, NADH, and FAD are fluorescent molecules with a myriad of functions tied to the metabolic state of the cell and the ratios of protein bound to unbound forms is indicative of cellular metabolism [63, 64]. FLIM is incredibly sensitive due to the natural sensitivity of the fluorophores to their local chemical environment; bound forms of these molecules show a significant increase in lifetime (for NAD(P)H, 3.2 ns) over their unbound, free solution forms (for NAD(P)H, 0.8 ns) and can thus be used to metabolically profile cells [65, 66]. More recently FLIM has been used in 3D tumor organoids to assess cell proliferation [67]. Because this is a microscopic technique, it also offers high spatial resolution, permitting insight into the subcellular ratios of

each of these fluorescent molecules. NADH and NADPH are nearly identical spectrally, but there have been some inroads into differentiating their FLIM signals, which is an important distinction due to the distinct roles of NADH in energy production and NADPH in biomolecule production and redox state maintenance [68, 69]. FLIM, however, is limited to those metabolites which are inherently fluorescent, and requires relatively expensive equipment.

By far the most robust technique for studying metabolism is Liquid Chromatography-Mass Spectrometry (LC-MS). LC-MS allows for the detection and quantification of nearly the entire metabolome of a sample. In 2D cell culture, cells are grown in culture medium, fixed (usually with ice cold methanol), and the metabolites are extracted using a mixture of organic and aqueous solvents before being passed through a chromatography column and analyzed by mass spectrometry. The combination of LC-MS allows for the separation of metabolites based on both the retention time (LC) and mass to charge ratio (MS) that allows for high resolution detection and identification of each metabolite. By comparing the metabolomes or specific metabolites of two nearly identical samples grown in different conditions, known as differential metabolomics, enriched pathways dependent on these differences can be elucidated. In addition, isotope labeling allows for the tracking of metabolites through different pathways through the detection of strong isotope peaks and the use of metabolic flux analysis using isotopically labeled metabolites yields a more complete picture of the metabolic network. The main shortcoming of this technique is that this represents an “average” metabolome for the sample, so extending this technique to 3D tissues results in a total loss of spatial resolution and a lack of cell specificity. To ameliorate the loss of specificity, cell sorting techniques can be used although this also presents its own set of challenges and disadvantages in sample handling.

Mass Spectrometry Imaging (MSI) is a Matrix Assisted Laser Desorption/Ionization (MALDI) variant that uses a laser and specific analyte preparation to desorb and ionize metabolites from finely sectioned tissues before running typical mass spectrometry and analysis. The details of MSI preparation and analysis are active areas of research and have been recently reviewed [70]. However, the result of an MSI experiment recreates the metabolome at each point with sub-micrometer resolution while retaining the spatial organization of the analyzed tissue [62]. By analyzing adjacent tissue sections histologically, metabolic differences between adjacent cells are resolvable as well. Very recently, MSI has been used to investigate tumor organoids in 3D to investigate topics such as drug delivery/penetration and the impact of hypoxia [71–73].

All three techniques outlined here have inherent strengths and weaknesses. Both XF and FLIM techniques can repeat measurements on the same sample and require little preparation, but offer somewhat limited information about the system, while LC-MS and MSI offer more complete information, but require more time, effort, and preparation to execute and analyze and are terminal experiments. As this is still a developing field of research, future advances may offer greater ease or scope for these techniques or new techniques altogether but metabolic studies of complex 3D tissues are finally possible. Being able to analyze cellular metabolism in tumor organoids will create a more complete picture of tumor angiogenesis.

6 Biomechanics in Tumor-Associated Angiogenesis

6.1 Overview

In addition to changes in metabolism and oxygen tension, biomechanical forces can regulate angiogenesis [74, 75]. Physical forces, including extracellular matrix (ECM) stiffness, compressive/contractile forces exerted due to cell proliferation and other cellular activities, and fluid forces exerted by blood and plasma flow have all been shown to be critical regulators of angiogenesis (Fig. 7) [76–82]. The composition, and therefore effective stiffness, as well as the organization of the ECM surrounding the tumor also plays a role in mechanically regulating angiogenesis [83–88]. Increased peritumoral ECM stiffness correlates with increased potential for angiogenesis and metastasis. Furthermore, both tumor cells and endothelial cells

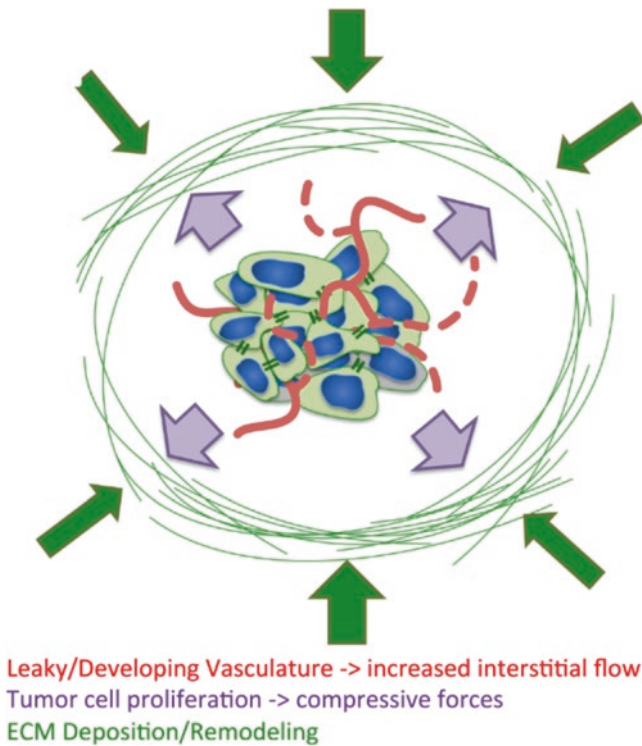


Fig. 7 Macromechanical forces in tumor progression. Typically, three different types of forces are considered important in tumor progression. Interstitial flow (*red*) generated by developing vasculature within the tumor promotes vessel growth at the periphery of the mass. Compressive forces and strains (*purple*) increase as tumor cells divide uncontrollably, putting pressure on the surrounding ECM. Finally, ECM surrounding the tumor can apply compressive forces (*green*) as more ECM is deposited by tumor and stromal cells, effectively creating a dense capsule of tissue containing the tumor mass

can actively remodel the ECM to facilitate enhanced angiogenesis as well as promote metastasis. Compressive forces in the tumor microenvironment are a result of the unchecked proliferation of tumor cells that are constrained by surrounding ECM and stromal cells [14, 89]. Finally, at the cellular level, contractile behaviors of cells in the tumor microenvironment can alter angiogenesis and tumor progression [90–93]. Enhanced mechanosignaling from cancer-associated fibroblasts (CAFs) and tumor cells alter ECM alignment and remodeling, promoting both enhanced angiogenesis and tumor cell metastasis [94–99]. Recent work has demonstrated that paracrine regulation to limit the number of tip cells occurs through the Notch pathway, which has been shown to be mechanosensitive [100, 101]. Furthermore, the shift from quiescent vascular endothelial cell to migrating tip cell mimics the phenotypic change seen in epithelial to mesenchymal transformation (EMT). In both cases cell-cell junction proteins are dramatically downregulated and cells become more migratory. Several groups have demonstrated that EMT is regulated through active biomechanical forces including cell-cell tension and contractile forces [92, 102–104].

Endothelial cells are also exquisitely sensitive to fluid flow including intraluminal flow, interstitial flow over and around the basolateral surface, and intercellular (transmural) flow between cell junctions. While interstitial flow of plasma can enhance angiogenic signaling in endothelial cells as well as invasive pathways in tumor cells, luminal flow of blood through vessels limits the angiogenesis [30, 105, 106]. Importantly, the leaky and tortuous nature of blood vessels in the tumor microenvironment impacts the magnitude and variance of all of these flows. Luminal flow exerts shear stress that regulates nitric oxide production by endothelial cells, which in turn limits endothelial cell sprouting and angiogenesis [30]. The interstitial flow of plasma leaks across the capillary wall and is reabsorbed in post capillary venules. Thus, the interstitial flow exerts transmural shear forces on the endothelial junctions, pressure forces from apical to basal, and basal to apical sides of the endothelial tube. The transmural flow has been shown to facilitate angiogenesis in a shear stress dependent manner [107]. Transmural flow is characterized by Starling's Law, in which the driving force is the arithmetic sum of hydrostatic and oncotic pressure differences across the wall of the vessels. The transmural flow exerts shear stress, the magnitude of which not only depends on the flow across the vessels but also on the size of vessel perforations. Interestingly, the vessel perforations in organs throughout the human body vary, indicating differential potential of organs for angiogenesis in response to transmural flow. The basal to apical interstitial flow, like that in post-capillary venules, has been shown to activate and directionally guide angiogenesis [108]. However, apical to basal flow, like that in capillaries, does not activate angiogenesis, indicating the direction of flow is important in activating the angiogenic program (Fig. 8).

Finally, tumor cells themselves can sense shear stress, leading to changes in the production of soluble mediators that directly impact angiogenesis such as VEGF, HIF1, and matrix metalloproteinase 9 [109, 110]. The shear forces are transmitted in cells by several mechanisms, including integrin signaling pathways and surface glycocalyx signaling. Integrins on the surface of endothelial cells attach to the ECM

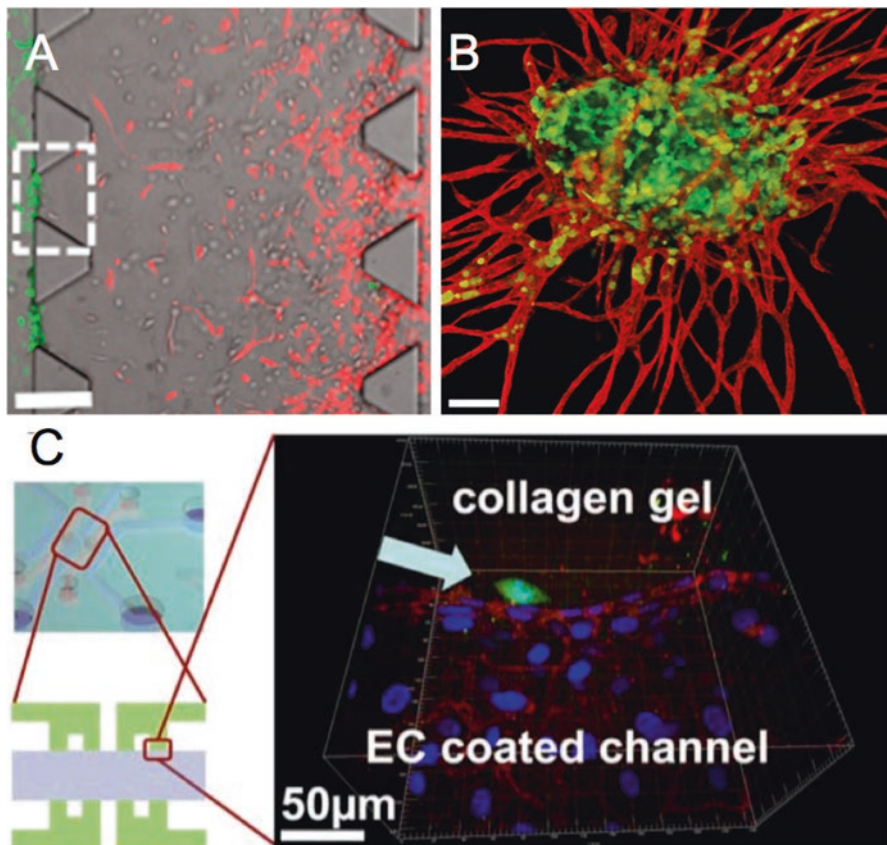


Fig. 8 Angiogenesis models for studying metastasis. (a) A microfluidic device that simulates features of intravasation includes a collagen filled central chamber with HT1080 fibrosarcoma cells (red) migrating towards an endothelial cell (green) lined fluidic line. Scale bar 30 μm (reprinted with permission from [155]). (b) An alternate 3D model of intravasation includes a spheroid of cells comprised of both endothelial cells (red) and SW620 colon carcinoma cells (green) embedded in a collagen gel that contains fibroblasts. Microvessels sprout from the spheroid and cancer cells intravasate and migrate within the vessel lumen. Scale bar 100 μm (reprinted with permission from [9]). (c) A model of tumor cell extravasation using a similar microfluidic device as in (a). Here endothelial cells line a fluidic channel (gray) and a collagen gel (green) is placed adjacent to the abluminal surface at specified regions. Tumor cells (breast cancer, MDA-MB-231) are introduced through the microfluidic channel, and evidence of extravasation is demonstrated (white arrow). Red label is VE-cadherin, and blue is the DAPI stained nuclei of the endothelial cells (reprinted from [160] and is covered under Creative Commons Attribution (CC BY) license)

surrounding the cells and begin the formation of focal adhesions (FAs) inside the cell. Over 200 proteins have found to be associated with FA formation and many of these or their downstream effectors are either mechanosensors or mechanoregulators. A full review of FA and glycocalyx signaling in tumor-associated angiogenesis is outside the scope of this book chapter but can be found in other excellent reviews [81, 111–116].

6.2 *Angiogenesis Models to Elucidate the Impact of Mechanical Forces*

Early understanding of biomechanical pathways involved in tumor-associated angiogenesis came exclusively from 2D monolayer cultures, either on uncoated or ECM-protein coated tissue culture plastic or glass. Another level of complexity in 2D monolayers is derived when polyacrylamide (PA) gels are functionalized with collagen or fibronectin before cells are seeded onto the substrate [117–119]. The advantage of this system is that the PA gels can be synthesized over a wide range of stiffness values with great control. ECM proteins, either collagen or fibronectin, can then be covalently bonded to the PA gels permitting cell culture. Numerous groups have used these gels to probe the mechanoregulation of cell behavior including FA formation and regulation of cell contractile behavior. The disadvantage of this widely used system is that it is limited to 2D studies. Recently, work from the Takyama lab has utilized this protocol in conjunction with PDMS-soft lithography and 3D collagen gels to generate hybrid platforms that allow for spatial control over cell seeding with the benefit 3D cell culture in ECM-based gels [120]. The 2D PA system has also been utilized to study the effects of matrix stiffness, specifically crosslinking of collagen by lysyl oxidase, on the upregulation of VEGF in hepatocellular carcinomas [119].

Additionally, there have been several studies that demonstrate remarkable differences in 2D and 3D signaling behavior, especially of endothelial cells [121]. A key demonstration of this occurs in integrin signaling regulation where FAs in 3D are formed and degraded much more quickly than in 2D. FAs are dynamic in 3D and the inhibition of specific integrins can either promote or inhibit faster migration [87, 113, 122, 123]. Results from studies such as these have prompted a new wave of tumor angiogenesis research incorporating the techniques used in tissue engineering to generate 3D organoids to replicate the native tumor microenvironment. These models have gone through several iterations, at first only including tumor cells and then adding more cell lines to recapitulate the complex multicellular environment. This includes stromal or support cells, immunological cells, and endothelial cells in the surrounding blood vessels. While numerous research groups have adopted 3D, multicellular approaches to study specific tumor types, metastatic potential, and cellular pathway regulators, there has been limited investigation of biomechanical regulation of tumor progression, specifically angiogenesis, in this type of model.

Tissue engineering protocols allow for several methods of spatial control over cellular seeding in either synthetic polymer or ECM-derived materials. One of the approaches is a “self-assembled” technique, in which cells are simply mixed together for co-culture before casting of the matrix in a premade mold or dish, and sometimes includes external mechanical stimulation from moving culture platforms including orbital shakers or rotating vessel wall bioreactors [85, 124–127]. Furthermore, many of these studies represent nascent research in developing novel platforms and are limited in their ability to interrogate the role of biomechanics in angiogenesis associated with tumor progression. Bates et al. demonstrated that

blocking integrin function in such self-assembled organoid models of colorectal cancer blocked tumor progression [128]. Vascularized liver buds have been generated via these same techniques, with a possible dependence on stromal cell contractile behavior for tissue assembly [129–131]. The advantages of this protocol are that the cells naturally orient themselves in a manner replicating the native *in vivo* tumor microenvironment. However, there is incomplete spatial control of this process, limiting the type of results that can be gleaned from such studies. However, these models are still important tools to understand tumor angiogenesis, and have demonstrated the importance of CAFs in promotion of tumor associated blood vessel growth through factors including VEGF, HIF-1a, caveolin-1 [31, 97, 132].

Another approach to investigate the role of biomechanics on angiogenesis in 3D tumor organoids involves the use of animal models. A recent study demonstrated the mechanosensitive nature of angiogenesis using the avian chorioallantoic membrane (CAM) model in the developing avian embryo [133]. Rings containing a collagen gel were implanted on top of the membrane, with tension induced in only the outermost layer of tissue. Harvested gels showed invasion of blood vessels due to tensile forces generated by the implant. This same model was utilized by another group to explore how crosslinks affecting biomechanical properties of the collagen gels altered VEGF production in tumor spheroids seeded onto the CAM [134].

Microfluidic model systems, as described above, provide a novel technique to study the effects of luminal and interstitial flow in a 3D tumor microenvironment containing self-assembled vasculature network with the further advantage of high levels over spatiotemporal seeding, flow conditions, and ease of visualization in real time during experiments. By controlling device parameters and fluidic pressures in feeding chambers, the precise direction and magnitude of interstitial flow can be manipulated, allowing for creation and monitoring of soluble signaling factor gradients that alter angiogenesis in the tumor microenvironment. We have developed multiple microfluidic devices that controls interstitial flow and permits tumor growth in the presence of self-assembled vasculature [29, 38, 135–137]. Others have utilized similar techniques to study the effects of ECM composition and stiffness, effects of diffusion of growth factors, alterations in cell phenotypes in co-culture and nascent vasculature biomechanical properties such as permeability [138–142]. The continued advance of model systems will continue to enhance our understanding of the role of mechanical forces on angiogenesis in the tumor microenvironment.

7 Intravasation and Extravasation in Tumor-Associated Angiogenesis

7.1 Overview

Numerous outstanding recent reviews are available that cover the general metastatic process of cancer [143–146], as well as those focusing specifically on the role of the endothelium including intravasation (tumor cells entering the circulation) and

extravasation (tumor cells exiting the circulation) [147–150]. In addition, a recent review describes *in vitro* and *in vivo* models that have been developed to probe the metastatic process including some of the very recent advances in microfluidic and “organ-on-a-chip” technologies [151]. Thus, this section will succinctly review the metastatic process, features of this process that involve the circulation which have been captured with *in vitro* models, and then focus on features of the metastatic cascade which specifically involve the circulation that have proven difficult to simulate including possible strategies moving forward.

Tumor metastasis is the process by which a primary tumor is able to successfully move, or metastasize, to another location in the body. While complex, many steps in the metastatic cascade have been described. For an epithelial-based tumor (which comprise approximately 80% of all tumors), the process can be summarized in five steps: (1) dedifferentiation from an epithelial phenotype to a migratory mesenchymal cell phenotype, usually termed epithelial-mesenchymal transition (EMT), (2) intravasation of the mesenchymal phenotype tumor cell, or clusters of tumor cells, from the primary tumor into the circulation, (3) survival within the circulation, (4) attachment to an endothelial cell at a distant site and extravasation from the circulation, and (5) survival and differentiation in a receptive stroma from the mesenchymal tumor cell phenotype back to an epithelial cell phenotype, termed mesenchymal-epithelial transition (MET). Engaging the circulation is a necessary step for successful metastasis. Intravasation, survival in the circulation, and extravasation (steps 2–4) all uniquely require or utilize the vascular network. Although specific features of these events have been demonstrated *in vitro*, several important features have not, and numerous important questions remain unanswered.

Intravasation occurs in what is generally referred as the metastatic niche, a tumor microenvironment that contains the necessary factors for successful migration and entry into the circulation. The development of the metastatic niche is complex but involves the release of growth factors and trophogens from the endothelium that encourage the clustering of tumor-associated myeloid cells, platelets, and tumor cells towards the vascular supply. For example, endothelial cells in cancer-associated blood vessels have differential expression of adhesion molecules, P- and E- selectin, that recruit attachment of leukocytes to the metastatic niche [74]. Also, increased release of stromal derived factor-1 (SDF1) from endothelial cells leads to recruitment of endothelial progenitor cells to the metastatic niche. The recruitment of additional cell types, along with altered expression on endothelial cells, leads to a cascade of cell secreted factors, such as VEGF, endostatin, and other pro-tumor growth factors that characterize the metastatic niche and contributes to metastasis [74, 152]. Using murine and zebrafish models, and 3D organotypic microvascular niches, Ghajar and co-workers demonstrated that endothelial tip cells of cancer-associated blood vessels have decreased expression of pro-dormancy factor, thrombospondin-1, and enhanced expression of pro-tumor factors, periostin and TGF- β 1, that encourages tumor cell migration [153].

7.2 *Methods to Investigate Intravasation and Extravasation of Tumor Cells*

Intravasation from within the metastatic niche requires the tumor cell(s) to cross the basolateral side of the endothelial cell. This necessitates overcoming the endothelial basement membrane and intercellular functional proteins. There is general consensus that intravasation occurs in vessels that are part of the tumor microenvironment and thus have characteristic features which are different from vessels in non-cancerous tissue including increased permeability and less basement membrane. Several relatively sophisticated 3D in vitro models of intravasation have recently been presented. At least two groups have utilized soft lithography to create a microfluidic device to mimic intravasation. In both cases tumor cells could migrate across an ECM hydrogel (collagen or fibrin) and then engage the abluminal surface of an intact layer of endothelial cells [154, 155]. Zervantonakis et al. coated a microfluidic line on the other side of the ECM that was lined with a confluent layer of endothelial cells [155]. The tumor cells could then penetrate the abluminal surface of the endothelial cells, thus mimicking intravasation. Strengths of this approach include the 3D migration of tumor cells in response to controlled gradients, and the controlled migration of tumor cells across an endothelial monolayer. The models are also easily adaptable to include other cells such as macrophages [155], and stromal cells [154]. In either case, the endothelial cell phenotype was not conditioned by the tumor microenvironment and only immortalized cell lines were utilized.

Ehsan et al. presented an alternate strategy to create a 3D in vitro model of intravasation by co-culturing endothelial cells and tumors cells in a spheroid and placing this spheroid in a fibrin gel [9]. A spontaneous vessel network formed within the spheroid and also sprouted from the spheroid. A colon cancer cell line intravasated into the vessel network, and they showed this process was related to EMT and the expression of the transcription factor SNAIL. Strengths of this model include the creation of a vascular network in close proximity to the tumor, and a true 3D vascular network. The surrounding matrix did contain stromal cells, but their work was also limited to immortalized cancer cell lines and there was no flow within the vessel lumens.

Mimicking the step of tumor cell survival in the blood vessel is problematic due to the complexity of blood and its components (e.g., platelets, leukocytes, clotting factors) and the mechanical microenvironment. Most cancer cells in the circulation do not survive; those cancer cells that do survive are able to overcome the shear stress and immune system by aggregating together and/or interacting with platelets [149]. These events have generally been captured using in vivo mice models and post-sacrifice observations of metastasis [156, 157]. This approach has been useful to identify some of the key cells and proteins involved, but lack temporal and spatial resolution. No in vitro model to date has been able to capture these dynamic events.

Extravasation requires the tumor cell(s) to cross from the luminal side of the endothelial cell, and thus crossing the intercellular junctional proteins and basement membrane, in that order. In contrast to intravasation, extravasation occurs at sites

distant from the primary tumor and thus the endothelium is generally considered to be normal, but specific to the organ. Many details of how a tumor cell attaches and transmigrates the endothelium have been worked out using Transwell© [158] chambers and 2D laminar flow chambers [159]. Some of the mechanisms parallel the steps of neutrophil adhesion and paracellular transmigration including the expression of PECAM1 and E-Selectin on the endothelial cells and $\alpha_v\beta_3$ integrin and CD44 on the cancer cell. A more advanced 3D microfluidic model was recently reported and demonstrated flow and extravasation of cancer cells through a microfluidic channel lined with endothelial cells [160]. The major weaknesses in the current *in vitro* models of extravasation are the lack of organ endothelial specificity. Most models have utilized human umbilical vein endothelial cells (HUVECs) [158, 161] cultured on a fibronectin coated membrane of collagen gel, and thus they do not contain organ specific features of the endothelial cell [162] or vascular architecture [163]. Overcoming these challenges in the mimicry of tumor cell extravasation represents a tremendous opportunity to enhance our understanding of tumor progression.

8 Summary and Future Directions

In this chapter, we have discussed the process of angiogenesis in tumor organoids, the development of novel model systems for its study, as well as numerous results garnered from such studies. Increasingly, researchers are trying to recapitulate the complex native *in vivo* tumor microenvironment to provide an enhanced understanding of tumor progression for the purpose of developing novel therapeutic strategies. Initial research strategies utilized simple co-culture systems, either 2D or 3D, or mouse models, both of which present limited spatial or temporal control in elucidating the cues that regulate angiogenesis and tumor progression. The recent trend is the generation of sophisticated organ-on-a-chip systems where researchers can control spatial and/or temporal patterning of cells in matrices that mimic the native tumor tissue. Furthermore, use of microfluidic systems based on optically transparent materials permits real-time analysis of angiogenesis in the tumor microenvironment. Modular control over factors including hypoxia, shear flow, biomechanical properties, and gradients of growth factors permit interrogation of tumor progression at previously unimaginable resolution and physiological relevance.

As this field develops further, we predict that we will generate models combining the numerous factors discussed in this chapter, to generate *in vitro* systems that fully recapitulate the complex, native tumor microenvironment. By enhancing our understanding of how these features alter not only tumor cell behavior, but also endothelial cells of tumor-associated vasculature and the processes accompanying tumor development including intravasation/extravasation, we increase the likelihood of a breakthrough scientific discovery that will allow for development of novel anti-cancer treatment strategies, targeting the processes we are only beginning to fully understand due to our refined models of tumor organoid angiogenesis.

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