Advanced Biomaterials and Microengineering Technologies to Recapitulate the Stepwise Process of Cancer Metastasis

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ABSTRACT

Cancer is one of the leading causes of death globally according to the World Health Organization. Although improved treatments and early diagnostics have reduced cancer related mortalities, metastatic disease remains a major clinical challenge. The local tumor microenvironment plays a significant role in cancer metastasis, where tumor cells respond and adapt to a plethora of biochemical and biophysical signals from stromal cells and extracellular matrix (ECM) proteins. Due to these complexities, there is a critical need to understand molecular mechanisms underlying cancer metastasis to facilitate the discovery of more effective therapies. In the past few years, the integration of advanced biomaterials and microengineering approaches has initiated the development of innovative platform technologies for cancer research. These technologies enable creating biomimetic *in vitro* models with physiologically relevant (i.e. *in vivo*-like) characteristics to conduct studies ranging from fundamental cancer biology to high-throughput drug screening. In this review article, we discuss the biological significance of each step of the metastatic cascade and provide a broad overview on recent progress to recapitulate these stages using advanced biomaterials and microengineered technologies. In each section, we will highlight the advantages and shortcomings of each approach and provide our perspectives on future directions.

Keywords: Biomaterials, Microengineering Technologies, Microfluidics, Tumor model, Angiogenesis, Intravasation, Extravasation

1. Introduction

Cancer is currently the second leading cause of death in the United States claiming the lives of over half a million Americans annually [1]. Poor patient prognosis and treatment outcomes are in large part due to the biological complexities of this devastating disease [2-5]. Cancer metastasis progresses through a stepwise cascade of events, including tumor growth (Fig. 1A), angiogenesis, stromal invasion, intravasation (Fig. 1B), extravasation, and colonization at secondary sites within the body (Fig. 1C) [3]. It is well recognized that the tumor microenvironment plays a substantial role in the metastatic process through dynamic biochemical and biophysical signaling cues. Due to these complexities, there is an overwhelming need to develop a fundamental understanding of cancer metastasis [4-10].

Animal models have been instrumental for studying the cellular and molecular basis of the metastatic process [11, 12]. However, due to the presence of confounding factors and physiological variability between humans and animals, it has been challenging to evaluate cause-and-effect relationships between specific biological cues and the resulting cancer cell behavior [13, 14]. In particular, these differences have led to failures in translational research towards therapeutic drug development [12]. Additionally, the effectiveness of anti-cancer drugs can substantially vary between patients due to heterogeneities in their molecular profiles [15]. Thus, there has been a heightened initiative to develop *in vitro* tumor models that closely mimic cancer pathophysiology, thereby providing more predictive platforms for personalized medicine.

To date, *in vitro* three-dimensional (3D) culture models (e.g. spheroids and cell-laden hydrogels) have been widely utilized to represent individual steps of the metastatic cascade [16]. Contrary to conventional two-dimensional (2D) assays, encapsulation of cancer cells within 3D biomaterials better recapitulates several aspects of the *in vivo* tumor microenvironment with more precise control over biophysical and biochemical signaling cues [17]. Specifically, cancer cells grown in 3D matrices self-organize similarly to their *in vivo* architecture [16, 18]. These models also enable studies with physiologically relevant gene/protein expression, gradients of small molecules/cytokines, and autocrine/paracrine signaling [17]. However, a critical component in the development of 3D models is synthesis and selection of proper scaffolding biomaterials that closely recapitulate the native tumor extracellular matrix (ECM).

Natural biomaterials, such as collagen and basement membrane extracts (e.g. matrigel), have been widely utilized in cancer related studies due to their abundance within the native

tumor microenvironment [19, 20]. Additionally, the relative bioactivity of these materials provides instructive cues that promote a similar cytoarchitecture to the *in vivo* organization [19, 20]. However, the use of natural matrices is associated with batch-to-batch variability and limited control over material properties (e.g. stiffness, matrix architecture) [20]. For instance, extracting basement membrane proteins from different *in vivo* locations (e.g. adipose tissue, parenchymal tissue) leads to inconsistent protein composition of the matrix [21] and variability in their mechanical and biochemical properties [22]. Consequently, natural matrices have a defined physical architecture that cannot easily be tailored to study the influences of biophysical cues on tumor progression. Alternatively, synthetic biomaterials enable precise tunability of biophysical properties such as matrix architecture and stiffness; however, their bioactivity is limited due to the lack of necessary cell-binding moieties and biochemical cues, thereby misrepresenting the *in vivo* physiology [23]. Due to these limitations, recent studies have focused on utilizing hybrid or composite materials with tunable matrix properties that mimic the native complexities of the tumor ECM [24, 25].

While 3D scaffolding biomaterials have led to significant advances in our understanding of tumor biology, they do not allow for precise modulation of critical factors involved in tumor progression, such as spatial organization of stromal and cancer cells, spatiotemporal gradients of cytokines, etc. [26]. To overcome these limitations, there has been a strong initiative to integrate advanced biomaterials with microengineering technologies (e.g. microfluidics and micropatterning), to provide an in-depth representation of various steps of cancer metastasis [26]. In this review article, we highlight recent developments of 3D tumor models that integrate advanced biomaterials with microengineering technologies. We outline the use of different types of biomaterials, including natural, synthetic, and composite hydrogels. Additionally, we will cover various microengineering technologies, such as microfluidics [27], micropatterning [28], micromolding [29], and bioprinting [30], used to model critical biological aspects of the metastatic cascade. Within each section, we will provide perspectives on current challenges and future directions to improve these platform technologies and gain unique insight into cancer biology.

2.1 Tumor Initiation and Microenvironmental Interactions

Tumor growth involves genetic and epigenetic mutations in cancer cells, which lead to upregulation of oncogenes and downregulation of tumor suppressors [31]. This phenomenon

initiates tumor formation at varying locations in the body and results in unpredictable phenotypic variations from patient to patient [32]. Despite the heterogeneity inherent to tumor development, all cancers typically share common “hallmarks” as defined by Weinberg and Hanahan [33]. Specifically, cancer cells of any origin may demonstrate uncontrollable replication, insensitivity to growth suppressors, resistance to cell death, and self-sufficiency in growth signaling. Furthermore, they exhibit increased cellular motility/metastatic potential as well the ability to regulate the formation of new vasculature (i.e. angiogenesis) [31]. Highly proliferative cancer cells have a competitive advantage within the microenvironment, becoming the dominant contributors to tumor growth by a process known as clonal selection [34]. Although genetic and epigenetic alterations force malignancy, the microenvironmental cues unique to each cancer type cause high heterogeneity in the progression of metastasis [35].

Typically, within normal epithelial tissue, cells are spatially organized with tight intercellular junctions and apical-basal polarity which maintains tissue homeostasis [36, 37]. Tumor expansion disrupts the organization of the parenchyma within the surrounding normal tissue [38]. During abnormal transformation of tumor and stromal cells, tissue polarity and tight intercellular junctions are lost by a process known as the epithelial-mesenchymal transition (EMT). Throughout the EMT, cancer cells downregulate epithelial markers (e.g. E-cadherin), and upregulate mesenchymal markers, such as N-cadherin [39]. Cells transition to a mesenchymal phenotype, becoming highly motile with an increased number of protrusions and enhanced myosin-based contractility [40]. The formation of the tumor also initiates a cascade of biochemical and biophysical signaling cues that recruit surrounding stromal components (e.g. capillaries, fibroblasts, immune cells), which in turn provide a favorable environment for tumor expansion [41].

The crosstalk between stromal fibroblasts and cancer cells specifically leads to secretion of various pro-fibrotic cues, such as TGF- β [42]. These stimuli initiate a process known as the mesothelial-mesenchymal transition (MMT), wherein stromal fibroblasts adopt a myofibroblast-like phenotype and differentiate to cancer associated fibroblast cells (CAFs) [4, 42]. Similar to myofibroblasts recruited during an acute injury (i.e. wound-healing response), CAFs exhibit a significantly higher proliferation rate and deposit excess collagen within the local tumor microenvironment [42]. In addition, CAFs have been shown to upregulate cancer cell proliferation and invasion by secreting growth factors such as stromal cell-derived factor 1

(SDF-1 α) [43]. These cells are also capable of promoting tumor angiogenesis through the secretion of pro-angiogenic factors (e.g. vascular endothelial growth factor (VEGF)) [44], indicating their synergistic role in driving cancer metastasis. On the other hand, immune cells have a diverse impact on cancer progression [45]. Several studies have demonstrated that CD8⁺ T cells and natural killer (NK) reduce tumor metastasis by inducing cancer cell death. Alternatively, tumor-infiltrating immune cells such as macrophages and regulatory T cells suppress the cytotoxic activity of CD8⁺ T and NK cells, thereby allowing cancer cells to survive and proliferate [45, 46]. The following section highlights some of the pioneer microfabricated 3D platforms integrated with hydrogel-based matrices for specific studies on tumor initiation and growth.

2.2 3D Microengineered Models to Study Tumor Initiation and Growth

Conventional 3D *in vitro* models for studying tumor growth were initially developed by encapsulating cancer cells in bulk hydrogel matrices to study cellular responses to various biochemical and biophysical cues [47]. Although these platforms allow for studying single-cell response to various external factors, they are relatively low throughput in nature and depend on the ability of cancer cells to self-organize into their native architecture [17]. To overcome this limitation, researchers developed multicellular tumor spheroids (MCTS), which are generally high-throughput and provide *in vivo*-like cellular organization to recapitulate tumor morphology. To create MCTS, numerous efforts have promoted the aggregation of cancer cells using a myriad of approaches such as low attachment plates [48], hanging drop [49, 50], rotating bioreactors [51], and liquid overlay [52, 53]. The resulting tumor spheroids lead to gradients of hypoxia and pH with significant deposition of ECM proteins [54]. While these models have gained popularity for mechanistic biological studies, these approaches do not reproducibly create a well-controlled biophysical architecture due to irregular deposition of ECM components [18]. Therefore, they are not optimal for controlled studies to dissect the critical factors that influence cancer cell behavior.

Several studies have utilized soft lithography and micromolding techniques to fabricate microwells in various materials including polydimethylsiloxane (PDMS) [55], polyethylene glycol dimethacrylate (PEGDMA) [56], and agarose [57]. Using these approaches, it was possible to construct 3D tumor spheroids embedded in a well-characterized matrix (e.g. collagen, PEGDMA) for better control over the resultant biophysical architecture. For instance, concave

microwells molded using PDMS or hydrogels (e.g. collagen [58]) provide cell anchoring sites in a curved architecture, which subsequently promote formation of tumor spheroids with high fidelity [18, 55, 58]. While MCTS are excellent platforms to recapitulate tumor growth and establish gradients of oxygen, nutrients, and various biomolecules, they are limited in terms of representing the spatial architecture and multicellular nature of the *in vivo* tumor microenvironment [59]. Additionally, these models do not enable incorporation of critical biophysical cues such as mechanical stress and interstitial fluid flow on cancer cell behavior [47].

To overcome the limitations of MCTS, other approaches have utilized an integrated strategy using hydrogel-based biomaterials and microengineering technologies (i.e. microfluidics, micromolding) to develop tumor entities with well-defined cell-matrix and cell-cell interactions [55, 60]. For instance, Boghaert *et al.*, employed SU-8 photolithography technique to create elastomeric stamps of PDMS on which they molded collagen to form 3D ducts for the co-culture of mammary epithelial and tumor cells [61]. The tissue surrogates were capped with a flat layer of polymerized collagen to form hollow ducts similar to the lobular architecture within the breast (Fig. 2A (i)). The cells were seeded such that a single tumor cell was present amongst normal mammary epithelial cells within each tissue surrogate. Their findings demonstrated that non-invasive tumor cells proliferated at a significantly higher rate at the edge of the ducts as compared to other locations within the tissue surrogate. On the other hand, invasive cells migrated more rapidly when they were localized at the ends of the ducts with suppressed invasion at all other locations (Fig. 2A (ii)). Computational (i.e. finite element) analysis revealed higher mechanical stresses at the proximal ends of the tissue surrogates as compared to the centers of the ducts, suggesting that biophysical parameters, such as tensional stress, contribute to cancer cell motility and proliferation. Thus, this technique offers the ability to modulate the tissue geometry and mechanical stress to assess the role of these factors on tumor expansion and correlate them to subsequent biochemical signaling.

In addition to micromolding techniques, UV-based photopatterning has also been widely used to develop *in vitro* 3D models for studying tumor growth and progression. For instance, Kaemmerer *et al.* utilized photocrosslinkable gelatin methacrylamide (GelMA) hydrogel to study the growth of ovarian cancer cells [62]. GelMA hydrogel has been demonstrated to be advantageous for tissue engineering and 3D culture of numerous cell types due to the high

concentration of matrix metalloproteinase-(MMP) sensitive degradation sites [63-67]. Additionally, the photocrosslinkable properties of GelMA provide distinct advantages over purely natural hydrogels by enabling the development of tissue constructs with well-defined microarchitecture [64]. In this study, the authors modulated the concentration of GelMA to regulate several biophysical attributes (i.e. stiffness, pore size, MMP-cleavable sites) and assess the role of these factors on the morphology of the tumor. Encapsulated ovarian cancer cells uniformly assembled as loose aggregates at lower (2.5% (w/v)) concentration of GelMA, while at higher hydrogel concentration (7% (w/v)) spheroids were mostly non-uniform and displayed decreased metabolic activity. On the other hand, at 5% (w/v) GelMA, the spheroids exhibited tight intercellular junctions along with the highest metabolic activity and spheroid size. Inhibition of MMP production decreased the spheroid size, confirming that the presence of MMP-cleavable sites provides sufficient cues amenable for tumor formation [62]. Thus, photopatterning of hydrogels provides a unique flexibility to tune biophysical attributes and study their role on tumor expansion. However, a potential drawback of traditional photocrosslinking techniques is the exposure of the cells to UV light, which could lead to cytotoxicity and/or mutagenesis. Despite this drawback, many studies have confirmed physiologically relevant cell behavior when embedded within GelMA hydrogel [62, 64, 67]. Furthermore, there have been recent initiatives to create visible light photoinitiators, which can potentially eliminate the need for UV light in photocrosslinkable hydrogels [68, 69].

Over the past few years, various approaches have also utilized microfluidics technologies to develop 3D models for studies on tumor initiation and growth [70-79]. For instance, Sung *et al.*, utilized a Y-shaped microfluidic platform with two adjacent channels loaded with cancer and stromal cells to mimic the native tumor microenvironment (Fig. 2B (i)) [79]. The aim of this study was to assess the transition of breast cancer from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) in the presence of mammary fibroblast cells. They utilized MCF10A.DCIS cells which were suspended in a 1:1 mixture of collagen:matrigel hydrogel and injected them into one of the channels, while human mammary fibroblasts (HMFs), embedded in the same ECM, were injected into the adjacent channel. Their results demonstrated that at the ECM interface between cancer and fibroblast cells, MCF10A.DCIS cells transitioned from normal mammary or DCIS morphology (i.e. circular morphology) to a more invasive phenotype with distinct cellular protrusions (Fig. 2B (ii)) [79]. Downstream analysis correlated the changes

in cellular morphology with remodeling of ECM at the invasive cellular sites, demonstrating downregulation of E-cadherin and breakdown of basement membrane proteins. The invasive transition was also maintained when both cell-laden hydrogels (one contained MCF10A.DCIS and the other comprised of HMFs) were separated by a spacer hydrogel of a similar matrix composition. The findings of this study suggested that both soluble signaling molecules and direct cell-cell contact mediated disease progression from DCIS to an invasive state [79]. Similarly, in another study, Jeong *et al.*, fabricated a seven-channel microfluidic device such that colorectal carcinoma tumor spheroids, embedded in collagen hydrogel, were co-cultured in close proximity to normal colon fibroblasts separated by media channels (Fig. 2C (i)) [71]. The authors observed that when colon cancer cells were co-cultured with fibroblasts, the diameter of tumor spheroids significantly increased compared to the mono-culture condition. Further analysis revealed that the increase in diameter of the spheroids was not due to cellular proliferation, but rather due to the deposition of fibronectin within the spheroids. Interestingly, when paclitaxel, a microtubule stabilizer, was introduced into the microfluidic device, it diffused more slowly through the hydrogel in the co-culture condition due to the presence of fibronectin. Additionally, colon fibroblasts demonstrated increased expression of α -SMA and higher cell motility (Fig. 2C (ii)).

Cellular microcapsules (or microspheres) is another emerging technology that has been utilized to assess the impact of biophysical stresses on tumor growth. Cellular microcapsules can be engineered using various types of biomaterials comprising either cancer cells alone or in co-culture with stromal components [80-82]. Using a microfluidic extrusion device, Alessandria *et al.* engineered alginate-based cellular microcapsules where colon carcinoma cells were suspended in an aqueous core enclosed in an alginate shell (Fig. 3A (i)) [80]. This platform utilized embedded mechanical sensors to assess the pressure exerted on the surrounding microenvironment (i.e. microcapsules) as the tumor grew and underwent structural reorganization. Upon cellular confluency, the endogenous biophysical stresses within the microcapsules induced a reduction in tumor growth kinetics. During the post-confluency phase, tumor spheroids exhibited a distinguishable necrotic core due to minimal diffusion of nutrients (Fig. 3A (ii)) with marked alterations in cellular organization. The confluent spheroids were further digested from the alginate shell and encapsulated in collagen hydrogel to observe the changes in their morphological signatures. The cells at the periphery of the spheroids had more

motile characteristics with an elongated morphology when compared to cells in the core of the spheroids, indicating the role of microenvironmental stress and hypoxia in triggering cellular invasion within a growing tumor mass (Fig. 3A (iii)) [80]. In another study, Jang *et al.* utilized a droplet based encapsulation technique to assess the influence of cell-ECM interactions on different types of gastric cancer cells [83]. In this study, a microfluidic device was utilized to engineer collagen beads embedded with either AGS (intestinal type) or Hs746T (diffuse type) gastric cells, which were surrounded by fluorinated oil. By varying the flow rate of the collagen and oil, it was possible to vary the diameter of the microbeads. Over 7 days of culture, both cell types exhibited high proliferation rate within the 3D microtumor model. Cell morphological analysis further revealed that while AGS cells formed compact spheroids within the microbeads, Hs 746T cells demonstrated a more motile pattern with F-actin rich protrusions. When compared to 2D platform, both cell types in 3D models demonstrated higher mesenchymal markers (N-cadherin, vimentin) than epithelial markers (E-cadherin). The morphological differences between the cell types resonated well with the *in vivo* characteristics of the cells, confirming that the proposed droplet based model could be a valuable tool to study growth kinetics of cancerous cells [83].

Various other studies have also utilized magnetic force-based cell patterning to control the spatial arrangement of cancer cells and perform subsequent biological studies on tumor growth [84, 85]. For instance, Okochi *et al.* utilized this strategy to develop an array of multicellular melanoma cells and study their interactions with 3T3 cells. Cells were labeled with magnetite cationic liposomes (MCLs), which contained 10 nm magnetite nanoparticles and thereby enabled patterning using a magnetic pin holder. When invasive melanoma cells were patterned within collagen I matrix, they formed spheroids, but demonstrated no invasive behavior. Furthermore, the authors explored the impact of fibroblasts on tumor growth by co-culturing human M-1 melanoma cells with 3T3 cells using three different patterns including (i) mixed with 3T3 fibroblast cells and embedded in matrix, (ii) embedded separately from fibroblasts in distinct hydrogel layers, and (iii) placed adjacent to a collagen sheet with embedded fibroblasts (Fig. 3B (i)) [84]. Their results demonstrated that stromal fibroblasts enhanced tumor invasiveness regardless of the spatial architecture, indicating that these cells can influence cancer cell motility in both contact- and soluble-factor- (i.e. non-contact) dependent modes. Additionally, the presence of fibroblasts upregulated several key genes indicative of

increased cell motility [84]. Overall, the outcome of this study highlighted the advantages of magnetic force based cell patterning in precisely controlling cellular arrangement.

The recent emergence of advanced fabrication technologies such as bioprinting have also enabled the development of 3D *in vitro* models for studies on tumor growth and expansion [86, 87]. For instance, Zhao *et al.* utilized bioprinting with a shear-thinning hydrogel “ink” comprised of gelatin, fibrinogen, and alginate embedded with Hela cells to engineer a cervical cancer model [88]. The cell and hydrogel solutions were printed in a layer-by-layer approach to construct 3D lattice structures with interconnected channels. Their results indicated that bioprinting can be utilized to create tumor constructs with high cellular viability, attributed to the shear-thinning properties of the selected biomaterial that protect the cells during extrusion. Numerous other techniques including acoustic cell patterning, piezoelectric cell printing, microdroplet chain arrays, and nanomagnetism have also been developed to pattern cells with well-defined spatial organization [89-94].

2.3 Perspectives

Synthetic and natural biomaterials with well-characterized properties have been instrumental in studying the influences of biophysical and biochemical cues on tumor initiation and growth [61, 62]. However, due to the high heterogeneity within the tumor microenvironment, the resulting stromal remodeling, and variation between different sub-types of cancer, one singular biomaterial cannot accurately recapitulate an environment with *in vivo*-like properties. For example, tumors initiating from pancreas exhibit a different ECM composition compared to the tumors initiating in breast [35]. In this regard, a major requirement to conduct physiologically relevant studies is to synthesize suitable hydrogel matrices that closely mimic the properties of the ECM components specific to the sub-type of cancer. For instance, Sokol *et al.* evaluated *in vivo* breast tissue and observed that the native ECM is composed of a complex structure of protein fibrils interweaving glycosaminoglycan (GAG) carbohydrate chains [95]. Subsequently, they composed a hybrid hydrogel with constituents of the native breast tissue including laminin, fibronectin, hyaluronic acid, and collagen [95]. Their initial studies compared the growth of cancer cells within the breast tissue mimetic ECM to various other natural hydrogels including collagen and matrigel. Interestingly, the proposed ECM matrix induced tissue organization similar to the histological slices of human breast [95].

Another approach to construct a native-like microenvironment relies on extracting the ECM from *in vivo* tumor and further seeding it with various cell types including cancer cells, endothelial cells to study tumor growth and progression. For instance, Lopez *et al.* utilized a decellularized ECM extracted from both healthy and cancerous colon tissue and prepared hydrogels through enzymatic digestion [96]. The tumor-extracted ECM had a unique protein composition with significantly higher amounts of collagen IV, laminin, fibronectin, and thrombospondin-2. Furthermore, the tumor-extracted ECM had a high storage and loss modulus compared to the healthy tissue, suggesting that the differential protein composition has a relationship with the rheological properties of the hydrogel. Additionally, the authors seeded both materials with colon cancer cells and observed enhanced growth in the tumor-extracted ECM as compared to normal ECM [96].

These studies demonstrate that organ specific hydrogels that can closely mimic the *in vivo* ECM have more suitability to mechanistically study tumor growth as compared to traditional hydrogel based matrices such as collagen I, matrigel etc. However, variabilities in ECM extraction protocols can further introduce alterations in hydrogel compositions which leads to inconsistent results. Additionally, organ specific hydrogels need to be integrated with various microengineering technologies that create spatial arrangement of cells and allow researchers to evaluate the effects of various biophysical factors such as mechanical stress and interstitial fluid flow.

Besides selecting a suitable hydrogel, choice of an appropriate 3D model is equally critical. In a recent study done by Brancato *et al.*, normal fibroblast and cancer associated fibroblasts were individually molded in two different 3D models, namely microtissues, by seeding cells on gelatin porous microbeads and a spheroid based model [97]. Characterization of ECM protein deposition demonstrated marked difference between NF and CAF specifically in the case of collagen, fibronectin and hyaluronic acid. On the other hand, in spheroid based model no difference was observed between the two cell types [97]. This clearly indicates that the specific 3D model for a given study should be wisely selected to recapitulate *in vivo* physiology of specific tumor microenvironment. With the advent of microengineering technologies and innovative biomaterials; integration of these two would be more advantageous to model native tumor architecture with various stromal components. We also envision that the tumor growth models should incorporate vascularized networks as opposed to avascular entities. Various recent

studies have demonstrated that in the presence of vascularized networks, tumor growth kinetics better mimic *in vivo*-like conditions for further drug diffusion studies [98].

3.1 Tumor Angiogenesis

Angiogenesis is the physiological process of neovascularization that is essential for tumor growth and survival by providing sustenance through diffusion of nutrients and oxygen [31]. In the realm of cancer, aberrant angiogenesis is closely associated with poor prognosis and thus, is a promising area of study to inhibit cancer progression [99, 100]. Tumor angiogenesis relies on a constitutively activated “angiogenic switch,” which initiates a cascade through the release of multiple overlapping and overabundant signaling factors such as VEGF, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and angiopoietin (Ang) [31, 101-103]. However, unlike angiogenesis in a normal physiological state, where there would be well-orchestrated gradients of angiogenic factors, the tumor microenvironment forces an unbalanced expression of pro- and anti-angiogenic factors that overall contribute to persistent but poor and irregular vascular formation [104]. These pro-angiogenic factors are secreted from many different cell types ranging from cancer, stromal, and inflammatory cells contributing sustained angiogenesis and aberrant vascular function [101, 104, 105]. For example, tumor-derived cytokines instruct macrophages to adopt an M2 phenotype (tumor-associated macrophages (TAMs)) subsequently causing TAMs to aid tumorigenesis by suppressing destructive immune functions, promoting endothelial cell proliferation, and enhancing the release of pro-angiogenic factors [106-110]. Infiltrating immune cells, along with the resident CAFs remodel the matrix by activating MMPs degrading the surrounding ECM for further exposure of pro-angiogenic growth factors [111].

Vascular endothelial growth factor, a prominent growth factor released by both cancer and stromal cells within the tumor microenvironment induces hyperpermeability in existing blood vessels causing the leakage of plasma proteins, such as fibrinogen and clotting factors, into the tumor microenvironment [104]. The deposited plasma proteins form a provisional matrix facilitating new vessel formation through addition of adhesion moieties and sequestered growth factors. However, the newly formed vasculature tends to lack both perivascular coverage and basement leading to immature and leaky vessels [104]. Taken together, these complex interactions demonstrate that a multitude of biochemical and biophysical components along with instructive cues from the ECM control of tumor-associated angiogenesis. As such, there needs to

be a greater understanding in tumor exploitation mechanisms by recapitulating the vascular microenvironment to interrogate the individual interactions and identify effective therapeutic strategies.

3.2 Modeling Vascularization *In Vitro*

Initial angiogenesis models started with seeding endothelial cells on a variety of matrices, such as collagen or matrigel, to form endothelial tubules. However, these models were limited to only quantifying tubule length, diameter, area, and number of junctions, and did not account for relevant endothelium physiology *in vivo*, such as control over biomolecular gradients and shear stress from interstitial flow. In fact, several labs disputed to whether or not these tubules contained open lumens, which is a critical defining feature of the blood vessel [112, 113]. This led to the establishment of sandwich and bead assays in order to form 3D tubules that would better mimic the *in vivo* situation [112, 113]. However, these models were difficult to scale-up with limited control over the critical elements of the vascular microenvironment, such as perfusion of fluids and intra/extravasation of cells. Eventually, the need for high-throughput and scalable methods with better control over the microenvironmental cues led to development of physiologically relevant microengineered technologies integrated with advanced biomaterials along with tissue engineering strategies. Specifically, these platforms attempt to faithfully represent vascular formation by utilizing tunable hydrogel-based matrices with bioactive properties while controlling the spatial organization of cells [114-116]. Once created, the models are typically used to assess the influence of angiogenic stimuli and various cell-cell/cell-matrix interactions on vascular formation. Use of advanced hydrogels offer expanded control over scaffold topography, geometries, and biomechanical cues that have been demonstrated to regulate endothelial cell behavior [114-116]. Several of these models have not been adapted to study tumor-associated angiogenesis, but various pioneer microengineered approaches for *in vitro* tissue vascularization will be reviewed in the following section.

Through collaborations between the Fischbach and Stroock groups, a novel microfluidic platform was utilized to develop microvascular networks in collagen I hydrogel to study angiogenesis using pre-formed geometries [102, 117-119]. They used soft lithography combined with micromolding technique to form well-defined channels that were later seeded with endothelial cells. The resulting microchannels were assembled within the PDMS mold to form a perfusable vascularized network [119]. The findings of these studies demonstrated that

angiogenic sprouting was dependent on collagen mass fraction, endothelial cell density, and vessel geometry [103, 117]. With decreasing collagen mass fraction, there was an increase in matrix porosity, which subsequently resulted in higher frequency of lumen formation with larger luminal areas [117]. The authors noted that the integration of similar co-culture models with complex perfusable vascular networks could potentially be used in future studies for anti-cancer drug transport. They also developed a similar platform to assess tumor angiogenesis under both hypoxic and normoxic conditions [120, 121]. They utilized a micromolding technique where they embedded invasive MDA-MB-231 breast cancer cells within collagen I and casted the cell-laden matrix into PDMS microwells (Fig. 4A (i, ii)) [121]. Subsequently, they seeded endothelial cells on top of the collagen and evaluated angiogenic sprouting in both hypoxic and normoxic conditions. Their findings revealed that both hypoxia and 3D culture significantly increased angiogenesis and upregulation of VEGF and IL-8 [121], as the key factors which have been shown to synergistically increase the rate of neovascularization [122].

The Kamm lab developed a microfluidic device that consisted of three straight channels connected by two compartments for acellular collagen scaffolds for cancer angiogenesis studies [123]. The central channel was coated with additional collagen ECM and seeded with human microvascular endothelial cells (HMVECs), which formed a confluent luminal structure mimicking the architecture of a perfusable blood vessel. They added control media in one channel flanking the central channel and VEGF supplemented media in the treated channel. They noted spontaneous angiogenic sprouting of HMVECs within the central channel towards the VEGF gradient. Subsequently, they added other cell types including rat mammary adenocarcinoma MTln3 cells, human glioblastoma U87MG cells, and mouse smooth muscle 10T 1/2 cells within the treated channel. Interestingly, they observed increased angiogenic sprouting towards MTln3 cell type, no significant difference in neovascular formation towards U87MG cells, and significantly inhibited angiogenesis in the presence of 10T 1/2 cells (Fig. 4B (i, ii)) [123]. Overall, their results confirmed that the heterogeneity of cancer cell types influences angiogenesis.

Similarly, the Jeon group developed a versatile microfluidic device that recapitulated both vasculogenesis and angiogenesis as a function of cell seeding configuration (Fig. 4C (i)) [124-126]. The two outer channels were used to culture lung fibroblasts, which provided soluble growth factors to aid in endothelial network formation. After encapsulating human umbilical

vein cells (HUVECs) within fibrin matrix (Fig. 4C (ii)), they spontaneously formed an intricate vascular network (Fig. 4C (iv)). However, when endothelial cells were seeded on the side of the fibrin matrix (Fig. 4C (iii)), they formed angiogenic vessels that migrated towards the media channel (Fig. 4C (v)). Additionally, the channels could induce interstitial pressure within the model by modulating the height of the media reservoirs [124, 125]. Due to these properties, they could mimic leaky vasculature and assess the effects of numerous growth factors including VEGF-A, VEGF-C, bFGF, and S1P on angiogenesis. Using the model, they could determine the optimal growth factor “cocktail” that induced the greatest angiogenic response. Furthermore, they incorporated glioblastoma cells in their microvascular chip to study the influence on tumor angiogenesis [124]. Highly malignant human glioblastoma multiforme cells were introduced into the outer channel of the same platform, which induced slight differences in the tubule length [124]. Moreover, there were stark differences in vascular phenotype between the fibroblast-induced vascularization compared to cancer cell-induced ones (Fig. 4C (vi)). The sprouts affected by soluble factors from the glioblastoma cells were found to form inefficient vasculature with immature lumens as well as aberrant fusions and convolutions between the angiogenic sprouts [124].

The Chen lab utilized various micromolding and bioprinting techniques to fabricate endothelialized tissue constructs and patent channels [115, 127-129]. Endothelial cells were encapsulated within collagen I hydrogel and placed on a micropatterned PDMS mold, and the constructs were polymerized at 37 °C. Using this technique, the authors visualized endothelial cord formation in real-time to capture the dynamics of tubulogenesis. Moreover, they found that manipulating the collagen concentration influenced the tube diameters suggesting the crucial role of matrix mechanical stiffness and ligand density on the behavior of the endothelial cords. More recently, they explored a technique where they casted collagen over a needle and seeded endothelial cells upon removal of the pin within the remaining hollowed structure (Fig. 5A (i)) [128]. They demonstrated that the developed structure was perfusable, had the ability to integrate with other tissue constructs, and formed angiogenic vessels in response to gradients of growth factors (Fig. 5A (ii)) [115, 127, 128].

The Lewis lab developed a bioprinting technique using Pluronic F127 as a ‘fugitive ink,’ or sacrificial mold, for tissue vascularization (Fig. 5B (i)) [130]. Pluronic F127 is a triblock copolymer consisting of a hydrophobic poly(propylene oxide) (PPO) segment and surrounding

poly(ethylene oxide) (PEO) segments giving it a PEO-PPO-PEO arrangement. This material can form thermally reversible micelles at concentrations above 21 wt% and temperatures above 10 °C transitioning from gel-to-fluid in aqueous conditions [131]. To take advantage of this behavior, they utilized a hybrid hydrogel consisting of gelatin, fibrinogen, and transglutaminase to print cell-laden features and cast bulk matrix that surrounded the solid Pluronic F127 patterns (Fig. 5B (i, ii)). Using this technique, spatial organization of cells could be controlled by using multiple nozzles to deposit several cells at different locations. After polymerization of the bulk matrix, the temperature was reduced to evacuate the remaining Pluronic F127 (Fig. 5B (ii, iii)). Post-evacuation, hollowed channels remained, which were subsequently seeded with HUVECs and led to the formation of a perfusable lumen. The printed structure was connected to a peristaltic pump, allowing for perfusion and maintenance of cell viability for up to three months (Fig. 5B (iv)) [132, 133]. Additionally, they explored printing pluronic in a complex lattice structure, which enabled the creation of an intricate endothelial network within the bulk hydrogel [132]. The endothelial network facilitated sufficient diffusion of nutrients within the bulk hydrogel. Mesenchymal stem cells were encapsulated in matrix and printed adjacent to the engineered blood vessels, while human neonatal dermal fibroblasts-laden matrix was casted over the resultant printed structure (Fig. 5B (v)) [132]. Maintenance of high cellular viability demonstrated the feasibility of this approach in the development of thick tissue constructs (>1cm). This technology can be used in the future towards recapitulating various aspects of cancer angiogenesis (i.e. interstitial flow, leaky vessels), or developing tumor microenvironment models (i.e. multiple cell types, parenchyma organization).

The Werner lab developed a 3D *in vitro* bioengineered tumor angiogenesis microenvironment within a novel glycosaminoglycan-based (starPEG-heparin) hydrogel culture system for breast and prostate tumor vascularization (Fig. 5C) [134, 135]. The 3D cultures using the developed starPEG-heparin hydrogels were compared with cultures within matrigel and were found to be more representative of the *in vivo* situation. They found that cancer cells grown in the starPEG-heparin hydrogel over a long period (7-10 days), maintained uniform morphology whereas those grown in matrigel did not [134]. This suggested that the composition variability in instructive cues (i.e. growth factors and stiffness) within matrigel changed over time while the starPEG-heparin hydrogel composition remained intact leading to better control of the microenvironment. The same group developed a novel study using a tri-culture model of cancer

cells, HUVECs, and MSCs forming tumor-vascular contacts representative of early tumor development phase. More importantly, they performed drug studies using 2D, mono-culture 3D and tri-culture 3D models with varying responses from the cells owing to the differences in 2D and 3D microenvironments as well as complex cell-cell interactions [134, 135]. They found that results from their tri-culture model, especially the prostate tumor model, were the most similar to *in vivo* drug response studies [136, 137]. In a follow-up study, they fabricated three different hydrogels decorated with either RGD, IKVAV (isoleucine-lysine-valine-alanine-valine, laminin-111 motif) or GFOGER (glycine-phenylalanine-hydroxyproline-glycine-glutamic acid-arginine, collagen I motif) integrin binding motifs to study the angiogenic and growth effect on MCF-7, MDA-MB-231, PC-3, and LNCaP cells [138]. They controlled the biophysical and biochemical cues independently by modulating the number of peptides functionalized to the PEG-heparin hydrogels. Within 14 days, different cancer cell types, except for MDA-MB-231, formed spheroids in the biohybrid hydrogel. In addition, they found that IKVAV and GFOGER motifs enhanced cancer growth and invasiveness in PC-3, but not in MCF-7 and LNCaP. Moreover, they repeated the same tri-culture assay from the previous study and found that these integrin motifs did not enhance angiogenic network development. Notably, IKVAV and GFOGER enhanced tumor-vascular interactions with vascular sprouts penetrating the tumor spheroids of MCF-7, PC-3, and LNCaP. Taken together, the Werner developed a robust biohybrid PEG-heparin hydrogel that could represent the tumor-vascular *in vivo* situation by modulating the different integrin motifs combined with tri-culture of cancer (MCF-7, PC-3, LNCaP, MDA-MB-231), stromal (MSCs), and endothelial cells (HUVECs) (Fig. 5C (ii)) [135].

3.3 Perspectives

The integration of advanced biomaterials and microengineering techniques has demonstrated great promise in recapitulating tissue vascularization and furthered the study of tumor angiogenesis [102]. Nonetheless, there are few established microengineered models that attempt to recapitulate tumor-associated angiogenesis, where most of the previous vascularization studies have been geared mainly toward the general theme of tissue engineering and regenerative medicine [102, 115, 117-119, 127, 128, 139]. In fact, a majority of previously developed 3D models are often focused on using geometrical cues or biochemical factors to drive angiogenesis, rather than influences from cancer-specific interactions. For instance, numerous groups have utilized micromolding and bioprinting technologies to study angiogenic

sprouting through complex perfusable channels [128]. These models could be fine-tuned and adapted to study tumor angiogenesis by introducing cancer cells and/or cancer-secreted growth factors to generate biochemical gradients or tumor-specific microenvironments that could modulate morphological and sprouting behavior of endothelial cells.

Additionally, 3D microengineered models with micromolded or pre-fabricated channels for lining endothelial cells, such as those developed by the Stroock [119] and Chen lab [115], have largely been less utilized in the recent years. Technologies that instead stimulate angiogenic sprouting and vasculogenesis within hydrogels, such as collagen, matrigel, and fibrin, have increased in popularity and are seen as a more *in vivo*-like representation of blood vessel formation. The ECM-like hydrogels, typically matrigel or collagen, used to encapsulate the ECs provide instructive cues that regulate endothelial polarization, migration, and the overall vascularization process [104]. Recently, the Fischbach lab [140] utilized various mixtures of collagen and matrigel within their microwell technology as demonstrated in Figure 4A to understand how different compositions and architectures of ECMs influence microvascular formation. They varied the compositions of matrigel to collagen I within their microwells by changing the polymerization times and temperatures to produce different ECM compositions and architecture. Notably, a hallmark of vessel maturation and stability *in vivo* is the presence of the basement membrane surrounding the vascular endothelium [104, 134, 141]. In this regard, they found that compositions containing matrigel polymerized at lower temperatures tested within their microwell technology contained higher amounts of collagen IV, a basement membrane protein. The authors suggested that higher matrigel content contained more instructive cues, such as growth factors and cytokines that affected the deposition of basement membrane proteins, like collagen IV, and promoted vascular morphology [140]. Moreover, this specific technology allowed access to the cell culture media to probe for levels of cytokines, such as IL-8, through enzyme-linked immunosorbent assay (ELISA). They demonstrated that cell-secreted IL-8 [140], which has been linked to tumor neovascularization [122, 142], varied depending on the ECM composition. Moreover, blockade of IL-8 affected the morphology of the microvascular network demonstrating the suitability of this platform for studying signaling pathways altered by ECM composition [140]. The proposed hydrogel within the microwells were not fully enclosed allowing for probing of the hydrogel stiffness through technologies such as AFM [28], however, the authors did not explore this specific area. In this regard, it would be greatly beneficial to

interrogate how the stiffness along with varying matrix compositions of collagen and matrigel affect vasculogenesis using a microengineered platform as matrix stiffening has been linked to tumorigenesis [143]. In a separate study, the Reinhart-King lab [144] leveraged nonenzymatic glycation to form advanced glycation end product (AGE) cross-links to stiffen collagen without increasing collagen concentration and studied the resulting stiffening effects on vascular formation. This study was able to recapitulate the scenarios where tumors undergo stiffening without an increase in collagen density [8, 38]. This approach could be utilized together with microtechnologies such as microwells to create a robust high-throughput platform technology capable of probing matrix stiffness, vascular morphology, and biochemical signaling pathways in the tumor vascularization niche.

In addition to matrix composition and architecture, embedding endothelial cells along with helper cells, such as pericytes or smooth muscle cells, also provides the necessary cues to create an *in vivo*-like representation of a microvascular network within the tumor microenvironment. Specifically, the George lab, as described above, developed a microphysiological systems (MPS) co-culturing ECs and helper cells to study how changes in interstitial flow affected vasculogenesis [145]. Viability data showed living vessels beyond even 40 days. Moreover, perfusion of beads through the device demonstrated open lumens while use of FITC-dextran diffusion showed that the vasculature was not leaky. The spontaneously forming networks from endothelial and stromal cells encapsulated within fibrin matrix could be modified by incorporation of a tumor region to understand how tumors or tumor-secreted factors affects the functionality and structural integrity of the vessels. In addition, this microfluidic platform could be adopted to study anti-cancer drug influences on endothelial cell behavior due to its resemblance of microcapillary networks and ability to perfuse fluids through this system. In comparison to the previous microwell technology used by Stroock and Fischbach [140], the capability of perfusing fluids through microvascular networks offers a unique ability to investigate endothelium permeability, angiogenesis, and metabolic activity.

In addition to including a variety of stromal cells to investigate angiogenesis and vasculogenesis, the source of endothelial cells must be expanded. Most of the above studies were limited to HUVECs as the sole model for vascularization. However, endothelial cells obtained from different organs have unique properties that may influence tumor progression [146]. For instance, experiments involving colorectal carcinoma cells showed selective adhesion to the

hepatic microvasculature suggesting unique organ-specific interactions [146]. Moreover, most studies have typically used HUVECs, rather than organ-specific endothelial cells, which may have biological implications when used to study angiogenesis or vasculogenesis on a chip. For example, capillaries from differing organs contain varying amounts of fenestrae, which are small pores allowing for rapid exchange of molecules [147]. Skin, lung, brain, and heart capillaries are generally non-fenestrated limiting diffusion while liver capillaries, not only contain fenestrae, but also feature discontinuous endothelium allowing for larger exchanges of molecules [147]. In this regard, the heterogeneity in endothelial phenotype could alter drug responses providing a lower predictive value. Therefore, microengineered drug screening assays may need to utilize an organ-specific approach by requiring all cells within the model to be from the same organ.

Investigating changes at the cellular or sub-cellular level in large and complex vascular networks presents significant limitations in terms of their ability to elucidate causal biological relationships between tumor angiogenesis and the microenvironment. To this end, models requiring functional vasculature should be tailored to specific physiological processes being studied, such as tumor angiogenesis and vasculogenesis, cancer invasion towards the blood vessels (reviewed in section 4), intravasation (reviewed in section 5), or extravasation (reviewed in section 6). In this regard, much of the above described models focused solely on development of the vascular network rather than the precise cell-cell interactions between tumor and endothelial cells. We envision that future models would incorporate both components to discover and analyze potential therapeutic targets for disrupting tumor angiogenesis.

4.1 Cancer Cell Motility and Invasion

Cancer invasion is one of the primary steps of metastasis, where a multitude of biochemical (i.e. release of cytokines) and biophysical (i.e. breakdown of basement membrane, ECM remodeling) cues lead to intravasation through the neighboring blood vessels [148]. Previous studies have demonstrated that cancer cell migration follows similar mechanisms to that of non-neoplastic cells such as immune cells, keratinocytes, and fibroblasts [149]. However, the imbalance of pro-migratory and stop signals in neoplastic cells abrogates the cell adhesion and promotes invasion [150].

Amongst various factors affecting tumor cell invasion, the role of the ECM has been considered to be paramount [148]. The initial microenvironment typically has a composition similar to that of native epithelial tissue [149]. However, in the presence of tumor cells the

associated stroma undergoes excessive remodeling leading to marked architectural and phenotypical changes along with altered expression of growth factors and chemokines [149]. For instance, native fibroblasts within the stroma differentiate to myofibroblast-like phenotype (i.e. CAFs), and deposit excessive amounts of collagen in the vicinity of tumor microenvironment [151]. Enhanced deposition of crosslinking proteins such as lysyl oxidase (LOX) further increases the fibrillary density and stiffness of the stromal ECM [8, 149]. During early stages of cancer invasion, collagen fibers realign parallel to the tumor boundary and thereby provide tracks to facilitate cancer cell invasion into the surrounding tissue [152]. The spatial arrangement of fibroblasts causes radial deposition of LOX throughout the stroma, which results in gradients of matrix stiffness within the tumor microenvironment [152]. Through a process known as durotaxis, cancer cells follow these gradients towards areas of higher stiffness [153].

The tumor microenvironment also presents different biochemical cues that can regulate cell invasion [149, 150]. For instance, a critical function of the ECM is to immobilize tumor cells by providing anchoring sites to their receptors. Within the tumor microenvironment, the decrease in pore size and increase in stiffness, caused by collagen and LOX deposition, induces integrin-mediated focal adhesion kinase (FAK) signaling in both cancer cells and CAFs and lead to loss of cellular polarity and differentiation towards a mesenchymal phenotype [149]. Furthermore, integrins can aid in recruitment of various proteases such as MT-MMP1 at the cell attachment site, which cleave and degrade the matrix, thereby promoting cellular motility [149]. ECM components such as proteoglycans and GAGs immobilize growth factors/cytokines, thereby act as localized reservoirs for biochemical cues. Altered cell-ECM interactions in tumor microenvironment induce gradients of soluble cues, which further promotes cancer cell invasion in a process commonly known as chemotaxis [153]. Specific chemokines of interest include CXCL12 (SDF 1 α), epidermal growth factor (EGF), and colony stimulating factor 1 (CSF1), which have been demonstrated to elicit a significant migratory response due to the presence of associated surface receptors on most types of cancer cells [153]. Furthermore, surface receptors such as CD44 can bind various ECM components such as hyaluronic acid and collagen, which facilitates invasion [153].

In addition to tumor ECM, stromal cells such as CAFs, TAMs, and adipose cells play a significant role in cancer invasion through secretion of numerous biochemical stimuli [154]. CAFs have been demonstrated to play a prominent role in stromal remodeling and eventual break

down of the ECM through the secretion of various proteases such as MMP-3 and MMP-1 [4]. Adipocytes also promote cancer cell migration through the secretion of MMP-11, and proinflammatory cytokines, such as IL-11, that enhance invasion through activation of the STAT 3 pathway [154, 155]. TAMs in this regards have been known to promote cancer invasion by releasing chemokines such as CSF1 [156]. TAMs also participate in ECM remodeling due to secretion of proteolytic enzymes such as cathepsins, MMPs, and serine proteases, which subsequently promote invasion [156].

The tumor microenvironment has a significant influence on cancer cell motility, where biochemical cues and ECM interactions dictate cell migration modality (i.e. mesenchymal or amoeboid migration) [157]. Mesenchymal mode of migration is primarily employed by cancer cells that invade through connective tissues such as adipose, muscle, and bone [152]. Through this process, cells are polarized resulting in the formation of actin-rich flat 2D (lamellipodia) and rod-like 3D thin protrusions (filopodia) at the leading edge. The focal contacts at the leading edge of the cell interact with the integrin binding sites present in the ECM to provide a mechanical linkage between the actin cytoskeleton and the matrix [158]. Finally, the traction force generated from the contraction of stress fibers at the rear of the cell displaces the cell towards its anchoring points at the leading edge [159]. On the other hand, cancer cells adapting the amoeboid form of migration depend on the cortical actomyosin network to generate the motive force [159]. The hydrostatic pressure within the cell body results in detachment of the actomyosin network and formation of spherical membrane herniations, more commonly referred to as “membrane blebs.” After the membrane blebs are formed, the actomyosin network beneath the membrane contracts and translocates the cell. As this mode of migration is not stress-mediated, amoeboid movement does not require strong cell-ECM interactions. Instead, cyclical expansion and contraction through restabilization of the actomyosin network allow the cells to squeeze through gaps and pores of the surrounding ECM [159]. Amoeboid migration is typically faster than mesenchymal migration and is commonly observed in breast cancer, lymphoma and small-cell lung carcinoma [151].

The modality of cancer cell migration is dependent on dynamic remodeling of the ECM [149]. For example, multicellular streaming is a unique case of migration where leader cell create microtracks by proteolytic degradation of ECM, and follower cells migrate on these microtracks [150]. Tumor cells also can undergo collective migration, in which a cell sheet or cluster moves

in concert without disruption of cell junctions [8]. This process is directed by “tip cells” to protrude and generate migratory traction by their interaction with the ECM [149]. Although cancer cells adopt different modes of migration depending on the location of the tumor, cells frequently switch between both migration modes due to the plasticity of tumors and heterogeneity of the surrounding microenvironment [150]. Such a phenomenon is also observed due to the induction of drugs that target one particular mode of migration [149]. In order to develop a mechanistic understanding of cancer cell invasion, various microengineered models integrated with innovative biomaterials have been utilized. Some of the pioneer work is reviewed in next section.

4.2 3D Microengineered Models to Study Cancer Invasion

Various studies have demonstrated the suitability of tumor spheroids, encapsulated in 3D matrices, as proper models to study the mechanisms of cancer cell invasion [160-163]. For instance, the Reinhart-King group utilized tumor spheroids with heterotypic co-culture of highly invasive MDA-MB-231 and non-invasive MCF10A cells, encapsulated in collagen, to study the role of tumor heterogeneity in collective cell migration [161]. Their results showed that collagen fiber alignment and proteolysis of the matrix played a significant role in invasion of different population of cancer cells. When spheroids of normal mammary MCF10A cells were utilized, collagen fibers were oriented parallel to spheroid boundary, but the addition of invasive MDA-MB-231 cells led to perpendicular realignment of collagen fibers thereby promoting invasion. Using confocal imaging, cellular microtracks were observed when MCF10A cells were co-cultured with MDA-MB-231 cells. Time lapse imaging revealed that MDA-MB-231 cells initially degraded the surrounding matrix, and MCF10A cells were able to invade the matrix by following the microtracks generated by MDA-MB-231 cells [161]. This study was crucial in elucidating the interactions between “leader” and “follower” cells within a 3D microenvironment indicating the suitability of this model to be utilized as an invasion assay. In a recent study, a novel spheroid based model was utilized in which tumor cells (MCF10A and MCF10A-HRas) were surrounded by basement membrane extract (BME) followed by encapsulation in collagen I hydrogel [164]. This model provided an interesting tool through which the effect of BME on cancer cell invasion in presence of a stromal component. The proposed invasion assay demonstrated that when the non-invasive MCF10A spheroids were encapsulated in collagen I hydrogel with no BME shell; the cells demonstrated a sheet like expansion. On the other hand,

when the spheroids shelled with BME and encapsulated in collagen I, the epithelial cells did not breach the BME and abolished the migratory activity. In contrast, spheroids made of the oncogenically transformed MCF10A-HRas cells showed enhanced breaching of the BME and invasion into the stroma (Fig. 6A (i)) followed by reorganization of collagen fibers at the invasion front (Fig. 6A (ii)). Further inhibition of MMPs demonstrated significant reduction in invasion distance in the presence of the surrounding basement membrane layer, while there was no alterations in invasion distance when cells were encapsulated only in collagen I (i.e. without BME) [164]. In another study by McLane *et al.*, a similar platform was developed using spheroids of MCF10A or MDA-MB-231 surrounded with BME [163]. The retrieved spheroids were then encapsulated either in collagen (soft matrix) or collagen suspended with poly(ethylene glycol)-di (succinic acid N-hydroxysuccinimide ester) (stiff matrix) in presence of fibroblast. The goal of this study was to investigate 1) the effect of tumor spheroids and stiffness of the matrix on fibroblast activation and 2) the effect of fibroblast on tumor invasion. Their findings demonstrated that while in the stiff gel the spheroids caused fibroblast cells to show higher contractile activity, the expression of markers such as alpha smooth muscle actin (α -SMA) and paladin decreased the suppressive influence of spheroids on fibroblast activation. Additionally, increased stiffness of the matrix allowed the tumor cells (MDA-MB-231) to markedly disseminate with limited influence on invasion of normal mammary epithelial cells (MCF10A) [163]. These spheroid-based models provided precise spatial arrangement of cells thereby establishing a unique tool to isolate the effect of ECM and biophysical and biochemical factors on cancer cell invasion in presence of stromal components.

While spheroid based models are popular in cancer related studies, other microengineering techniques can be employed to enable spatial control over spheroids in a high-throughput scale. For instance, in a recent study, Kraning-Rush *et al.* created a tumor microarray model encapsulated in collagen hydrogel using micromolding technique by PDMS stamps (Fig. 6B (i)) [165]; a technique pioneered by Nelson *et al.* [29, 166]. Using photolithography, silicon wafers were first fabricated with unique microarchitectures. Elastomeric stamps were then used to imprint collagen and engineer arrays of microtracks (Fig. 6B (i)) [165]. This technique allowed for rapid fabrication of a high-density array of microtracks with desired geometry and spatial arrangement on a single chip to study the cancer cell migration within tracks similar to tumor microenvironment (Fig. 6B (ii)). Their results demonstrated increased cell migration rate

and distance, in MCF10A and MDA-MB-231 cells in pre-formed microtracks compared to when encapsulated within nonpatterned collagen (Fig. 6B (iii)). To further investigate the effects of matrix architecture (i.e. microtracks) on cell migration, MT-MMP1 activity was inhibited using si-RNA transfection. The loss of MT-MMP1 activity decreased the speed of invasion within unpatterned collagen, however it had no significant difference on migration speed of cells inside the microtracks. These results suggested that once microtracks are created by leader cells, the follower cells migrate along these tracks and do not need to breakdown the ECM. This phenomenon could be also a possible explanation to the relatively low success rate of MMP inhibitors in clinical trials, however further investigations are required to make solid conclusions [165]. In a follow-up study, the same group utilized this platform (Fig. 6B (i)) to gain a mechanistic understanding on the role of cytoskeletal dynamics on the migratory behavior of cancer cells in collagen microtracks. They showed that when cells were seeded on bulk collagen hydrogel of varied densities, the motile fraction of cells decreased at higher concentrations of collagen. However, in presence of microtracks, collagen density had no significant influence on motile fraction or individual speed of the cells. Inhibition of cell-matrix interactions in collagen microtracks did not significantly decrease the cell speed. However, actin contractility inhibitors including ROCK, myosin light chain kinase and myosin II inhibitors (CT04, Y27632, ML7, blebbistatin) significantly reduced the motile fraction of the cells within the collagen microtracks. These findings suggest that cytoskeleton dynamics play an active role in cellular migration regardless of MMP activity [167]. Other studies have also utilized similar micromolding techniques to engineer high-density arrays of tissue constructs to assess cancer cell invasion [58, 168, 169] suggesting easy adaptation of this technology.

Photocrosslinkable biomaterials have also gained enormous popularity to create 3D tumor models with high fidelity and architecture for cancer invasion studies [28, 67, 170]. For example, Peela *et al.* utilized GelMA and a two-step photolithography process to micropattern a 3D tumor model with defined tumor and stromal regions [28]. Specifically, this technique enabled the creation of a high-density array of circular tumor constructs of cell-laden GelMA, surrounded by acellular GelMA hydrogel. As the crosslinking density is dependent on exposure time, their model enabled precise control over the stiffness of the tumor and stromal regions. The authors encapsulated three breast cancer cell lines (MDA-MB-231, MCF-7, and MCF10A) within the model and observed significant differences in their migratory behavior, where non-

invasive cells clumped to form tumor spheroids and metastatic MDA-MB-231 cells invaded into the surrounding stroma (Fig. 6C (i)) [28]. Real-time imaging further revealed that due to the stiffness differential between the tumor and stromal regions, migration speed was significantly increased upon cellular invasion into the stroma (Fig. 6C (ii)). Additionally, cells exhibited collective migration with a specific directionality while inside the high-stiffness tumor constructs, but lost these cues upon invasion into the stroma. Thus, integrating of GelMA with photopatterning technique enabled the authors to isolate the effect of stiffness on cancer cell migration into the stroma.

Advanced micropatterning techniques have also been utilized to create complex 3D structures that have the ability to decouple biophysical cues (e.g. fibrillary density, pore size, stiffness) independently of each other and investigate the effects of such parameters on cancer cell invasion. For instance, Hoffmann *et al.* patterned PEG hydrogels conjugated with cell adhesive RGD peptides to study migration of HT1080 fibrosarcoma cells within 2D and 3D microenvironments [171]. Similarly, Spagnolo *et al.* fabricated cage-like scaffolds using femtosecond-pulsed laser at varied powers to create microstructures with various geometries (e.g. pillars, ridges) [172]. Using these 3D-complex cage-like microstructures, a pore-size threshold that distinguished the migration between metastatic breast cancer cells and normal epithelial cells was determined. Although this technology provides unprecedented control over cellular microenvironment, it requires specialized and expensive equipment that limit their use to specialized labs and low-throughput experimentation.

Numerous PDMS-based microfluidic devices have also been engineered using soft lithography techniques to interrogate the biological mechanisms of cancer invasion [27, 77, 173-179]. For example, the Konstantopoulos group utilized a microfluidics platform to study mechanisms of single-cell motility by engineering miniscule microchannels embedded with singular invasive MDA-MB-231 cells. The size of the microchannels inhibited actin polymerization and thereby prevented both mesenchymal and amoeboid modes of migration [180]. Cells embedded within the channels were osmotically stimulated, adopted different cellular lengths and velocities depending on the stimulation parameters (Fig. 6D). They unveiled that cells within the microchannels moved similar to an osmotic engine, where water was pumped throughout the cell body thereby driving migration opposite to the osmotic gradient. The authors suggested that this mechanism of migration is typically facilitated *in vivo* within

connective tissue when cells are tightly packed in longitudinal tracks of bundled collagen. In a recent work, Truong *et al.* developed a microfluidic device to model tumor invasion in response to transient gradients of EGF [27]. The model allowed constructing 3D tumor and stromal regions side-by-side with interconnectivity between matrices (Fig. 6E (i)). The proposed design enabled extensive analysis on chemotactic invasion, demonstrating that the cells invaded with greater speed upon addition of EGF. Interestingly, non-invasive MCF7 cells transitioned to an invasive phenotype in the presence of EGF. Furthermore, their results indicated that EGF-stimulated cells exhibited significant EGFR localization near the nucleus as compared to the non-stimulated cells where the receptors were found primarily on the cell membrane. Analysis of phosphorylated EGFR further depicted the activation of EGF pathway. Lastly, the authors showed the novelty of this platform to study cancer invasion in the presence of stromal cells such as CAFs (Fig. 6E (ii)), which further enhanced the invasion of tumor cells [27].

Microfluidics technology has also been employed to study ECM alterations during active invasion. For instance, Gioiella *et al.* utilized a microfluidic model to study ECM alterations during the tumor invasion into the stroma. In particular, the tumor region incorporated microtissue of MCF7 cells, whereas the stroma region contained microtissue of normal fibroblast (NF) [178]. Mono-culture of microtissues made of either NF or CAFs alone, were used as controls. In co-culture condition, the tumor cells invaded the surrounding stroma within 12 hours. Upon 48 hours, fibroblast cells within the stroma surrounding the tumor region exhibited marked expression of α SMA as well as PDGF similar to the CAF incorporated condition, suggesting activation of NFs. Furthermore, there was an enhanced expression of MMP-9 in co-culture condition as compared to mono-culture of NF. Extracellular matrix proteins including collagen, hyaluronic acid and fibronectin were also observed to be overexpressed in presence of the tumor microtissues. Using second harmonic imaging, ECM remodeling was observed in co-culture condition confirming the activation of NF when co-cultured with tumor micro-tissues [178]. These studies illustrate the ease and popularity of microfluidic platforms to study various aspects of cancer invasion.

Paper-based devices have also emerged as intriguing platforms that have numerous advantages including facile manipulation, rapid prototyping, and low cost for biological studies [181, 182]. The Whitesides group has developed numerous 3D paper-based microfluidic devices to interrogate various biological phenomena including cancer cell invasion [181, 183]. In their

platform, they observed that cells can migrate between adjacent layers of papers. Furthermore, by stacking sheets of paper on top of each other, thick tissue-like constructs could be prepared to mimic human pathophysiological conditions (Fig. 6F) [184]. Each paper could carry different types of cells and ECM molecules, so the approach enabled infinite combinations of composite constructs with heterogeneous cell populations [89, 181-183, 185]. In addition to pristine spatial arrangement, paper-based devices have been used as a powerful tool for studying hypoxia-induced responses in numerous cell types [181]. By placing paper-based construct in a holder to limit diffusion, a gradient of oxygen and nutrients is generated from the media source, to investigate the growth and invasion of tumor cells as well as their response to radiation therapy [186]. In another study, to precisely quantify the migration of tumor cells, a single sheet of paper coated with a cancer-cell laden hydrogel was sandwiched between two sheets of paper with acellular hydrogel coatings [187]. At defined time points, the sheets could be separated to quantify the number of cells invading into the adjacent papers. Combined with quantitative PCR, this assay could further be used to assess the downstream genetic consequences of cellular invasion. Other technologies such as acoustic cell patterning, piezoelectric cell printing, etc., have been used to similarly assess co-culture interactions with well-defined spatial organization [84, 85, 91, 188, 189].

4.3 Perspectives

Microengineered models provide significant advantages over conventional 3D and *in vivo* models as they recapitulate the tumor microenvironment at the initial stage of cancer cell invasion while enabling precise control over the biochemical and biophysical properties. However, there are still several unmet needs to better enable studies on cancer invasion. Current models have unveiled different modes of cellular motility in response to various cell-cell and cell-ECM interactions, but there is a lack of models that study the influence of ECM components on tumor cell plasticity during invasion. To this end, Schneider *et al.* utilized nanoimprint lithography to print micropores on a basal scaffold and investigate the heterogeneity in cell migratory patterns [190]. The authors demonstrated that with changes in pore sizes, shape, and deformability, cancer cells can demonstrate stark differences in both migration speed and the mechanisms of motility [190, 191]. In a similar context, another study led by the Ferrari lab utilized a nanoprinted model to unveil the mechanisms by which cancer cell adopt invasion strategies based on various phases of cell cycle [192]. Specifically, they engineered a unique

model of interstitial cancer migration assay using electrohydrodynamic 3D colloidal gold nanoprinting to develop gates with small pore size. This approach allowed them to restrict cancer cell migration and study nuclear deformation at various stages of cell cycle. At large pore size, the cells strived to penetrate the gates by extending their membrane protrusions. However, when the dimensions of the pores were reduced to limit cellular passage; most of the cells could not penetrate through the pores due to the need for nuclear deformation. To gain further insight on the relation between cell cycle and nuclear deformation, the cells were transfected with cell cycle reporter “Fucci” [193]. Higher frequencies of pore penetration were observed for those cells, which were in M/G1 phase (early postmitotic phase) as compared to later stages of the cell cycle. This model clearly suggested the critical role of cell cycle on nuclear deformation that can further modulate cancer cell invasion [192]. We envision that in forthcoming 3D models, biochemical cues along with biophysical factors will be incorporated to gain a compressive understanding of tumor cell plasticity during invasion.

Another significant driver of tumor plasticity is differentiation of cancer stem cells during tumor progression. In the tumorigenesis model, it is accepted that cancer stem cells are tumor initiating cells that contribute to chemotherapy resistance as well as metastasis [194]. Although different factors such as mechanical stress, hypoxia, and chemical mutagens affect the regulation of cancer stem cells, very few microengineered models exist to assess migration of cancer stem cells in response to various microenvironmental factors [194]. Recently, Lee *et al.* utilized photocrosslinkable polyacrylamide gels to create mechanical stress within micropatterned tissue constructs of various geometries and further demonstrated that cells isolated from these constructs express upregulated stem cell markers. Interestingly, when implanted within an *in vivo* model, the cells exhibited high frequency of metastatic events [195]. While this study elucidated the role of several factors that could affect the upregulation of stem cell markers and invasion, very few studies have explored these biological phenomena in a mechanistic manner.

One important aspect of cancer invasion is the interaction of cancer cells with stromal cells. While interactions between CAFs and cancer cells have been investigated on conventional 2D assays and 3D macroscale models [196, 197], they have not been extensively characterized in 3D microengineered models with precise spatial arrangement of tumor and stromal cells [17]. The Nikkhah lab made significant progress towards this goal with their microfluidic device, which contained separate tumor and stroma compartments [27]. However, this phenomenon has

not yet been mechanistically investigated in detail. In this regard, there are very few studies assessing the effects of combinatory stromal components (e.g. immune cells and CAFs) on cancer cell invasion [198]. We envision that future studies of cancer cell invasion will integrate microengineering technologies and advanced biomaterials to explore cellular invasion in response to the dynamic release of cytokines and growth factors from other cell types, as well as in response to parameters such as interstitial fluid flow and dynamic stromal remodeling.

As another critical shortcoming, the majority of current 3D models of invasion have utilized established cell lines. While these models have provided a detailed understanding of the underlying biology mechanisms during invasion, cell lines often lose their *in vivo*-like phenotype [191]. Incorporation of patient-derived cells within microengineered models will enable a) development of physiological understanding of invasion that also accounts for tumor heterogeneity, b) better design of therapeutic compounds and c) fine-tuning of the models for applications geared toward personalized medicine.

5.1 Intravasation

Intravasation, as defined by the National Cancer Institute (NCI), is the invasion of a cell through the lining of blood or lymph vessel such that it can enter the circulating stream and translocate to various tissues of the body [199]. This phenomenon has been observed to play a key role in numerous physiological functions [199]. For instance, during an injury or infection, immune cells migrate from the lymph nodes to the site of infection by intravasating into either the blood stream or lymphatic vessels upon receiving signals from the site of injury [199]. However, during cancer metastasis, intravasation is utilized by the tumor cells as a part of highly orchestrated invasion-metastasis cascade leading to colonization at distant tissues [200, 201].

Cancer intravasation can be broadly classified into two major categories, namely active and passive intravasation. During active intravasation, tumor cells migrate towards the blood or lymphatic vessels due to the gradient of chemokines [202]. On the other hand, some cells can undergo passive intravasation by shedding from tumor masses growing into the surrounding vessels, which collapse due to high stress and poor network formation [203]. While active intravasation involves cytoskeleton activity and integrin/MMP upregulation, passive intravasation can occur solely from the selective advantage of tumor cells in a stressed environments, which ultimately help them develop a large tumor mass and intravasate the nearby fragile vessels [203].

Tumor cells can intravasate through either blood or lymphatic vessels [204]. The intravasation of tumor cells through blood vessels is known as hematogenous spread, which mainly occurs through angiogenic sprouts as opposed to pre-existing vasculature [204]. On the other hand, lymphogenous spread entails intravasation through neovascularized lymph vessels [204-206]. While tumor cells utilize ligands VEGF-A and -B for hematogenous spread, lymphangiogenesis entails VEGF-C and -D binding to the VEGF-3 receptor and hence promotes lymph node intravasation [202, 205]. Tumor cell intravasation has been shown to be enhanced by stromal cells, such as TAMs, CAFs, etc. [156, 199, 207]. In particular, it has been observed that TAMs secrete EGF, which promotes tumor cell migration toward the blood vessels [199, 208], and form invadopodia within the vicinity of the endothelial barrier and aids in transendothelial migration (TEM) [199]. Tumor necrosis factor- α (TNF- α) released by TAMs increases blood vessel permeability thereby assisting in TEM [199, 208]. CAFs, on the other hand, facilitate tumor cell migration through blood vessels by secreting chemotactic factors such as CXCL12 [199]. Neutrophils present in the stroma also secrete a variety of proteases, such as MMP-9, that can degrade the surrounding ECM therefore releasing additional pro-angiogenic factors [199]. Alternatively, for lymph node intravasation, tumor cells express CCR7 which responds to gradients of CCL21 and CCL19 secreted by lymph endothelial cells to guide the chemotactic movement of tumor cells toward lymph vessels [205].

Once reaching the endothelial barrier, tumor cells intravasate through the endothelial junction by paracellular or transcellular processes by breaching surrounding basement membrane [199, 208-211]. In the paracellular process, tumor cells remodel endothelial junctions by secreting MMPs in regions where protease activated receptors are present on endothelial cells. Alternatively, they can demonstrate receptor shedding of vascular endothelial cadherin (VE-cadherin) and angiopoietin 1 (Ang-1) to disrupt endothelial junctions [208]. By remodeling cell-cell junctions on the lining of the vessel, tumors cells can intravasate into the circulating stream in a paracellular fashion. Additionally, cancer cells can engage in transcellular migration (TCM) where they cross the endothelial cell cytoskeleton and membrane by creating transitory pore-like structures [208].

Taken together, *in vitro* models that can recapitulate a physiologically relevant environment with controlled chemokine gradients are necessary to study the underlying mechanisms involved in intravasation. To date, numerous approaches have utilized

microfabrication technologies and innovative biomaterials to model intravasation with a great promise towards elucidating the mechanistic understanding of this process. Some of the pioneer studies have been reviewed in the following section.

5.2 Microengineered *in vitro* models to study tumor intravasation

Tumor intravasation models have been microengineered using techniques such as microfluidics [198, 212-215], pre-vascularized tumor spheroids [216], magnetic force based cell patterning [217], etc. For instance, Lee *et al.* utilized a seven-channel microfluidics platform to study tumor intravasation [214]. The platform was vascularized by embedding HUVECs and lung fibroblasts in a fibrinogen hydrogel and subsequently loading these cell-laden hydrogels into the respective channels. The device was then incubated for 7-8 days to allow for the 3D formation of microvessel. MDA-MB-231 cells were then seeded and allowed to attach onto the fibrin wall. The media channels were positioned in a manner that created gradient of growth factors radiating from the vessels toward the cancer cells, leading to chemotactic migration of the cells toward the endothelium. Over the course of the culture period, increasing amount of cancer cells intravasated into the vessel lumen. Furthermore, treatment of microvessels with TNF- α enhanced the permeability of the vessels resulting in higher rates of intravasation [214]. Similarly, the Kamm group utilized a novel microfluidic device to elucidate the role of TNF- α and TAMs in regulating endothelial barrier permeability and tumor cell intravasation (Fig. 7A (i)) [198]. Their device was fabricated so that two PDMS channels flanked a central layer of ECM (i.e. collagen). The endothelial channel (right) was vascularized in the presence of tumor cells (left), and TAMs were embedded within collagen hydrogel to study their effect on vessel permeability and tumor intravasation. In the presence of TAMs, microvessels had higher permeability allowing tumor cells to intravasate to the apical region of the endothelial monolayer with ease (Fig. 7A (ii)). To specifically study the role of TNF- α secreted by TAMs, EGF was perfused through the vascular channel to induce a chemokine gradient for cancer cell migration while adding additional TNF- α to study its influence on endothelial permeability and tumor intravasation. Their results clearly demonstrated that in the presence of TNF- α , the endothelial permeability significantly increased resulting in higher rates of intravasation. They also knocked down TNF- α in the presence of TAMs and observed that intravasation levels did not drop to the baseline, suggesting that TAMs may secrete other soluble factors or exhibit cell-cell interactions that permeabilize the endothelial barrier independent of TNF- α [198]. Recently, Han *et al.*,

utilized a microfluidic platform to study the effect of oriented collagen fibers on tumor cell migration across the basement membrane surrounding the vessels during intravasation [218]. In order to recapitulate the interface of interstitial matrix (mainly collagen I) and basement membrane surrounding the blood vessels in native tumor microenvironment; the device was loaded with matrigel followed by collagen I leading to sandwich of two hydrogels (Fig. 7B (i, ii)) [218]. The collagen fiber orientation was generated due to swelling of matrigel and strain developed into the system. In particular, the ECM channel was first filled with matrigel followed by injection of fluid collagen. During the polymerization matrigel volume swelled while collagen volume shrunk. This in turn led to development of strain inside the device that caused collagen fibers to orient vertically at the interface of collagen-matrigel hydrogel toward the vascular channels (Fig. 7B (iii)). Confocal microscopy revealed that the two hydrogels did not fuse into each other due to viscosity of matrigel, which subsequently allowed collagen fibers to locally orient within the device. MDA-MB-231 cells within the sandwiched gel began intravasating the gel interface (collagen-matrigel interface) as early as 48 hours, while by 144 hour most of the cells invaded and crossed the matrigel region. On the other hand, when the collagen fibers were oriented homogenously, MDA-MB-231 cells could not invade matrigel suggesting that the fiber alignment enabled the cells to gain high migration persistence and breaking through the basement membrane (Fig. 7B (iv)). In contrast, MCF-7 cells (less invasive) when introduced into the composite gel only rearranged into spheroids and by 168 hours could not enter the gel interface. This study thus indicated that cancer cells utilize the collagen fiber orientation to migrate across the basement membrane surrounding the media channel [218]. These findings clearly demonstrate the advantage of microfluidic devices to investigate the mechanistic role of ECM architecture, stromal components and biochemical cues on cancer cell intravasation.

In another interesting study, Ehsan *et al.* utilized a co-culture spheroid based model of cancer and endothelial cells to develop a pre-vascularized tissue. They further embedded the tissue in a fibrin hydrogel containing fibroblasts (Fig. 7C (i)) [216]. The model was utilized to investigate intravasation along with neovascularization and EMT. In the presence of tumor cells, the vessel sprouts were more irregular and shorter as compared to spheroids containing only endothelial cells and fibroblasts. Moreover, SW620 breast cancer cells intravasated into the lumens of the vessel (Fig. 7C (ii)). Notably, this effect was observed in hypoxia conditions where the intravasation rate was enhanced (Fig. 7C (iii)). Further analysis confirmed that Slug, a

marker for EMT, was highly upregulated within the hypoxic environment and played a critical role in intravasation [216]. While this study demonstrated the ease and suitability of spheroid-based models to study the mechanism of intravasation; still not many labs have adapted this technology to model the physiological process of TEM.

Other attempts have utilized magnetic force cell patterning as an alternate method to study tumor intravasation. For instance, Yamamoto *et al.*, seeded HUVECs on matrigel to initiate the formation of microvessels [217]. Next, B16F1 melanoma cells, labelled with magnetic cationic liposomes, were patterned using magnetic force into spheroids on top of the pre-formed microvascular network. The patterned construct was subsequently embedded within collagen hydrogel. Tumor cells in close proximity of the vessel attained an elongated invasive morphology as compared to cells at further distances ($> 80 \mu\text{m}$), which maintained their original morphology. Additionally, due to the presence of tumor cells, the intercellular junctions of endothelial cells were disrupted, prompting the migration of cancer cells and subsequent intravasation. Downstream gene analysis revealed that intravasation of B16F1 cells could be due to significantly higher expression of MMP-9, IL-6 and MDR-1, which are known to be contributors to matrix remodeling, inflammation, angiogenesis, and proliferation [217]. This specific study demonstrated that the cell-cell interactions between cancer (i.e. melanoma) and endothelial cells play a significant role in promoting intravasation of melanoma cells [217].

5.3 Perspectives

Current 3D *in vitro* models have generated a great wealth of knowledge about the mechanisms of intravasation, with a prime focus on hematogenous spread. However, there are various aspects in this regard, which still need to be further explored. For instance, *in vivo* intravasation also occurs through lymphatic vessels. Various groups have observed the phenomenon of lymphangiogenesis within *in vitro* 3D settings using different methodologies including microfluidics [125], culturing lymphatic endothelial cells (LECs) on an engineered connective tissue [219], as well as by embedding biopsies of rat thoracic within collagen [220, 221]. However, the incorporation of tumor intravasation with lymphangiogenesis within a microengineered model has yet not been thoroughly explored. In this regard, Pisano *et al.* utilized a microfluidic device built from boyden chamber to establish interstitial, transmural and luminal flow shear stress, within lymphatic capillaries, that can influence tumor intravasation [212]. The model was designed to have three components including microfluidic channels for

establishing LEC culture, regions to house the 3D tissue constituting tumor cells (i.e. MDA-MB-231 cells mixed in collagen I and matrigel) and fluidic inlets/outlets to control the flow rates. A pressure gradient was formed in the device, inducing interstitial flow throughout the cell culture region, and transmural flow towards the engineered lymphatic capillaries. The LECs in the engineered capillaries also faced shear stress due to the luminal flow of the culture medium through the channels. Using live cell imaging, the effect of different fluid flows on transmigration of tumor cells was recorded and quantified. Higher number of MDA-MB-231 cells transmigrated across the lymphatic capillaries as the luminal flow rate was increased compared to static conditions. Additionally, combining the luminal flow with transmural flow further enhanced the number of transmigrated cells. Downstream analysis demonstrated that increased transmigration of MDA-MB-231 cells was primarily due to elevated expression of CCL21 chemokine, which participates in lymphatic metastasis [212]. In another study, the Yoon lab utilized a microfluidic device to isolate highly chemotactic cells from a population of heterogeneous tumor cells that can migrate across confined spaces of lymphatic capillaries [222]. The model had two inlet channels along with multiple geometrically constricted channels with pore size similar to lymphatic capillaries *in vivo*. The left inlet was utilized to flow a stream of MDA-MB-231 cells into the device whereas chemoattractants were added in the right inlet to trigger migration of cells through intermittent channels. The findings demonstrated a significant difference in the migration of wild type MDA-MB-231 cells as compared to knockdown p38 MDA-MB-231 cells. The wild type cells elongated their pseudopodia beyond the small pores to establish attachment to substrate indicating mesenchymal mode of migration. On the other hand, the p38 knockdown cells could only squeeze through without significant migration. These results confirmed that p38 gene of mitogen activated protein kinases (MAPK) family play a significant role in cell motility and lymphatic endothelial metastasis [222] consistent with *in vivo* studies [223]. While these models elucidated the role of biophysical factors on transmigration of cancer cells across lymphatic capillaries; there are still very few models exploring the role of biochemical factors on lymphogenous spread. We envision that with the use of microfluidic models and LECs; lymphatic capillaries can be engineered and the role of various stromal components and chemokines could be mechanistically assessed in tumor intravasation [224].

The majority of the previous studies have elucidated the phenomenon of intravasation through paracellular mode of migration disrupting the intercellular endothelial junctions in order

to facilitate their entry into the circulatory system [198, 199, 214, 216]. However, transcellular intravasation, where cancer cells enter through the endothelial cell body, has not been extensively studied. In this regard, the Chew lab specifically utilized 2D [225] and 3D [226] platforms to initially model a vascular network by seeding/encapsulating endothelial cells on collagen and subsequently adding invasive breast cancer cells to investigate various modes of intravasation. Using fluorescent resonance energy transfer (FRET) imaging, they observed that the endothelial myosin light chain kinase played a significant role in transcellular invasion, but not in paracellular invasion [226]. In their follow up study, the authors studied the mechanism through which transcellular pores are created by invading cancer cells. The pores were further strengthened when they got encapsulated within myosin-rich structures termed transcellular circumferential invasion arrays (TCIA). TCIAs were densely packed with actin fibers, unique sarcomere alignment, and tensional stresses that regulate the reorganization of ECs actin-myosin network during transcellular intravasation [225]. While the Chew group primarily leveraged imaging technologies to elucidate biophysical mechanisms behind transcellular intravasation, the use of microengineered models combined with biochemical stimuli can provide further insight into the mechanism of intravasation. We believe that the future microengineered models will begin to incorporate additional stromal components to study transmigration of tumor cells through lymphatic or angiogenic vessels.

6.1 Tumor Extravasation and Colonization

For cancer cells to disseminate to other organs and form secondary tumors, they must first migrate through the blood vessel wall into the surrounding tissues. This specific process requires a complex cascade of step-wise events starting with the initial arrest of cells onto the endothelium, either through physical occlusion within a vessel or cell adhesion sites [149]. Following attachment, tumor cells undergo TEM, which involves a series of cell-cell and cell-ECM interactions where cells intravasate into vasculature and eventually extravasate to secondary sites [149].

Fluid flow and receptor-ligand interactions between cancer cells and the endothelium wall are major influencers of extravasation [149]. Circulating tumor cells (CTCs) under adequate flow within the blood stream demonstrate a leukocyte-like rolling behavior to enhance successive pairing of receptors on the CTC surface to the ligands on the vessel wall. Major contributors to maintaining these transient adhesive contacts are cancer cell surface

glycoproteins sialyl Lewis-a/x, surface ligands on the endothelium, such as P-, L-, and E-selectin, and shear stress due to the constant cyclical flow of blood [227]. In addition to these factors, adhesion strength is also regulated by other receptors, such as intracellular adhesion molecule 1 (ICAM1), vascular adhesion molecule 1 (VCAM1), and integrins. On the other hand, tumor cell arrest also involves mechanical trapping within blood vessels of diameters smaller than the CTCs, which results in enhancing receptor-ligand pairing leading to higher chance of extravasation [228].

Secondary sites of cancer metastases have been observed to be rather specific for each cancer subtype suggesting that metastasis is not at all a random process. In fact, Stephen Paget made the same observations in 1889 after examining over several hundreds of autopsy records from different patients [229]. He eventually described his findings as the ‘seed and soil’ hypothesis, where these different areas within the body were comprised of unique microenvironments (soil) catering to certain cancer metastases (seed). His theory was challenged in 1929 by James Ewing who proposed that cells instead break off from invading tumor masses to become a tumor emboli that would be subsequently carried away by the flow of interstitial fluid [230]. Based on the anatomy and circulatory patterns of the vascular system, cancer dissemination throughout the body could be described as the emboli becoming mechanically lodged in specific secondary sites [230]. Presently, it is accepted that these two ideas are not mutually exclusive and that cancer dissemination also involved mechanical trapping in the vascular system and specific biochemical interactions with the microenvironment of the secondary site [231].

Although, the subset of secondary sites for each specific cancer has been well-characterized in the recent years, the mechanisms supporting organs specific metastasis is still unclear. For instance, colorectal or pancreatic cancers primarily metastasize to the liver and lungs, while breast and lung adenocarcinoma metastasize with a further range that includes the bone, brain, lung and liver [232]. Research into metastatic tissue tropism for colorectal carcinomas have shown to favor the liver due to the proximity of the permeable liver capillary sinusoids to the mesenteric circulation, which is further evidenced by the 70% of patients with colorectal metastases in the liver [233]. However, experiments with colorectal carcinoma injection intravenously, intra-arterially, or intraportally all showed selective adhesion to the hepatic microvasculature suggesting that specific biomolecular interactions are involved

influencing the site of metastasis for each subtype of cancer [146]. In addition, studies on other types of cancer, such as breast and prostate, determined that expression of cell surface receptor CD44 increased cell attachment and enhanced the formation of confluent layers of human bone marrow endothelial cells [234]. On the other hand, microenvironmental factors such as diffusible chemokines and growth factors have also been demonstrated to influence the types of metastases grown at secondary sites [235, 236]. CXCL12, which is constitutively expressed in areas such as the bone marrow and lymph nodes, typically promotes the homing of hematopoietic stem cells expressing CXCR4 [235, 236]. Interestingly, breast cancer cells expressing CXCR4 preferentially metastasized to areas around the body rich in CXCL12 such as those mentioned above [236]. Overall, extravasation and organ-specific colonization is a highly dynamic process governed by many factors such as mechanical forces, chemokines, and cell-cell and cell-ECM interactions [232]. As such, there needs to be a greater understanding in tumor extravasation and colonization mechanisms by recapitulating the microenvironment of secondary sites in order to interrogate the individual interactions and identify effective therapeutic strategies.

6.2 Modeling Tumor Extravasation and Colonization *In Vitro*

Conventional studies of extravasation have mostly relied on *in vivo* models, such as mice tail-vein injections and zebrafish embryos, through the use of intravital microscopy [17, 233]. Although these models allow for organism-level physiological responses, their main disadvantages are lack of recapitulating human specific interactions and precise control of the microenvironmental cues [237]. On the other hand, conventional *in vitro* platforms, such as Transwell assays, offer some ability to investigate cancer extravasation with better control of the microenvironmental components than animal models [237]. For instance, adhesive interactions between cancer and endothelial cells could easily be studied using tumor-endothelium adhesion assays. In addition, transwell assays have been modified with 3D collagen gel inserts to study cellular behavior within different ECM compositions [238]. However, these simple assays are still limited by the lack of control over multi-cellular interactions and complex recapitulation of 3D cytoarchitecture along with restricted imaging capabilities in these platforms [237].

To address the above issues, a number of groups have made efforts to recapitulate *in vivo*-like microenvironment by microengineering *in vitro* 3D models integrated with advanced biomaterials [239, 240]. In particular, these advanced platforms provide specific advantages over simpler models by incorporating tunable biophysical (i.e. matrix stiffness, interstitial flow) and

biochemical cues (i.e. growth factors, drugs) coupled with high-resolution imaging within a precisely defined tumor microenvironment (i.e. cytoarchitecture, cell-cell interactions) to elucidate specific mechanisms of cancer extravasation [241].

In recent work by Zhang *et al.* [239], invasion distance and TEM of salivary gland adenoid cystic carcinoma (ACC) cell aggregates were investigated within a microfluidic device containing an endothelium barrier on top of an ECM (basement membrane extract) containing CXCL12 chemokine (Fig. 8A) [239]. Overtime, CXCL12 was diffused from the ECM beyond the endothelium and into the channel containing the ACC cell aggregates. The findings from this work demonstrated that CXCL12 stimulation was necessary to induce cancer cell aggregates to invade across the barrier. Moreover, the Qin lab presented AMD3100 as a CXCR4 antagonist to block uptake of CXCL12 that resulted in inhibition of ACC cell aggregate invasion and TEM. This work promoted the effectiveness of using a microfluidic model coupled with biomaterials to define a specific tumor extravasation microenvironment to study the influence of CXCL12/CXCR4 pathway as a target for disrupting TEM. Similarly, Song *et al.* [240], developed a two-layered microfluidic device with a permeable polyester membrane in between to study TEM under constant media flow along with the influences of basal (i.e. below the endothelial layer) or apical (i.e. above the endothelial layer) chemokines (Fig. 8B) [240]. The top PDMS layer contained a single channel connected to an inlet and outlet for media flow and CTCs while the bottom PDMS layer comprised of two sections labeled as upstream and downstream regions that intersected with the top channel [240]. Endothelial cells that were seeded on top of the polyester membrane could be selectively activated by treatment with biomolecules introduced into either the upstream or downstream regions in the bottom channels. TNF- α and CXCL12 were added individually into these regions and caused circulating MDA-MB-231 cancer cells to selectively adhere to the activated endothelial monolayer, which demonstrated precise control of the vascular endothelium to promote metastatic potential [240]. Similarly, AMD3100 treatment decreased attachment of MDA-MB-231 breast cancer cells onto the endothelial wall. Overall, this work demonstrated that crosstalk with CXCL12 enhanced cancer cell attachment by activating the endothelial cells and that blocking CXCR4 could prove to be a potential treatment for cancer metastasis. In addition to endothelium attachment, mechanical stresses during extravasation was studied by Chaw *et al.* in a microfluidic model that featured transmigration chambers along with several ports for cell input and read-outs (Fig. 8C)

[242]. Within circulation, CTCs must first undergo a series of deformations within narrow capillaries prior to adhering to the vascular endothelium. In this device, the incorporated microgaps of varying sizes mimicking blood capillaries for which the cells must pass through under physiological flow before reaching the transmigration chamber, which contained an endothelial barrier with or without matrigel. Importantly, there was 150 μm between the microgaps and the endothelium to allow for quantification of changes in cell surface area and deformation indices [242]. They tested different cancer cell lines, including HepG2, HeLa, and MDA-MB 435S, and found alterations in cell viability and doubling time after deformation. However, they did not find any significant changes in cell motility after having the cells undergo deformation. Still, the results from this particular study demonstrated that an endothelial cell and matrigel lining inhibited cancer cell extravasation, highlighting the importance of an intact basement membrane for cancer therapy [242].

Bersini *et al.*, developed an *in vitro* 3D co-culture model consisting of vascularized bone-mimicking tissues [243]. They utilized a Design of Experiment (DoE) approach to systematically analyze the most optimal and synergistic conditions for producing such an environment. Using HUVECs, MSCs, and osteo-derived MSCs along with fibrin and collagen matrices, they demonstrated a model with hollow functional vascularized network penetrating within the bone-mimicking matrix. They could observe active vessels growing within the matrix as well as active re-modeling of ECM by staining for changes in collagen and fibrin fibers. In that regard, this specific model holds the potential to be adopted as a cancer extravasation to bone models. For instance, channels adjacent to perfusable vascularized networks could be added to characterize the extravasation to bone behavior of CTCs from different cancer types (i.e. breast, lung, liver, etc.) Moreover, the bone matrix could be utilized to study leukemia and its different forms in order to understand how this cancer initiates and progresses to metastasis.

In addition to these models, the Kamm group has recently developed a series of advanced microfluidic models to study the different steps of the metastatic cascade ranging from cancer cell adherence to a vascular endothelium to secondary tissue tropism and colonization [244-246]. For instance, Jeon *et al.* utilized a microfluidic platform to create an endothelium barrier on a collagen scaffold wall to study tumor cell extravasation at different seeding densities (Fig. 9A) [244]. Moreover, they showed that the endothelium had heightened levels of permeability after introduction of tumor cells indicating heterotypic crosstalks leading to disruption of the

endothelial monolayer [244]. Following this study, they developed a five-channel vascularized MPS featuring cell loading areas in the second and fourth channels (gel-channels) while the other three channels were designated for media only (Fig. 9B) [247]. In this study, one of the two gel-channels contained HUVECs while the other was comprised of NHLFs and both were suspended in a fibrinogen and thrombin mixture. This specific configuration enabled communication between the two cell types leading to diffusion-dependent paracrine signaling giving rise to *in vivo*-like complex microvascular networks. To study extravasation, tumor cells were circulated throughout the device, and extravasation events were subsequently observed and evaluated using real-time microscopy. Moreover, the endothelium was treated with TNF- α to study the effect of pro-inflammatory cues on the endothelial barrier, which subsequently resulted in increased permeability and cancer extravasation rate. Moving forward, the developed extravasation model was utilized in mechanistic studies to understand the specific drivers and mediators of cancer extravasation. For instance, the same group studied organ-specific extravasation by developing bone- and muscle-mimicking microenvironments using specific cell and ECM compositions. This model was used to elucidate the role of $\beta 1$ integrin during extravasation where they discovered that $\beta 1$ integrin knockdown cells were unable to produce protrusions into the sub-endothelial matrix [248]. $\beta 1$ integrin was found to be necessary for migration beyond the endothelium which overall demonstrated the potential of this microfluidic model for elucidating the mechanisms underlying cancer extravasation. In addition to these studies, Gilardi *et al.*, utilized the same microfluidic model to further investigate the mechanism of cancer extravasation [249]. Specifically, they focused on the role of Talin-1 in invasion of MDA-MB-231 and HT1010 cancer cells. Using siRNA to silence Talin-1, they utilized the device to monitor cancer cell adhesion on the mimetic human vascular network. They showed that lack of Talin-1 expression dramatically reduced adhesion efficiency and extravasation for both cell lines. Additionally, these models were used to determine the effect of monocytes and macrophages on cancer cell extravasation. For instance, Boussommier *et al.*, integrated macrophages and HUVECs into the microfluidic device (Fig. 9) which formed functional vascular networks along with macrophages embedded in the fibrin matrix [250]. The permeability of the microvascular network was measured with fluorescent rhodamine dextran and found to be similar to reported values from *in vivo* studies. In addition, the presence of macrophages was discovered to not have a significant increase in cancer cell extravasation. In a follow-up study, monocytes derived from

patients were perfused into the device and allowed to extravasate in order to mimic monocyte-derived macrophages (MDMs) [251]. Although no difference in extravasation rate was found with or without MDMs, cancer cell migration away from vessels in the presence of MDMs was significantly increased. Overall, the models developed by the Kamm lab have been able to discern certain steps of the metastatic spread, allow for imaging at high resolution, and facilitate precise control of the microenvironment over more traditional assays.

Aside from the above models, one aspect, which has not been well addressed, is fate of the cancer cells after extravasating. To that end, Cui *et al.*, developed a perfusable microfluidic TEM assay integrated with a biocompatible porous membrane that also contained several independently controlled microchambers to isolate cells that have migrated through the endothelium [252]. Extravasated MDA-MB-231 cells were then isolated and characterized. They found that the extravasated cells exhibited higher aspect ratio, faster 2D migration, less consistent stress fiber alignment, and a higher nuclear palladin expression, compared to the cells that did not migrate across the endothelium.

6.3 Perspectives

The above discussed microfluidic platforms have established great potential in modeling tumor extravasation in order to understand the mechanisms and drivers underlying metastatic spread from the blood vessels to the secondary site. However, of the technologies discussed above, the models developed by the Kamm lab have emerged as the leading platforms for studying tumor extravasation. These platforms consisted of functional vascular networks that have been validated for cancer adherence and extravasation [246]. In addition, the Kamm lab has pushed toward understanding the minute steps of cancer cell extravasation by manipulating microenvironmental conditions and utilizing advanced imaging in their models. Recent work and collaboration with the Weinberg lab has included the study of neutrophils and NK cells and their involvement in tumor extravasation [253]. For instance, the microvascular network described in Fig. 9B were treated with 4T1-bearing mice splenocyte-conditioned medium prior to circulating MDA-MB-231 cells. They found that the conditioned medium enhanced extravasation efficiency ~ 2.5 fold suggesting that neutrophils activated endothelial cells to promote TEM. Overall, this technology has great capability for recapitulating the complex vascular microenvironment in order to facilitate in-depth study of vascular physiology in conjunction with tumorigenesis.

Moving forward, there is still a major lack of available extravasation models developed to include organ- or tissue-specific tropism for different types of cancer coupled with TEM. The majority of the above microfluidic technologies focused on the specific mechanisms of extravasation limited only to interactions between cancer cells and the endothelial barrier. However, several articles point to complex crosstalks between the CTCs and the cells within secondary site contributing by paracrine signaling of chemokines or inhibitory biomolecules to tumor extravasation [254, 255]. To study the influences of the secondary sites, Barney *et al.* developed a novel cell-ECM screening method to predict breast cancer metastasis to the bone, brain, liver, and lung using a biomaterial platform with precise control of the ECM composition (Fig. 9C) [256]. The proposed biomaterials were coupled to glass surfaces using silane chemistry. Three different ECM microenvironments (bone, brain, and lung) were prepared to quantify cancer cell phenotypes first through changes in cell adhesion and morphology and later using migration metrics (i.e. speed, displacement, and chemotactic index). The *in vitro* analysis performed on this platform was able to predict tissue tropism for seven out of eight heterogeneous cell lines tested and further developed specific ECM fingerprints for bone-, brain-, and lung-tropic cancer cells. Overall, this specific technology provided screening of cell-ECM interactions across the different heterogeneous cancer cell lines to predict *in vivo* metastasis. Nonetheless, this approach does not incorporate TEM and chemokine gradients, which play important roles in tissue specific metastasis.

On the other hand, the Kamm group has developed a tri-culture microfluidic 3D model to recapitulate bone-specific metastasis of breast cancer (Fig. 9D) [245]. They lined an endothelial wall against a bone matrix containing collagen gel and osteo-differentiated human bone marrow-derived mesenchymal stem cells (hBM-MSCs). Breast cancer cells (MDA-MB-231) were later introduced into the endothelial cell coated channel and they found that the bone matrix induced significantly higher extravasation than collagen gel only. In addition, in placement of the hBM-MSCs, they introduced CXCL5, a major chemokine secreted by osteoblasts, and still found higher rates of extravasation compared to the control group. Blocking the paired receptor, CXCR2, reduced extravasation but did not show any difference in extravasation distance suggesting a non-important role in breast cancer cell migration [245]. Despite all of the significant progress, microfabricated models to assess tumor extravasation in other types of cancers and their subsequent secondary sites are still missing. As a potential avenue, specific

tissue matrices could be designed similarly to those developed by Barney *et al.* and incorporated with microfluidic models, such as those from the Kamm group, in order to develop multi-parametric studies of cell-cell and cell-ECM drivers of extravasation and colonization [256]. Moreover, MPSs containing microvascular networks developed by the Kamm or George (discussed in section 3) lab have largely been focused to only understanding the interactions between cancer cells and the vasculature. In future studies, we hope to see an expansion of extravasation to specific tissues in the microfluidic platforms described above for better development of therapeutic and understanding the mechanisms of extravasation across different types cancers and the subsequent influences of the secondary sites.

7.1 Anti-Cancer Drug Studies

Recent studies demonstrated that drugs frequently exhibited poor efficacy *in vivo* compared to the corresponding *in vitro* studies [17, 54, 257]. Additionally, stromal cells along with ECM and soluble factors dynamically alter drug response and resistance of cancer cells, however they are not well-represented in traditional *in vitro* assays [257]. To address these issues, 3D platforms have been developed by integrating advanced biomaterials and microscale technologies to offer more flexibility through the incorporation critical components of the tumor microenvironment (e.g. perfusable vasculature) to reproduce physiologically relevant effects such as enhanced permeability and retention (EPR) [258-260]. Moreover, tissue-engineered 3D models recapitulate critical characteristics of solid tumors including cytoarchitecture, gradients of chemokines, physiological mass transport, and the presence of multiple cell types [54, 261]. Due to these unique characteristics, these models are more predictive of cellular responses to anti-cancer drugs compared to standard monolayer culture assays [59, 262-266]. In this regard, 3D tumor models can be leveraged to recreate such interactions and elucidate microenvironment-mediated functional regulation of anti-cancer drug response [48, 59, 267-271]. Herein, we highlight recent developments in the use of 3D models and advanced biomaterials for anti-cancer drug testing.

7.2 Innovations in Biomaterials and Microscale Models for Anti-cancer Drug Testing

To date, significant efforts have been made to mimic the composition and architecture of the native tumor microenvironment in order to optimally recapitulate physiological drug responses of cancer cells *in vitro* [262-264]. A wide array of natural and synthetic biomaterials

has been utilized in conjunction with microengineered platforms to create state-of-the-art 3D models for anti-cancer drug screening. Below, we will provide a detailed overview on the application of novel biomaterials and microengineered models that have been utilized for anti-cancer drug testing.

In a study by Hongisto *et al.*, JIMT1 breast cancer cells were seeded on top of polymerized matrigel, which aggregated and formed 3D clusters [272]. The breast cancer cells grew faster under this condition and were significantly more responsive to drug treatment when screened with two different drug libraries compared to their 2D culture counterparts. The higher drug sensitivity in the matrigel system indicated the critical role of the ECM in modulating drug responses of cancer cells. Furthermore, Lan *et al.*, engineered a tumor model consisting of alginate hydrogel for 3D culture of HepG2 liver cancer cells on top of a monolayer of MCF-7 cells to evaluate both hepatotoxicity and anti-cancer drug efficacy (Fig. 10A (i)) [273]. A polycarbonate disc was primarily designed to support the cell-laden alginate hydrogel in a well-plate setting. Breast cancer cells were seeded at the bottom of the well plate prior to insertion of the support disc. The HepG2-encapsulated alginate system metabolized acetaminophen leading to generation of toxic byproducts that significantly reduced the viability of MCF-7 cells. CT50 (50% cytotoxicity) values obtained from the 3D HepG2 drug screening correlated well with *in vivo* LD50 values as compared to the 2D condition without the presence of alginate hydrogel (Fig. 10A (ii)) [273]. This unique culture approach based on alginate discs provides an improved method for testing side-effects (i.e. hepatotoxicity) of anti-cancer drug compounds. The cells in 3D culture systems were more resistant to drugs and generated larger IC50 values [273], which was consistent with many reports that cells in 3D culture systems are often less sensitive to standard chemotherapy drugs due to limited diffusion through 3D hydrogel [274-276].

Alternatively, synthetic hydrogels conjugated with bioactive peptides have enabled investigating specific cell-ECM interactions mediated by biochemical (i.e. drug response) and biophysical cues [271]. In this regard, Yang *et al.* cultured 3D clusters of ovarian cancer cells using a synthetic peptide hydrogel RADA16-I, which provided the unique ability to control nanofibril density [277]. The ovarian cancer cell suspension was mixed with the RADA16-I peptide hydrogel and dispensed into a petri dish. The cells were homogeneously distributed within the 3D matrix. When screened against 5-fluoruracil, paclitaxel, and curcumin, the 3D ovarian cancer cell culture demonstrated increased drug resistance, as two- to five-fold higher

concentrations were needed to exceed a 50% inhibition activity level compared to 2D culture system.

PEG-based hydrogel integrated with biological components (i.e. cell integrin-binding motifs and cell-secreted proteases) have been also utilized to form 3D clusters of epithelial ovarian cancer cells [25]. Ovarian cancer cells were mixed with the PEG-based hydrogel precursor solution and polymerized to form 3D cultures. The drug response of the 3D cell cultures was evaluated using a common chemotherapeutic, paclitaxel. The cell survival and proliferation was measured for treated 3D culture systems using both DNA content and metabolic activity-based assay. Results indicated that cell survival and proliferation was reduced by 40% when 3D cultures were treated with paclitaxel for 7 days, and by 60% when treated for 14 days. A larger viability reduction (80%) was observed in monolayer cultures treated with a similar concentration of paclitaxel for 3 days. The paclitaxel resistance observed in ovarian cancer cell spheroids correlated with *in vivo* responses [278].

Hyaluronic acid (HA) is a GAG found in the ECM that is secreted by CAFs into the extracellular space of tumors [279-284]. Certain cancer cells such as prostate cancer cells and triple negative breast cancer cells overexpress the CD44 receptor and CD44-HA ligation, which subsequently promote signaling that leads to increased growth, survival, and drug resistance [285-288]. A recent study demonstrated that HA derivatives promoted formation of 3D clusters of poorly-adherent prostate cancer cells [24]. To form 3D cultures, prostate cancer cells were encapsulated in hydrogel based matrices by direct mixing with HA derivatives leading to spontaneous crosslinking. Interestingly, prostate cancer cells in the HA hydrogel 3D culture displayed greater apoptosis when treated with camptothecin compared to the 2D culture of cells. Similarly, a combinatorial drug treatment with docetaxel and rapamycin showed greater toxicity to cells in the 3D hydrogel system compared to 2D culture. These results were attributed to differential gene expression and signaling between the two culture systems, highlighting the importance of implementing 3D cultures for drug testing studies. In another study, a hyaluronan-based hydrogel was utilized to alleviate the low viability issues of patient-derived xenograft (PDX) prostate cancer cells (Fig. 10B) [289]. Culturing prostate PDX tumor cells within the 3D hyaluronan-based hydrogels supported cellular viability. When treated with docetaxel, the 3D PDX prostate cancer model displayed much higher drug resistance compared to 3D cell line models, indicating the importance of utilizing PDX models for cancer drug testing. Due to the

simplicity and throughput of generating arrays of spheroids, numerous pharmaceutical companies and researchers have leveraged this technology for high-throughput drug screening [18, 54].

The use of a polymeric aqueous two-phase system (ATPS) has also been explored in great detail to microengineer 3D cancer cell cultures [290-292]. This technology developed 3D cancer cell culture systems by using the concentration-dependent immiscibility of aqueous solutions of PEG and dextran (DEX). Standard microplates were filled with the aqueous PEG phase solution, prepared with a culture medium. Cancer cells of interest were homogeneously mixed with the aqueous DEX phase solution and then submerged in cell culture medium. The DEX phase-cancer cells mixture was dispensed as submicroliter droplets into individual wells of a microwell plate. Subsequently, cancer cells became entrapped in the aqueous DEX phase drop and spontaneously aggregated to form 3D spheroids (Fig. 10C) [293]. This technology has been highly optimized to form uniformly sized 3D cultures for use in drug screening. Interestingly, it was demonstrated that 3D cultures of a triple negative breast cancer cell line (MDA-MB-157) were completely resistant to paclitaxel while monolayer cultures of the cells were highly sensitive to the drug treatment [274]. Similarly, 3D cultures of a skin cancer cell line (A431.H9) displayed higher resistance to paclitaxel and cisplatin treatment compared to its monolayer counterpart [294]. A recent study used the polymeric ATPS approach and demonstrated high throughput, dose-dependent screening of 25 anti-cancer compounds against 3D cultures of breast, brain, and colon cancer cells [293]. This screening used over 7,000 tumor spheroids and introduced a high-throughput method for ranking drug efficacy in 3D culture of cancer cells. These studies demonstrate the feasibility of using the polymeric ATPS technology for convenient high-throughput screening of anti-cancer compounds against cancer cells in 3D culture.

As stated earlier, spheroid culture mimics the *in vivo* condition better than 2D *in vitro* culture. However, the lack of perfusion reduces their ability to accurately model drug exposure and transport to cells. Thus, there have been other approaches based on the use of microengineered platforms to have a better control over drug distribution and response. For instance, Ruppen *et al.*, developed a perfusion microfluidic platform to assess chemosensitivity of spheroid, where they were able to probe the media using a standard plate reader [295]. This study utilized malignant pleural mesothelioma (MPM) cell line, which is rare, but highly aggressive and lethal thoracic cancer. The microfluidic device contained channels with eight

spheroid traps in which MPM spheroids were perfused and localized inside these traps (Fig. 11A (i)) [295]. Upon loading the spheroids, chemosensitivity assays based on proliferation and apoptosis, were used to calculate the GI50 (50% of maximal inhibition of cell proliferation) of Cisplatin (μM) within the MPM tumor spheroids (Fig. 11A (ii)). Interestingly, when compared to 2D models, the GI50 nearly doubled with the 3D model suggesting higher chemoresistance similar to the *in vivo* conditions.

In another study, Bruce *et al.*, fabricated a 3D microfluidic cell culture platform to investigate acute lymphoblastic leukemia (ALL) within the context of a bone marrow microenvironment [296]. Tri-culture of SUP-B15 leukemic cells, primary bone mesenchymal stem cells (bMSCs), and human osteoblasts (HOBs), were encapsulated in collagen I to form a bone mimetic microenvironment. They compared chemoresistance of Ara-C, a chemotherapy agent used mainly in the treatment of cancers of leukocytes, in 2D, static 3D, and dynamic 3D models (i.e. with constant media perfusion). Interestingly, the cells were less viable within the 2D compared to 3D model suggesting that the 3D microenvironment induced a chemoprotective effect on cancer cells. Perfusion of Ara-C maintained a constant drug concentration over time, which induced a lower viability compared to the static culture without perfusion. These observations are in agreement with clinical data suggesting that the bone marrow microenvironment acts as a sanctuary for leukemia [297]. In another study, Aung *et al.*, developed a microfluidic device with cancer spheroids of MCF-7 cells and HUVECs confined within GelMA hydrogel. HUVECs formed an endothelial layer surrounding the tumor spheroid [298]. They studied the potential of the platform as a drug screening assay by investigating the efficacy of doxorubicin (DOX) on the HUVEC layer and the MCF-7 tumor spheroids. Using fluorescence imaging, penetration of DOX with different concentrations (1, 10, and 100 $\mu\text{g mL}^{-1}$) was measured, confirming that higher amounts of DOX were needed to adequately penetrate through the tumor spheroid. Moreover, they were able to demonstrate a dose-dependent response as a function of spheroid size owing to the ECM and HUVECs acting as a physical barrier. Overall, these platforms highlighted the significance of producing a biomimetic environment within using microfluidic assays to accurately assess the efficacy of numerous anti-cancer drugs.

7.4 Perspectives

The use of biomaterials to develop 3D cell culture models has provided enormous benefits to the field of cancer drug research and development. Nonetheless, several challenges still exist. For instance, conventional 3D cell culture methods still present technical difficulties when creating models that have reproducible environments for cells [241]. Furthermore, due to throughput limitations, the pharmaceutical industry typically defaults to spheroids testing, which often lacks critical biological stimuli from ECM, interstitial flow, and interactions between multiple organs [54]. On the other hand, the use of microengineering technologies integrated with innovative biomaterials for 3D cell culture can alleviate these difficulties and present defined microenvironments that include manipulation of biophysical and biochemical cues for high-throughput applications [241, 299]. For example, this concept has allowed simultaneous examination of effects from multiple cancer drugs on 3D cultures in collagen gel by patterning of cultures as an array of cell panels in a silicon chip [300]. However, most of the above described novel biomaterials were not well-integrated with MPSs (described in sections 2 to 6) that would have been capable of leveraging both the advantages of the material properties and the microengineered platforms. For instance, there is a major lack of studies that simulate the effect of anti-cancer drugs at a tissue and/or organ level using microengineered technologies. These are traditionally more or less limited to isolated cells or spheroids with simple cell-ECM interactions. In addition, studying tissue interfaces such as of that between the vascular endothelium and surrounding parenchyma would provide much needed information on the pharmacology and pharmacokinetics of drugs at a cell and tissue level [301]. Typical pharmacokinetic studies describe drug distribution within the body by testing the specific drug concentration in the plasma and various tissues [302]. However, local drug penetration is often influenced by interstitial fluid flow, sequestration, hypoxia, pH and ECM composition that are often not accounted for [266]. Traditional anti-cancer drug models often do not incorporate functional vasculature and interstitial fluid flow, which have major influences on the effect of drug resistance through convective and diffusive mass transport [303]. Additionally, the ECM within the tumor microenvironment often acts as a physical barrier especially in solid tumors inhibiting anti-cancer drug penetration while sequestering drugs depending on their physicochemical properties [304]. Hypoxia has been also implicated in upregulating pro-survival and angiogenic pathways in cancer cells [305]. In specifics, hypoxia can induce a quiescent state leading to higher resistance against drugs that act on cell proliferation. Buildup of lactic acid due

to hypoxia as well as a lack of adequate vasculature has been shown to prevent weakly basic drugs from being effective on cancer cells [306].

The Kamm group has worked extensively to develop functional vasculature with perfusion in a variety of microfluidic configurations in order to recapitulate tumor vascular physiology and analyze vasculogenesis in response to anti-cancer therapeutics. In one instance, they assessed the effect of bortezomib on HUVECs to inhibit tumor angiogenesis at the ECM (collagen)-endothelium interface [307]. They found similar cytotoxic and anti-angiogenic effects as *in vivo* validating their microfluidic platform as a testing bed for angiogenesis inhibitors. In addition, several other groups have been attempting to incorporate complex functional and perfusable vasculature to couple this system with cancer spheroids for drug testing. For example, the George group created perfused solid tumors in a microfluidic device by coupling tumor spheroids of SW620 colorectal cells in co-culture with HUVECs and NHLFs within a fibrin matrix [145]. In this regard, the controlled presentation of chemicals in microfluidics allowed studying the efficiency of drug delivery to 3D culture of cancer cells. In another study by Xu *et al.*, lung cancer cells were cultured in 3D in basement membrane extract within a microfluidic device (Fig. 11B (i)) [308]. This device had the ability to control fluid flow to multiple unique cell culture chambers at defined volumes, thereby allowing the creation of separate concentration gradients of up to four different drugs simultaneously (Fig. 11B (ii)). In addition, co-cultured 3D models of lung cancer and stromal cell lines showed ~2x lower apoptosis compared to single culture of lung cancer cells in 3D, indicating the influence of stromal cells on drug responses of cancer cells, which exemplifies the need to model complex cell-cell interactions. Moreover, the use of 3D culture of primary cells in this device resulted in larger IC₅₀ with gefitinib than 3D culture of the lung cancer cell line suggesting that heterogeneity among primary cells and established cell lines would lead to a varying drug response [308]. Overall, this platform had the unique ability to test multiple drugs and combination therapies with high-throughput, indicating its potential to investigate drug sensitivity of cells from individual patients towards realizing personalized cancer therapy. In a recent study, Liu *et al.* developed a biomimetic microfluidic platform to recapitulate events of tumor-induced angiogenesis from salivary gland adenoid cystic carcinoma (ACC) and oral squamous cell carcinoma (SCC) cells [309]. To investigate the physiological similarity of their microfluidic device, a traditional nude mouse xenograft model was used in conjunction with the

tumor angiogenesis platform. Using bevacizumab, a recombinant humanized anti-VEGF monoclonal antibody, to disrupt angiogenesis, they found similar results between the microfluidic and mouse xenograft models. These findings highlight the ability for microengineered models to recapitulate specific metastatic steps, such as angiogenesis or intravasation, while allowing for drug screening results comparable to *in vivo*.

Nonetheless, interactions among different organs within the human body notably contribute to the effect of the anti-cancer drug response, while this critical phenomenon is often lacking even in the most complex of MPSs [310]. In that regard, it is paramount to develop advanced microengineered technologies consisting of multiple connected organ-on-a-chip devices with precise control over microenvironmental parameters, which are tailored to the targeted cancer type [311, 312]. Specifically, these systems contain units of cells that emulate human organ functionalities and recapitulating a biomimetic physiology and could provide tremendous predictive power in drug efficacy [313]. To that end, the Shuler group developed a micro cell culture analog (μ CCA) with 3-D hydrogel to study the cytotoxic effects of anti-cancer drugs in a connected organ-on-a-chip model using multiple cell lines including HCT-116, HepG2/C3A and Kasumi-1 (Fig. 11C) [314]. The device consisted of interacting tumor, liver, and bone marrow, connected by channels to establish a human physiologically-based pharmacokinetic (PBPK) model. Both matrigel and alginate were used for cell encapsulation, where matrigel provided a 3D scaffold for the anchorage-dependent cells (i.e. HCT-116 and HepG2/C3A cells) while alginate housed the more mobile Kasumi-1 cells. The combination of these biomaterials and their microengineered device enabled recapitulating similar cytotoxic effects as in animal and human clinical studies. Overall, this type of MPS seems to be a step-forward for the future of anti-cancer drug technologies. In addition, using innovative biomaterials to recapitulate organ-specific ECM in a multi-organ system may lead to more realistic drug responses of cancer cells. Compared to some of existing methods such as matrigel-based 3D culture systems that are limited by a relatively fixed ECM composition and batch-to-batch variability, the use of advanced biomaterials would offer unique advantages in terms of tunability of biochemical, mechanical, and transport properties, and reproducibility to facilitate mechanistic biological studies on ECM-mediated behavior of cancer cells [17]. Additionally, functional vasculature with perfusion is necessary to study the pharmacokinetics and pharmacodynamics of therapeutics to enhance prediction for drug response [303]. These design

criteria would better guide drug studies by improving the functionality of the organ-like constructs and enhance accuracy of the study results, which would expand the capabilities of 3D microengineered models.

8. Conclusion

State-of-the-art microengineered technologies that incorporate advanced biomaterials have emerged as unique alternatives to 3D bulk hydrogel and animal models, providing precise control over critical parameters required in cancer biology studies and drug testing. Conventional 3D cell culture methods such as transwell assays and sandwich culture platforms do not incorporate heterogeneous tissue level organization. Alternatively, organotypic culture models, including spheroids and tissue explants represent the architecture of the tumor microenvironment but lack precise control over physiologically relevant biochemical and biophysical cues.

Innovative microtechnologies integrated with 3D biomaterials for multi-cellular cultures can alleviate these challenges and present well-tuned microenvironments capable of modulating ECM composition, cellular cross talk, and distribution of soluble factors. In this regard, recent tumor models have enabled gaining fundamental insight into the biological aspects of the metastatic cascade, which has not been fully possible using conventional assays. These platforms enable controlled studies on how tumor cells respond to signaling cues through each stage of the metastatic cascade. Microengineered models also lead to faster and more reproducible findings with improved imaging resolution at a single cell level compared to studies using animals.

Despite significant progress, there are still numerous challenges in the development of tumor models to be able to fully represent the *in vivo* tumor microenvironment. For instance, a majority of the microengineered models described in this review article utilized a limited variety of cell lines to represent cancer. For instance, the MCF-7 cell line, commonly used as a model for invasive breast ductal carcinoma studies, was established in 1973 at the Michigan Cancer Foundation. However, current studies have demonstrated that tumor heterogeneity needs to be fully unveiled and understood prior to advancing toward translational cancer research. For instance, in a recent study by Yates *et al.*, they showed that multiple regions of a primary breast cancer, when sequenced, revealed differing spatial patterns in the transcriptome of each region. In addition, subclonal diversification did not follow a strict pattern and varied among different patients with a high correlation between the tumor growth and the degree of heterogeneity in

triple negative breast cancer [315]. In this regard, the incorporation of patient-specific cells and heterogeneous cell populations is crucial to predictively mimic the *in vivo* response.

There is still a great need to develop a truly predictive model of cancer metastasis that can be used as personalized diagnostic devices in clinic. Major roadblocks exist in pursuit of this goal, the first being that no current platform aims to recapitulate the multi-step metastatic process within a single device, which is vital to determining the physiological response for patients at the end-stage of metastasis. In this regard, it is also required to understand the specific interactions between tumor cells and other organs post-extravasation and colonization. Furthermore, the biomaterial selection and design within current models are typically arbitrary and not organ-specific, which could significantly influence cellular and molecular responses. To this end, studies on the development of organ-specific biomaterial compositions are required to better mimic cancer cell behavior.

While the advent of microengineering technologies has led to significant discoveries in tumor biology, multidisciplinary research still seems to face tangible barriers in adopting expensive techniques such as bioprinting, acoustic patterning, etc. due to the required expertise and cost. On the other hand, simpler and cheaper technologies such as microfluidics and micromolding are more likely to be adopted by scientists outside of the engineering arena (i.e. biologist). Despite their significant advantages over conventional assays (e.g. transwell), microengineered platforms still lack comparative benchmarks. Therefore, many biologists still rely on the use of standard experimental platforms and/or *in vivo* animal models. To fully embrace microengineered models, there must be significant initiatives toward interdisciplinary collaborations. Instead of just referring to animal models, data obtained from human studies should be also utilized as a critical step to guide the development of future state-of-the-art technologies. In this regard, the incorporation of high throughput genome sequencing can enrich the knowledge-base of various pathways influenced by different hydrogel compositions.

Overall, the integration of microengineered models and innovative biomaterials has led to the creation of powerful models that enable tight control over numerous biophysical and biochemical parameters that influence tumor progression. Further development of biomimetic models based and the physiological validation will continue to provide new insights into our mechanistic understanding of cancer biology and will enhance the efficacy of drug testing for clinical trials.

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10. References

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FIGURE CAPTIONS

Figure 1: Schematic of the metastatic cascade. Tumor growth and development results in ECM remodeling (A) as well as differentiation of cancer stem cells and fibroblasts. Subsequently, angiogenesis, cancer cell invasion, and intravasation (B) occur. Finally, surviving cancer cells and cancer stem cells circulate through the body, attach to blood vessels, and extravasate (C) to form secondary metastases.

Figure 2: (A) Schematic (i) of fabrication of microengineered tissue surrogates, and representative immunofluorescent images (ii) of demonstrating invasion of tumor cells from the end of the cavities. Adapted with permission from the National Academy of Sciences: [PNAS], copyright (2012) [61]. (B) Schematic of microfluidic model (i) to study transition of MCF10A. DCIS to Invasive Ductal carcinoma in presence of Human mammary fibroblasts, and F-actin structure (ii) of co-cultured group MCF10A.DCIS cells as compared to mono-cultured MCF10A.DCIS cells. Adapted with permission from the Royal Society of Chemistry (RSC): [Integr. Biol.], copyright (2011) [79]. (C) Schematic (i) of seven channel microfluidic device, and immunostained images (ii) of Alpha SMA and F-actin in mono-culture and co-culture conditions. Adapted with permission from PLOS: [PLOS ONE], open-access (2016) [71].

Figure 3: (A) Schematic (i) of engineering alginate based microcapsules containing cells, and Immunofluorescent images (ii) of Ki67, Fibronectin and Nuclei to demonstrate presence of necrotic core at center in alginate based spheroids as compared to control spheroids generated by agarose cushion technique. (iii) Invasive morphology demonstrated by tip cells in alginate microcapsules based spheroids as compared control spheroid when encapsulated in collagen. Adapted with permission from the National Academy of Sciences: [PNAS], copyright (2013) [80]. (B) Schematic (i) of magnetic force-based cell patterning to create three different co-culture arrays, and (ii) immunofluorescence images indicating invasive transition of melanoma (green) cells in response to stromal fibroblasts (red). Adapted with permission from Wiley Online Library: [Biotechnology Progress], copyright (2010) [84].

Figure 4. (A) Schematic (i) of a micromolded model that studied neovascularization in the presence of tumor cells. Fluorescent images (ii) demonstrating separation of endothelial monolayer from cancer cells upon construction. Adapted with permission from Elsevier: [Biomaterials], copyright (2015) [121]. (B) Microfluidic device demonstrating angiogenic response to MTLn3 cancer cells (i) and U87MG cancer cells (ii). Adapted with permission from RSC: [Lab chip], copyright (2009) [123]. (C) Schematic (i) depicts a microfluidic device with different cell seeding configurations for studying vasculogenesis (ii) or angiogenesis (iii). Fluorescent images of engineered vascular tissue (iv), angiogenic sprouting (v), and altered angiogenic sprouting in the presence of U87MG glioblastoma cells (vi). Adapted with permission from RSC: [Lab chip], copyright (2013) [124].

Figure 5. (A) Micromolding technique (i) developed by the Chen group demonstrating formation of a confluent endothelial monolayer (ii) within a patent channel and vessel sprouting in response to angiogenic gradients. Adapted

with permission from the National Academy of Sciences: [PNAS], copyright (2013) [128]. (B) Bioprinting technique developed (i-iv) by the Lewis lab utilizing Pluronic F127 as a sacrificial mold to thick (>1cm) tissues, and demonstration of nutrient diffusion to support differentiation of MSCs to osteogenic tissue. Adapted with permission from the National Academy of Sciences: [PNAS], copyright (2016) [132]. (C) Fluorescent images showing the ability of StarPEG-heparin hydrogel for studying the effect of mural cells and endothelial cells as well as for tumor vascularization. Adapted with permission from Nature Publishing Group (NPG): [Sci. Rep.], open-access (2016) [135].

Figure 6:(A) (i) Fluorescent images depicting individual and collective migration of MCF10A-HRas across basement membrane into collagen I in a spheroid based model. (ii) Confocal images showing reorganization of collagen I fibers during active invasion at the interface of basement membrane and stroma. Adapted with permission from Elsevier: [Biomaterials], copyright (2017) [164]. (B) Schematic (i) of fabrication of collagen microtracks, confocal reflectance microscopy image (ii) of cell tracks formed in non-patterned gel, and immunofluorescent images (iii) of cell migration in both 3D matrix and Collagen track. Adapted with permission from RSC: [Integr. Biol.], copyright (2013) [165]. (C) Tubulin (red) staining of MCF10A cells (i) indicating formation of spheroids, cell tracks (ii) of invasive MDA-MB-231 cells. Adapted with permission from Elsevier: [Biomaterials], copyright (2016) [28]. (D) Mouse S180 sarcoma cells were kept in isotonic medium or exposed to hypotonic shock at the leading edge of the cells (red arrows). Results indicated induced cellular motility opposite to the osmotic gradient as well as decreased cell body volume in the hypotonic condition. Adapted with permission from Cell Press: [Cell], copyright (2014) [180]. (E) Schematic (i) of co-culture invasion assay from Truong et al. and phase-contrast images (ii) demonstrating increased migration of SUM-159 cells in presence of CAFs. Adapted with permission from Nature Publishing Group (NPG): [Sci. Rep.], open-access (2016) [27]. (F) Paper-based invasion assay: a single layer of wax-patterned paper containing GFP-expressing A549-HGF-M cells (metastatic lung cancer cells) was suspended in a hydrogel (Layer 0) and positioned between sheets of paper containing only hydrogel (Layers 0 + n for layers above the seeded layer, and 0 - n for layers below the seeded layer). All layers of paper were stacked in an acrylic holder with the impermeable bottom to block diffusion of oxygen and nutrients. Fluorescent images of all layers of paper were taken after culture for 24 h. An array of circular zones was visible due to the autofluorescence of the wax. Within the circles, darker pixels correlate with a higher intensity of GFP (e.g., higher density of cells). Adapted with permission from Elsevier: [Biomaterials], copyright (2015) [184].

Figure 7: (A) Schematic (i) of microfluidic device for studying tumor intravasation, and real-time live cell imaging (ii) demonstrating intravasation at different time points into the formed lumen. Adapted with permission from the National Academy of Sciences: [PNAS], copyright (2012) [198]. (B)(i) Schematic representing fabrication of sandwiched ECM in the microfluidic device. (ii) Confocal image showing the location of collagen I and matrigel in the Z-y plane. (iii) 3D image of collagen 1 and matrigel composite ECM at the interface. (iv) Bright field and fluorescent image depicting tumor cell intravasation into sandwiched ECM due to collagen I fiber orientation. Adapted with permission from the National Academy of Sciences: [PNAS], copyright (2016) [218]. (C) Schematic

(i) of spheroid model for blood vessel sprouting and intravasation, immunostained images (ii) demonstrating sprouting of blood vessels and intravasation of tumor cells (green) into the newly formed blood vessel (red), and higher rate of intravasation (iii) observed at hypoxic conditions. Adapted with permission from RSC: [Integr. Biol.], copyright (2014) [216].

Figure 8: (A) Microfluidic device featuring a single endothelium in order to study cancer extravasation under a chemokine gradient. Adapted with permission from RSC: [Lab Chip], copyright (2012) [239]. (B) Platform fabricated by *Song et al.* containing two specific regions that could be selectively activated by a chemokine point source originating from either of the two. Cancer cell adhesion to the two regions was assessed under flow conditions. Adapted with permission from PLOS: [PLOS ONE], open-access (2009) [240]. (C) Device incorporated with microgaps in order to recapitulate cancer cells squeezing through small capillaries. Adapted with permission from RSC: [Lab Chip], copyright (2007) [242].

Figure 9. (A) Microfluidic device developed by the Kamm group in order to study cancer cell extravasation through an endothelium. Adapted with permission from PLOS: [PLOS ONE], open-access (2009) [244]. (B) A similar platform by the Kamm lab featuring microvascular networks Adapted with permission from RSC: [Integr. Biol.], copyright (2013) [247]. (C) Cell-ECM interaction screening method by *Barney et al.* used to study the effect of different cancer cell metastatic signatures. Adapted with permission from RSC: [Integr. Biol.], copyright (2015) [256]. (D) Bone microenvironment recapitulated within a microfluidic device to study breast cancer extravasation to bone. Adapted with permission from Elsevier: [Biomaterials], copyright (2014) [245].

Figure 10: (A) Anti-cancer model fabricated by seeding breast cancer cells on bottom of a well plate and encapsulating HepG2 cells in Alginate matrix above the confluent monolayer. Adapted with permission from Elsevier: [Toxicology and Applied Pharmacology], copyright (2011). (B) Patient derived prostate tumors were grown subcutaneously in SCID mice and then harvested and dissociated. The dissociated tumor cells were seeded in 6-well plates in which mouse-derived cells (red) attached to the well bottom. The remaining tumor aggregates (blue) were cultured as 3D tumoroids in the hyaluronan-based hydrogel (ii). Adapted with permission from ACS: [Mol. Pharmaceutics], open-access (2014) [289]. (C) Spontaneous formation of spheroid of triple negative breast cancer cell MDA-MB-157 after incubation for 24 hrs in aqueous two-phase system (ATPS) and resulting tumor morphology with increasing concentrations of anti-cancer drug (ii). Adapted with permission from ACS: [Mol. Pharmaceutics], copyright (2016) [293].

Figure 11: (A) (i) Schematic demonstrating trapping of the ovarian cancer spheroids within microfluidic channel. (ii) Bright field images showing perfusion of the spheroids at different culture periods. Adapted with permission from RSC: [Lab Chip], copyright (2014) [295]. (B) Schematic (i) of a microfluidic chip-based 3D co-culture with four unique drug inputs that each generate three distinct concentrations based on fluid flow. Phase contrast images with fluorescent overlay (i) demonstrated that ethidium treated non-small cell lung cancer (SPCA-1) cells stained

red with increasing concentration, indicating increased apoptosis and cell death. Adapted with permission from Elsevier: [Biomaterials], copyright (2013) [308]. (C) Schematic of micro cell culture analog with corresponding PBPK model of the liver, tumor and marrow compartment. Adapted with permission from RSC: [Lab Chip], copyright (2010) [314].

A(i)

(ii)

B(i)

(ii)

C(i)

(ii)

A(i) **(ii)** **(iii)** **B(i)** **(ii)**