HHS Public Access

Author manuscript

Biomaterials. Author manuscript; available in PMC 2017 December 14.

Published in final edited form as:

Biomaterials. 2016 November ; 108: 197–213. doi:10.1016/j.biomaterials.2016.08.052.

Heralding a New Paradigm in 3D Tumor Modeling

Eliza L.S. Fong[1,2,*], **Daniel A. Harrington**[3], **Mary C. Farach-Carson**[3,*], and **Hanry Yu**[1,4,5,6]

[1]Department of Physiology, National University of Singapore, Singapore

[2]Department of Biomedical Engineering, National University of Singapore, Singapore

[3]Department of BioSciences, Rice University, Houston, USA

[4]Mechanobiology Institute, National University of Singapore, Singapore

[5]Institute of Bioengineering and Nanotechnology, Agency for Science, Technology and Research, Singapore

[6]Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou, China

Abstract

Numerous studies to date have contributed to a paradigm shift in modeling cancer, moving from the traditional two-dimensional culture system to three-dimensional (3D) culture systems for cancer cell culture. This led to the inception of tumor engineering, which has undergone rapid advances over the years. In line with the recognition that tumors are not merely masses of proliferating cancer cells but rather, highly complex tissues consisting of a dynamic extracellular matrix together with stromal, immune and endothelial cells, significant efforts have been made to better recapitulate the tumor microenvironment in 3D. These approaches include the development of engineered matrices and co-cultures to replicate the complexity of tumor-stroma interactions *in vitro*. However, the tumor engineering and cancer biology fields have traditionally relied heavily on the use of cancer cell lines as a cell source in tumor modeling. While cancer cell lines have contributed to a wealth of knowledge in cancer biology, the use of this cell source is increasingly perceived as a major contributing factor to the dismal failure rate of oncology drugs in drug development. Backing this notion is the increasing evidence that tumors possess intrinsic heterogeneity, which predominantly homogeneous cancer cell lines poorly reflect. Tumor heterogeneity contributes to therapeutic resistance in patients. To overcome this limitation, cancer cell lines are beginning to be replaced by primary tumor cell sources, in the form of patient-derived xenografts and organoids cultures. Moving forward, we propose that further advances in tumor engineering would require that tumor heterogeneity (tumor variants) be taken into consideration together with tumor complexity (tumor-stroma interactions). In this review, we provide a comprehensive overview of what has been achieved in recapitulating tumor complexity,

[*]Corresponding Authors: Eliza L.S. Fong, MD9 #04-11, Department of Physiology, 2 Medical Drive, Singapore 117597, bieflse@nus.edu.sg and Mary C. Farach-Carson., Rice University, 6100 Main St., MS-140, Houston, TX 77005, USA, farachca@rice.edu.

and discuss the importance of incorporating tumor heterogeneity into 3D *in vitro* tumor models. This work carves out the roadmap for 3D tumor engineering and highlights some of the challenges that need to be addressed as we move forward into the next chapter.

1. INTRODUCTION

Oncology drug development remains challenging despite intense efforts by researchers and the pharmaceutical industry, with only 7.5% of drugs tested in Phase 1 clinical development eventually obtaining approval [1]. This begs us to ask why clinical trials in oncology are burdened with such high failure rates. Factors responsible for this have been suggested, including the inherent complexity of cancer, problems with clinical trial design (that is, trials are not driven by predictive biomarker hypothesis), and lastly, the use of standard preclinical models poorly representative of tumors in patients [2, 3]. Evolving from the initial greatly simplified assumption that a tumor is merely a mass of transformed, proliferating cancer cells, it is now widely accepted that there exists an evolving, three-dimensional (3D) network of stromal, immune and endothelial cells within a dynamic extracellular matrix (ECM) that supports and mediates tumor therapeutic sensitivity and resistance [4]. Considering this tumor-stroma *complexity* of solid tumors, it is logical to postulate that the traditional monolayer model on tissue culture plastic has inherent limitations in mimicking aspects of the *in vivo* tumor microenvironment and hence, drug response. Indeed, over the past decade, there has been a paradigm shift towards the development and use of 3D *in vitro* tumor models to better recapitulate the tumor microenvironment context that governs tumor behavior [5–7]. A growing number of technologies have been developed to model various complex aspects of the tumor microenvironment. These 3D systems range from simple, freely floating spheroids to more sophisticated engineered systems based on naturally-derived or synthetic scaffolds. A hope is to endow spatiotemporal control over cell-cell and cell-ECM interactions in a more physiologically relevant 3D context that will provide a more accurate preclinical model.

Besides context, the success of preclinical tumor modeling fundamentally depends on using patient-representative cancer cell sources. Since the development of the US National Cancer Institute-60 (NCI-60) anticancer drug screen in the late 1980s, cancer cell lines have become standard preliminary screens in the preclinical drug discovery and development process [8]. Although cancer cell lines grown as monolayers have contributed to a valuable repertoire of knowledge in cancer biology over the decades, the use of this cell source to represent patient tumors has been perceived increasingly as a major contributing factor to the dismal failure rate of anti-cancer drugs after they move from preclinical model to human trials [9, 10]. Underlying this notion is the increasing acceptance that cell lines, as a result of adaptation to artificial *in vitro* culture conditions, poorly retain the intrinsic heterogeneity and phenotypic signature of the original tumor from which they were derived [11]. It is now recognized that individual tumors are not masses of identical cells but rather mixtures of co-existing phenotypically and genotypically distinct cell populations able to demonstrate enormous plasticity. Such clonal diversity (tumor *heterogeneity*), contributes to drug resistance and disease progression [12], similar to that which occurs in bacterial populations that acquire drug resistance. Indeed, after more than 25 years of heavy use in cancer research, the

NCI-60 panel was very recently suspended as the gold standard in favor of patient-derived xenograft (PDX) models as a radical transformative push for cancer cell sources that more closely preserve the heterogeneity in individual patient tumors [13]. PDX models are generated by engrafting and expanding primary tumor fragments or cells in immunocompromised rodent hosts. Empirical studies have determined that PDX models largely recapitulate key characteristics of the original tumor - including histopathological, biological and genetic features - better than traditional cell line-based xenograft models (reviewed in Hidalgo et al. [14]). PDX models may be created from treatment naïve patient specimens, or from tumor specimens from patients with drug resistant, aggressive disease. In a parallel bid to address the limitations of cancer cell lines as cell sources for cancer research, technological advances in growing adult stem cells of several benign epithelial lineages in a matrix with specialized media (termed ‘organoid’ culture) were recently leveraged to successfully grow patient-derived organoid (PDO) cultures of pancreatic, prostate and colorectal cancers *in vitro* [15–17]. PDO cultures also have been shown, at least for colorectal cancer, to recapitulate the clonal heterogeneity of the original patient tumor [17].

Although great strides have been made in engineering the complex 3D tumor microenvironment *in vitro*, similar steps now must be made towards incorporating tumor heterogeneity. Moving past 3D versus 2D comparisons in tumor modeling *in vitro*, with these new cell sources (PDX models and PDO cultures), we are in an exciting position to model and understand cancer in an unprecedented fashion. This review aims first to summarize the importance of modeling tumor-microenvironment interactions, then to highlight the advances in engineering such complexity *in vitro* in 3D models. We next will discuss the concept of tumor heterogeneity, which is currently lacking in most 3D tumor models, and finally outline the challenges and opportunities ahead in embracing primary cell sources. We believe that the incorporation of both tumor microenvironment *complexity and* tumor *heterogeneity* into 3D tumor models (Figure 1) will ultimately enable us to address the mammoth challenge of recapitulating cancer *in vitro* for both therapeutic drug development and mechanistic studies.

2. Modeling the Complex Tumor Microenvironment in 3D

Originally proposed in 2000 by Hanahan and Weinburg [18], and modified in 2011 [19], eight hallmark capabilities are acquired by cancer cells during tumor progression from the normal to neoplastic state; they are: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction. Additionally underscored, was that the acquisition of these hallmark capabilities relied on heterotypic interactions between the cancer cell and multiple distinct non-malignant cell types in the tumor microenvironment [18]. Indeed, transformed tumor cells can initiate and orchestrate critical changes in the surrounding stroma from a prohibitive to supportive environment for tumor progression [4]. These changes include recruitment of cancer-associated fibroblasts, immune cell infiltration, ECM remodeling and establishment of tumor vascular networks [4]. Together, these culminate in the establishment of tumor-stroma interactions that modulate tumor sensitivity - conferring resistance or

synthetic lethality - depending on the class of therapeutics and the microenvironmental context [20]. An increased proportion of stroma relative to tumor content has been associated with tumor progression and decreased patient survival for a number of solid cancers, including colorectal cancer [21], pancreatic cancer [22] and prostate cancer [23]. As such, cell-autonomous tumor models have limited relevance in accurately modeling the disease and predicting drug response. In this next section, we will briefly detail components of the tumor microenvironment, and then discuss advances that have been made in recapitulating these components in engineered 3D *in vitro* tumor models.

2.1. The Tumor Microenvironment Shapes Tumor Progression and Therapeutic Response

2.1.1 The ECM of the Tumor Microenvironment—In normal homeostatic tissues, the ECM is a spatially defined 3D network of macromolecules composed of subsets of collagens, proteoglycans/glycosaminoglycans such as perlecan/HSPG2, elastin, fibronectin, laminin and other glycoproteins, with diverse physical, mechanical and biochemical functions associated with its composition [24–26]. Through binding to cell surface receptors, components of the ECM serve as ligands to regulate cellular functions such as survival, growth, migration and differentiation [25]. Additionally, the ECM sequesters growth factors, serving as a reservoir for biologically active molecules that can be released upon enzymatic trigger to influence epithelial cell proliferation and migration, and provides mechanical stability to tissue borders [26, 27]. Remodeling enzymes such as matrix metalloproteinases (MMPs) continuously modulate the amount, composition and topography of the dynamic ECM structure. Dysregulation of ECM dynamics, as is seen in the excessive ECM production and deposition of fibrosis, can increase the risk of cancer [27]. Malignant tumors, likened to wounds that never heal, typically develop at sites of chronic injury [28, 29]. In the presence of underlying tissue inflammation, stromal fibroblasts are activated and induced to transdifferentiate into myofibroblasts, which synthesize copious quantities of collagen and other ECM components, giving rise to the ‘desmoplastic reaction’ [30, 31]. Accompanying the deposition of collagen is an increase in lysyl oxidase activity, which crosslinks these fibers, thereby stiffening the ECM and enhancing focal adhesion assembly and signaling [32]. Moreover, tumor cells and myofibroblasts also secrete MMPs that not only degrade ECM to nullify its function as a physical barrier, but also generate ECM fragments that activate latent secreted growth factors that promote tumor survival and invasion [32, 33].

In addition to its integral role in tumor progression, the tumor-associated ECM also modulates the cancer cell response to drug treatment [34] by acting as a physical barrier to drugs [35]. For example, the accumulation of hyaluronan – a major glycosaminoglycan in the ECM linked to multidrug resistance and tumor progression – around tumor cells has been reported to protect malignant cells from immune cell surveillance as well as physically restrict the access of antibodies, hence contributing to resistance against monoclonal antibody-derived therapeutics [36]. Direct adhesive interactions between cancer cells and ECM also have been reported to confer drug resistance, a phenomenon termed cell-adhesion mediated drug resistance (CAM-DR). Binding to hyaluronan promotes multidrug resistance gene 1 expression, cytoskeletal protein-drug fluxes and chemoresistance in tumor cells [37, 38]. Integrin binding to ECM components such as collagen, fibronectin and laminin also confers protection against apoptosis-inducing agents [39]. Given the importance of the ECM

in cancer cell survival and drug resistance, several ECM-targeting approaches have been developed, ranging from inhibition of specific integrin interactions and activity of MMPs to the synthesis/degradation of ECM, as reviewed in Holle et al [35]. In summary, because the biochemical and physical features of the ECM in the tumor microenvironment modulate the cancer cell fate, favor progression and trigger therapeutic resistance, ECM should be incorporated into 3D *in vitro* models if accurate preclinical testing of new therapeutics is to become standard.

2.1.2. Cancer-Associated Fibroblasts—Activated during the normal wound healing process, resident fibroblasts differentiate into myofibroblasts to stimulate angiogenesis, increase ECM production and physically contract the wound [40, 41]. The presence of these myofibroblasts in chronic inflammation can result in pathological tissue fibrosis, such as in the case of the liver and pancreas, and increase the risk of cancer [42]. Myofibroblasts are not only present at sites of injury, but are also a prominent component of stromal fibroblasts in tumors; these stromal fibroblasts (consisting of myofibroblasts and fibroblasts) are specifically termed cancer-associated fibroblasts or CAFs [43]. Abundant in the reactive tumor stroma of many epithelial cancers, CAFs serve to promote cancer cell survival, angiogenesis, invasion and metastasis [44]. Besides originating within the tissue itself, CAFs also have been proposed to originate from endothelial cells, bone marrow-derived progenitor cells, and even cancer cells that have undergone EMT [44]. As such, CAFs have heterogeneous phenotypes and functions [44]. Primarily, they secrete multiple soluble factors such as stromal-derived factor 1 (SDF-1), transforming growth factor β (TGF-β), and hepatocyte growth factor (HGF) in their paracrine interaction with cancer cells to promote tumorigenesis [44]. Orimo et al. showed that CAF-derived SDF-1 not only enhanced tumor growth in breast carcinoma cells by direct paracrine stimulation via the CXCR4 receptor, but also recruited endothelial progenitor cells into the tumor mass, augmenting tumor angiogenesis [45]. Another major contribution of CAFs to the tumor microenvironment is their production of ECM components and proteolytic enzymes that result in extensive ECM remodeling [31]. Through force-mediated and protease-mediated matrix remodeling, it has been shown that fibroblasts generate tracks in the tumor matrix that lead the collective migration of cancer cells [46].

In addition to tumorigenesis, CAFs also contribute to the acquisition of therapeutic resistance via ligand-dependent activation of receptor tyrosine kinases [4]. It recently was shown that fibroblast-derived HGF could rescue *BRAF*-mutant melanoma cells from RAF inhibition through increased phosphorylation of the HGF receptor, MET, and reactivation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-OH kinase (PI(3)K)-AKT signalling pathways. Based on the understanding that CAFs play a tumor-supporting role, Mertens et al. demonstrated that the selective killing of CAFs in a cholangiocarcinoma model suppressed tumor outgrowth and improved host survival [47]. These examples underscore the protective role that CAFs play in the tumor microenvironment. Hence, co-targeting tumor and CAFs may be an effective means to improve drug response.

2.1.3. Vascular Endothelial Cells and Pericytes—Blood vessels typically function as conduits to enable the exchange of oxygen, nutrients and waste as well as immune cells between the circulatory system and tissues in the body [48]. However, in tumorigenesis, cancer cells co-opt host vessels and induce the formation of structurally and functionally abnormal vessels either via angiogenesis or vasculogenesis [49]. These vessels are leaky, branch and connect to one another randomly in a network, and have uneven lumen size. Because of this structural leakiness, interstitial pressure within the tumor increases and blood flow is impaired [48]. With reduced perfusion, oxygenation and nutrient delivery to the cancer cells is reduced, creating an environment that is hypoxic, acidic and even necrotic [49]. Additionally, poor perfusion hinders the delivery of tumor-targeting therapeutics and effector immune cells.

Oxygen-starved tumors have been correlated with poor prognosis; hypoxia-inducible factors (HIFs, or hypoxia-sensing proteins) produced in response to hypoxia have been reported to increase levels of c-MET and HGF signaling, which can increase cell motility, invasion and metastasis [50, 51]. Hypoxia and acidosis also contribute to angiogenesis, resistance to apoptosis, altered metabolism, induction of the cancer stem cell phenotype, genetic instability, and resistance to radiotherapy, chemotherapy and immunotherapy [52]. Based on the initial hypothesis that inhibition of blood vessel formation deprives tumors of nutrients and hence inhibits their growth, various anti-angiogenic agents have been developed and approved for clinical use [48]. One such example is bevacizumab, a vascular endothelial growth factor (VEGF)-specific antibody, which has been approved for use with chemotherapy or cytokine therapy for several late-stage advanced metastatic cancers [48]. Contrary to this hypothesis, Jain in 2001 proposed that anti-angiogenic strategies may actually normalize the tumor vasculature, slow tumor progression and increase tumor sensitivity to chemotherapy, immunotherapy and radiation therapy [48]. It has since been proven both preclinically and clinically that anti-angiogenic therapy does favor tumor normalization, but the complex relationship between this phenomenon and tumor drug delivery needs to be further elucidated [53].

Pericytes reside on the exterior of vascular endothelium, stabilizing, maturing and providing structural support to blood vessels. As pericytes secrete VEGF, pericytes support endothelial cell survival and contribute to resistance against anti-angiogenic VEGF-depletion therapies [54]. While this indicates that the abrogation of pericytes is a viable therapeutic strategy, the role of pericytes in normalization of the tumor vasculature also must be considered. In the absence of pericytes, vascular permeability and interstitial fluid pressure increases, decreasing tumor perfusion [55]. Consequently, the delivery of drug therapeutics and oxygen is hindered in tumor cells, which promotes cancer cell metastasis [55]. Because of the intimate relationship between endothelial cells and pericytes, the means by which pericyte coverage should be modulated is an active area of current investigation.

2.1.4. Immune Cells—Proposed more than a decade ago, the cancer ‘immunoediting’ hypothesis predicts that the immune system paradoxically both can prevent and promote neoplastic disease [56]. This unifying framework that currently guides cancer immunology encompasses three processes: elimination, equilibrium and escape [56]. In the first phase, the invasively growing tumor incites an inflammatory response that recruits cells of the

innate immune system to the tumor site. Infiltrating lymphocytes then are stimulated to produce interferon-γ that elicits tumor death and angiostatic chemokines. Further tumor cell killing is achieved via activated macrophages and natural killer cells, as well as dendritic cell-induced activation of tumor-specific cytolytic T cells of the adaptive immunity arm [56]. Entering the equilibrium phase, the host immune system continues to contain, but not fully eradicate, any surviving cancer cells. During this process, a new population of tumor clones with different mutations that confer them with decreased immunogenicity may spawn [57]. The third phase of escape occurs when recalcitrant tumor cells that develop resistance to immune detection and/or elimination surpass the host immune defense and progressively expand [57].

Understanding this tight tumor-immune system interplay has important ramifications for the development of novel strategies in cancer immunotherapy, such as activation of innate and adaptive immune effector mechanisms and neutralization of immune inhibitory and suppressive mechanisms [58]. One such example is that based on immune checkpoint therapy, of which three immune checkpoint agents, ipilimumab, pembrolizumab and nivolumab, were recently approved by the U.S. Food and Drug Administration. The tumoricidal properties of activated T cells are tightly regulated by stimulatory and inhibitory signals; immune checkpoint molecules serve to counteract stimulatory co-signals and restrain T cell responses in the tumor microenvironment. Two such immune checkpoint molecules are the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death-1 (PD-1). Because this therapy can achieve durable clinical responses and even long-term remissions in some patients, immune checkpoint therapy has become the newest of standard cancer treatment modalities, together with chemotherapy, surgery, radiation and targeted therapy [59]. Continued advances in our understanding of how the immune response is dynamically modulated in the tumor microenvironment are expected to improve the survival for many more cancer patients in the years to come. The ability to co-culture immune cells with primary cancer and stromal cells in 3D will provide another means to bring personalized immunotherapy one step closer to reality.

2.1.5. Adipocytes—Research into the role of adipose tissue on tumor progression is relatively new, primarily initiated by observations linking obesity to certain cancers (e.g. esophageal and endometrial). Adipocytes are typically a major cell type in the tumor microenvironment of some cancers such as breast and abdominally metastasizing cancers (e.g. ovarian and gastric) [60]. Chronic inflammation exists in obese individuals. A surplus of energy typically leads to an accumulation of triglycerides and hypertrophy of adipocytes, which upregulates the secretion of pro-inflammatory adipokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-18, and leptin [60, 61]. This results in the infiltration of inflammatory cells such as lymphocytes and macrophages, which, together with the adipocytes, contribute to the inflammatory milieu [60].

In the presence of cancer cells, adipocytes dedifferentiate and delipidate, adopting an activated state in which they secrete matrix metalloproteinases and pro-inflammatory cytokines; these fibroblast-like adipocytes were recently identified as a new stromal population, coined cancer-associated adipocytes (CAA) [62, 63]. CAA not only increases cancer cell invasiveness, but the metabolites that they release, such as free fatty acids, are

thought to fuel tumor growth [60]. In one example, Gazi et al. demonstrated that lipid translocation occurs from adipocytes to prostate cancer cells, suggesting that adipocytes may further the rate of prostate cancer progression [64]. These insights warrant a better understanding of the role of adipocytes in the tumor microenvironment, which may generate new metabolic targets for cancer prevention and therapy.

2.1.6. Interstitial Flow—Progress made in dissecting and understanding the tumor microenvironment in 3D has well established the roles of tissue architecture, ECM and tumor-stroma interactions in tumor progression. The role of physical factors such as interstitial pressure and fluid flow on tumor progression, however, remains poorly understood. As an in-depth review of the role of interstitial flow in cancer is beyond the scope of this review, the reader is encouraged to refer to an excellent review by Munson and Shieh [65]. In brief, abnormal tumor-associated angiogenesis increases the influx of fluids into the tumor stroma. However, the lymphatic system typically is unable to effectively balance this increase influx of fluid, resulting in a net interstitial flow from the tumor mass into the surrounding tissue [65]. Interstitial flow generates shear forces that not only alter tumor cell proliferation [66] but also establishes extracellular gradients of invasion-promoting proteases and cytokines [67–70]. Importantly, changes in interstitial flow also may alter the transport and distribution of therapeutics [65].

2.2. 3D Platforms that Mimic the Complexity of the Tumor Microenvironment

2.2.1. ECM

2.2.1.1 Natural Matrices: Some of the earliest and currently still widely employed matrices for cancer cell culture are that of basement membrane extracts and collagen gels. Derived from natural ECM, these materials inherently are able to support cancer cell-ECM interactions such as integrin binding, growth factor signaling, and 3D cell migration and invasion. Reconstituted basement membrane extracts, commonly marketed as Matrigel® or Cultrex®, are gelatinous protein mixtures derived from mouse sarcoma tumors and consist mainly of collagen type IV, perlecan and laminin, as well as a variety of different growth factors. Pioneered by Bissell's group, early studies into understanding the nature of the extracellular microenvironment that supports normal mammary morphogenesis and differentiation demonstrated that normal and malignant human breast cancer cells behave differently when cultured in reconstituted basement membrane. While normal breast epithelial cells maintained a structurally and functionally differentiated phenotype, malignant cells did not respond to the surrounding basement membrane [7]. By blocking β1-integrin, it was further shown that the normalization of integrins was able to revert malignant breast cancer cells to a normal phenotype, indicating the importance of the ECM in influencing malignant behavior [71]. Besides its role in cell differentiation, reconstituted basement membranes have since then been widely used to study different aspects of cancer biology including tumor growth, invasion, angiogenesis, cancer stem cell, dormancy and therapeutic response [72]. In one example, Yeung et al. showed that colorectal cancer cell lines, like other cancer cell lines, contain subpopulations of cancer stem cells that can be enriched through the use of an *in vitro* Matrigel®-based differentiation assay in conjunction with specific cell surface markers [73]. The identified cancer stem cells were able to self-renew, give rise to multiple colonic lineages and initiate tumors *in vivo*. Given that the

epithelial basement membrane serves as a barrier through which tumor cells must degrade to invade and metastasize, reconstituted basement membrane also has been widely used in the Boyden chamber invasion assay to determine the invasiveness of cancer cells and to identify factors that stimulate or inhibit cancer cell invasion [72].

Beyond the basement membrane barrier, the tumor-associated stromal compartment (reactive stroma) is typically characterized by an extracellular matrix that is rich in collagen type I fibers [74, 75]. Type 1 collagen serves as a physical barrier against cancer cell invasion. As such, another commonly employed natural matrix used to culture cancer cells *in vitro*, and particularly to study cancer cell invasion, is collagen type I. Using fresh primary breast cancer cells, Cheung et al. leveraged 3D collagen type I hydrogels to identify the most invasive cancer cells in heterogeneous primary breast tumors [76]. When these primary breast tumors were grown as organoids in invasion-promoting collagen type 1 hydrogels, specialized cancer cells with a basal epithelial phenotype were observed to lead the collective invasion front, reinforcing the important concept of tumor heterogeneity – that invasive behavior is determined by a specific subset of cancer cells in the primary tumor population. Given the correlation between aligned collagen fibers and breast tumor progression [77], Riching et al. generated 3D collagen gels containing aligned and randomly oriented fibers to elucidate the underlying mechanisms by which aligned fibers promote cancer cell migration. It is thought that alignment may increase matrix stiffness, thereby enhancing migration by means of durotactic guidance. Interestingly, it was reported that alignment facilitates cell migration by increasing directional persistence and limiting protrusions along aligned fibers, rather than increasing the speed of migrating cells. 3D collagen gels have also been used to elucidate the effect of matrix stiffness on cancer cell invasion. A landmark study by the Weaver group showed that collagen crosslinking-induced ECM stiffening supports the invasive phenotype by enhancing integrin signaling [78]. By using glutaraldehyde as a crosslinker to increase the stiffness of collagen gels independently from pore size or collagen concentration, Lang et al. showed that 3D invasion is dependent on pore size; while increased matrix stiffness promotes 3D invasion in gels with large pores (small steric hindrance), increased matrix stiffness hinders cell invasion in gels with small pores (large steric hindrance) [79]. In another interesting study, the Weaver group reported the development of a 3D tension bioreactor platform to facilitate studies on ECM stiffness; by mechanically loading collagen gels to induce gel stiffening (via collagen strain hardening) while maintaining composition and pore size, increasing matrix stiffness was also found to enhance tumor cell invasion [80].

Lastly, besides collagen and basement membrane-derived gels, the use of decellularized matrices to mimic the native tumor microenvironment *in vitro* also has been investigated [81–83]. Based on studies of organ reengineering for orthotopic transplantation in tissue engineering, it is thought that the preserved extracellular matrix and vasculature in the isolated matrix may provide a native environment for the growth of cancer cells. In one example, Moll et al. seeded decellularized porcine small intestinal submucosa with primary fibroblasts, microvascular endothelial cells, and a malignant peripheral nerve sheath cell line and cultured the resulting tumor construct under flow perfusion in a bioreactor [84]. As compared to traditional 2D culture, the 3D culture generated a more *in vivo*-like tumor tissue construct. A similar approach was taken by Dunne et al., who demonstrated the use of

decellularized adipose tissue scaffolds to mimic the microenvironment of mammary adipose tissue surrounding breast cancer cells [81]. In this platform, breast cancer cells exhibited similar growth profiles and cellular organization as compared to xenografts, and different drug resistance as compared to 2D-cultured cells.

2.2.1.2. Synthetic Matrices: While reconstituted basement membrane and collagen type I hydrogels are widely used in cancer research, the properties of these naturally derived matrices inherently are difficult to control and modulate, making it difficult to maintain reproducibility and rigor of results. To confer greater experimental control over mechanical and biochemical properties, semi-synthetic 3D systems have been developed; in these systems naturally derived materials are chemically modified or mixed with synthetic materials to enable tighter control over material properties. This approach has been employed for the generation of 3D hyaluronan-based hydrogels for cancer cell growth [85–89]. Through its interaction with hyaluronidiases and cell surface receptors such as CD44 and RHAMM, hyaluronan - a glycosaminoglycan that is elevated in several cancers - promotes tumor growth and metastasis [90, 91]. By modifying hyaluronan with complementary reactive groups, crosslinked hyaluronan hydrogels with controllable mechanical properties and pore sizes were developed [89, 92]. Mimicking the hyaluronan-rich bone microenvironment, 3D hyaluronan hydrogels have been shown to support the growth and metastatic phenotype of bone metastatic prostate and renal cancer cells in culture [89, 92, 93]. To further simulate the biochemical complexity of the tumor microenvironment *in vivo*, proteins and peptides have been incorporated either physically or through chemical conjugation into hyaluronan hydrogels. In one such study, fibronectin and laminin were incorporated into hyaluronan hydrogels to examine the impact of the brain metastatic melanoma microenvironment on therapeutic response [94]. Melanoma cells cultured in the presence of laminin or fibronectin exhibited differential sensitivity to inhibitors of BRAF and ERK, underscoring the importance of contextual drug assessment.

In another approach, traditional fully synthetic materials such as polyesters poly(lactic-co-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL), as well as poly(ethylene glycol) (PEG) have been widely investigated for use in tissue engineering due to their independently tunable biochemical and physical properties. Indeed, these synthetic scaffolds permit the systematic evaluation of the effects of tumor microenvironmental components on tumor progression. In recent years, PEG hydrogels have been used to probe the effect of matrix stiffness and ECM interactions in the tumor microenvironment. As PEG hydrogels resist non-specific protein adsorption and hence cell adhesion [95], these materials serve as ‘blank slates’ upon which different ECM moieties can be incorporated and investigated [96]. One of the first proof-of-concept confirmations of such an approach was a study by Loessner et al., which investigated the effect of matrix stiffness, integrin engagement and cell-mediated matrix remodeling on the growth of encapsulated ovarian cancer cells [97]. Proliferation in 3D was shown to be dependent on cell-integrin engagement and the ability of cells to proteolytically remodel their surrounding matrix. In another study, Gill et al. showed that PEG-based hydrogels modified with cell-adhesive and enzyme-degradable peptides induced murine lung adenocarcinoma cells to form lumenized polarized spheres, mimicking that observed with naturally-derived Matrigel® [96]. Additionally, controlled alterations in

matrix stiffness and concentration of cell-adhesion ligand were shown to influence the degree of lumenization and polarity as well as intra-spheroidal localization of proliferative and apoptotic cells. The use of PEG-based hydrogels to precisely simulate the tumor microenvironment *in vitro* has since then been demonstrated for glioblastoma [98] and pancreatic cancer [99]. Of mention, the Anseth group has also employed these PEG-based hydrogels to study tumor cell migration in 3D under controlled biochemical and physical *in vitro* conditions [100, 101]. In seeking to understand the differences in migration mechanisms between normal and malignant cells, the migration, morphologies, adhesiveness, expression of adhesion proteins and, cytoskeletal structure of malignant fibrosarcoma cells were compared against that of primary human dermal fibroblasts. As fibrosarcoma cells were found to adopt more rounded morphologies, exhibit decreased adhesiveness and increased directional motility as compared to dermal fibroblasts, and behave more similarly to melanoma cells, the authors propose that malignant cells migrate differently from normal cells as a result of their transformed phenotype.

The studies as described above largely have limited the exploration of biochemical cues to the use of integrin-binding fibronectin-derived RGD (Arg-Gly-Asp) amino acid sequence and a mutated version of a collagen-derived MMP-sensitive sequence, as originally employed by Lutolf et al. in 2003 [102]. However, the ECM itself is not limited to just fibronectin or collagen but is in fact, a complex tissue-specific mixture of other proteins, carbohydrates, enzymes, lipids and signaling molecules [103]. Moving forward, with such tunable synthetic 3D systems available for modification, future studies would greatly benefit from mining other ECM-derived motifs to expand our knowledge of the impact of various ECM components on tumor progression and to modulate tumor behavior. As an example, we recently showed that enzymatic cleavage of perlecan (a large heparan sulfate proteoglycan that organizes the basement membrane network underlying epithelial and endothelial cells) by matrix metalloproteinase-7 (MMP-7, an enzyme upregulated in invasive prostate cancer cells) serves as a trigger for cell dispersion and invasiveness [33]. Intact perlecan maintains tissue borders and also harbors a reservoir of heparan sulfate-bound growth factors critical for the normal wound healing response [26]. In a follow-up study, we verified this enzyme-substrate relationship in both tissue and serological prostate cancer patient specimens [104]. This example highlights the importance of developing spatiotemporally cell-responsive ECM matrices that can provide dynamic insight into tumor development. It would be interesting to leverage this discovery to design synthetic 3D matrices as a Matrigel® substitute which can be used to understand the role of epithelial tissue ECM borders as barriers against cancer cell invasion. Lastly, though it has not been demonstrated for cancer cells, we have shown that ECM-derived motifs can be used to guide spheroid formation *in vitro*. Specifically, we previously showed that 3D hepatocyte spheroids can be generated on the surface of 2D polyethylene terephthalate (PET) films, and tethered, by controlling the ratio of conjugated RGD and galactose ligands [105]. Additionally, the hepatocyte spheroid-promoting property of galactose also has been used to generate hepatocyte spheroids within macroporous cellulosic sponges [106]. Such an approach may be leveraged for understanding the relationship between cell-cell and cell-ECM interactions in cancer cells.

2.2.2. Co-Cultures—Besides interactions with the acellular ECM, reciprocal crosstalk between cancer cells with the surrounding stromal cells in the tumor microenvironment can profoundly influence the extent of tumor progression. In the following section, recent studies that probed the effect of tumor co-cultures with fibroblasts, endothelial and immune cells in 3D engineered systems will be described.

2.2.2.1. With Fibroblasts: In the simplest of 3D culture configurations, tumor and stromal cells can be encapsulated with direct cell-cell contact as mixed spheroids in the absence of any supporting matrix. Several methods have been developed to generate spheroids in culture; these include the use of spinner flask culture, hanging drops, microfluidic chips and culture over a non-adherent surface [107]. For example, Majety et al. developed a 3D co-culture model that allows direct cell-cell contact between pancreatic, breast or lung cancer cells, together with human fibroblasts or CAFs [108]. This mixed spheroid model was achieved by co-seeding tumor cells and fibroblasts on culture wells coated with poorly cell-adhesive poly(2-hydroxyethyl methacrylate). Through this study, it was shown that co-culture induced enhanced survival and differential expression of soluble factors in a cancer type-specific manner. To achieve greater control over tumor composition and densities, Jaganathan showed that tumor-fibroblast interactions can be forged using magnetic levitation [109]. As opposed to the passive formation of spheroids, breast cancer cells and fibroblasts actively formed large-sized aggregates in less than 24 hours in the presence of a magnetic field, the density and composition of which were controlled by the number and type of cells initially seeded.

Scaffold-based approaches also have been investigated for the development of tumor-fibroblast co-cultures, particularly to recapitulate the drug-diffusion ECM barrier in the tumor microenvironment. In one such study, liver carcinoma cells were mixed with liver stromal fibroblasts and encapsulated within collagen gels [110]. Using the anti-cancer drug doxorubicin, it was shown that co-cultures with stromal fibroblasts exhibited enhanced resistance to the drug over mono-cultures. Additionally, the surrounding collagen matrix also increased the resistance of the co-cultures over cancer cells alone in the absence of any collagen matrix. In a slightly different culture configuration, pre-formed colonic tumor spheroids were encapsulated in a collagen gel together with stromal fibroblasts [111]. Using RNA microarray analysis, it was shown that co-culture with stromal fibroblasts induced changes in transcript levels of genes involved in invasion, ECM remodeling, inflammation, and angiogenesis. However, this study was limited by the inability to identify whether the signaling pathway changes occurred in the tumor and/or stromal cells. Focusing specifically on bone, several groups have reported the development of tumor cell-bone cell co-cultures to engineer primary and secondary bone cancers *in vitro* [112–115]. For example, the Hutmacher group developed a tissue engineered bone construct that was used to investigate the interactions between prostate cancer cells and osteoblasts during prostatic bone metastasis [112, 113]. The engineered bone construct was fabricated by wrapping medical grade PCL-tricalcium phosphate with sheets of human osteoblasts. When prostate cancer cells were seeded directly onto the engineered bone, the resulting co-culture resulted in elevated levels of MMPs, steroidogenic enzymes and prostate specific antigen, characteristics of prostatic bone metastasis [112]. However, again, this co-culture study was

limited by the pooled biochemical analysis, which made it impossible to identify whether the changes occurred in the tumor and/or stromal compartment. This problem was circumvented in a later study by encapsulating the prostate cancer cells in PEG hydrogels, thereby enabling the spatial segregation of cancer cells from the osteoblasts for downstream analysis [113].

To confer greater spatial control over the organization of tumor cells with surrounding fibroblasts, several research groups have employed miniaturization technology, in the form of microfluidic chips, to create tumor-on-a-chip models [116–118]. Tissue structure is especially important for non-invasive forms of cancer, such as ductal carcinoma *in situ* (DCIS), where delineated boundaries between tumor and stroma still exist. Focusing on DCIS, Choi et al. developed a microsystem that enabled the co-culture of tumor spheroids together with normal mammary epithelial cells and fibroblasts in a compartmentalized multilayered microfluidic device [117]. In this set-up, paclitaxel was shown to exert a growth-inhibiting effect on the engineered DCIS lesions. In another interesting study, Bischel et al. developed a DCIS model with physiologically relevant 3D luminal structures in the presence of surrounding stromal fibroblasts [118]. Viscous finger patterning first was used to generate normal mammary epithelial cell-lined lumens through a collagen type I hydrogel. Following this, DCIS cells were added to fill the ducts to model a duct with DCIS. In the presence of mammary fibroblasts, DCIS cells were observed to invade into the surrounding stromal compartment.

2.2.2.2. With Vasculature: Although 3D models of angiogenesis and tumor development have been developed, co-culture models that combine both processes and allow the study of 3D tumor angiogenesis and cancer cell-endothelial cell interactions are still scarce [119]. The predominant mechanism for tumor angiogenesis is thought to be sprouting angiogenesis, characterized by the migration, proliferation, 3D organization and tube formation of endothelial cells from pre-existing vessels [120]. This neo-vascularization process is in turn, tightly regulated by various cell types (including fibroblasts, pericytes, and smooth muscle cells) that secrete soluble factors and establish cell-cell interactions [121]. The immense complexity of this multistep process therefore has confined much of the characterization to animal models [121]. To address this problem, Seano et al. developed an *ex vivo* 3D assay of sprouting angiogenesis using arterial explants from human umbilical cords [121]. Embedding the explants in reconstituted basement membrane supported the outgrowth of capillary-like structures consisting of endothelial cells. Additionally, sprouting was observed to be sustainable in the presence of prostate cancer spheroids and in the absence of exogenous growth factors. However, independent manipulation of ECM and individual cell types to dissect the mechanisms regulating tumor angiogenesis is challenging in this explant model.

As such, several groups have undertaken a more bottom-up approach to model tumor angiogenesis, by selectively combining tumor cells, endothelial cells and/or mural-like cells within a known matrix, such as reconstituted basement membrane, collagen type 1 gels or synthetic engineered systems [122–126]. In one such study, Correa de Sampaio et al. developed a minitumor model, combining breast cancer cells, endothelial cells and fibroblasts as a collagen type 1 gel-embedded heterogeneous spheroid. In this model, breast

cancer cells also were able to stimulate endothelial sprout formation even in the absence of exogeneous angiogenic factors. Importantly, independent transduction of the three different cell types with shRNA against membrane-type 1 MMP revealed that cancer cell-derived MT1-MMP is not likely to be critical in the activation of tumor angiogenesis; rather, the study suggests a role for fibroblasts in mediating endothelial cell sprout formation.

Advancing this bottom-up approach one step further, the Werner group developed a highly defined, modular and tunable hydrogel platform that was used to identify biophysical, biochemical and cell-cell interactions that optimally support angiogenesis *in vitro* [127]. This 3D hydrogel system consists of starPEG-heparin, angiogenic growth factors, cell-adhesive ligands and enzymatically-cleavable peptide linkers. The highly sulfated glycosaminoglycan heparin was incorporated to enable the sustained delivery of multiple pro-angiogenic growth factors such as basic fibroblast growth factor (bFGF/FGF2), VEGF and SDF-1α. Modulation of hydrogel stiffness and growth factor combinations led to the identification of soft (200 Pa) hydrogels with three co-delivered growth factors (bFGF, VEGF and SDF-1α) as the optimum matrix condition that best supported capillary network formation. In a subsequent study, breast and prostate tumor-vasculature co-cultures were developed using this same 3D platform [128]. 3D tri-cultures (cancer, endothelial and mesenchymal stem cells) were more resistant to chemotherapeutics as compared to 2D cultures, and further exhibited *in vivo*-like tumor regression. This modular system is a first step toward the systematic study of tumor angiogenesis, but would benefit further from the use of primary tumor cells as well as tissue-specific endothelial and vessel-supporting cells.

Microfluidic platforms that enable the precise integration of various biophysical and biochemical factors that govern tumor-vasculature interactions have also been developed [129–131]. In one such study, Bai et al. co-cultured a highly invasive bladder carcinoma cell line as aggregates embedded in a collagen scaffold in close proximity to an endothelial cell-lined channel mimicking a blood vessel to screen a panel of therapeutics (and combinations thereof) that can inhibit EMT [131]. Using the degree of tumor aggregate dispersion as a measure of drug efficacy, it was found that the presence of endothelial cells enhanced the drug resistance of the bladder carcinoma cells. Further analysis revealed that the increased invasiveness of these cells were a result of growth factors (HGF and FGF-2) secreted by the endothelial cells. This microfluidic co-culture platform may be useful for the evaluation of anti-metastatic therapeutics and understanding the mechanism behind the vasculature-driven invasive tumor phenotype.

In addition to the use of microfluidic platforms to study tumor migration and drug response, microfluidic platforms aimed at studying the interplay between endothelial barrier function and tumor intra- and extra-vasation have also been developed [132, 133]. In one example, Zervantonakis et al. engineered a microfluidic system consisting of two hydrogel-interconnected channels that enabled the real-time monitoring of cancer cell intravasation into an endothelial monolayer [133]. In the presence of macrophages, the frequency of tumor intravasation through the endothelial barrier was enhanced. Additionally, the effect of macrophages on tumor intravasation was attributed to an increase in endothelial permeability caused by macrophage-secreted TNF-α. While these microfluidic platforms have greatly advanced our ability to study the dynamics of tumor-endothelial cell interactions, the

physiological relevance of these artificial vessel analogues generated by lining channels with an endothelial monolayer is limited. However, the generation of functional, perfusable 3D vascular networks has been demonstrated [134, 135]. The use of such *in vivo*-like vascular networks can enable us to better understand the processes of tumor angiogenesis, tumor cell motility across blood vessels as well as the impact of physiologically relevant fluid forces on tumor-endothelial interactions.

2.2.2.3. With Immune Cells: The immune system is inherently capable of recognizing and eradicating 'non-self' transformed cells until an equilibrium phase is reached. Understanding the mechanisms employed by cancer cells to escape this equilibrium is crucial to the development of rational therapeutics that promote the anti-tumor immune response [136]. Despite the importance of understanding the tumor-immune system interaction, most of the data available thus far have relied on traditional 2D co-culture models, and only a limited number of studies have carried out such investigations using more physiologically relevant 3D models. The infiltration of immune cells into tumors can paradoxically result either in an anti-tumor response, or the active facilitation of tumorigenesis and blocking of a protective immune response [137]. In seeking to develop a 3D model of T cell infiltration in lung tumor, Alonso-Nocelo et al. co-cultured a human lung adenocarcinoma cell line together with lymphocytes in a cross-linked porous polystyrene scaffold and characterized the changes in the secretome profile as an output measure of the heterocellular crosstalk [138]. Analysis of the secretome indicated that the incorporation of infiltrated T lymphocytes increased the secretion of proteins that favored the malignant phenotype. Additionally, differences in active pathways also were found between 3D and 2D co-cultures; the presence of complement proteins in 3D, but not 2D, suggests that the 3D environment maintains a continuous pro-inflammatory state. Besides T cells, tumor-associated macrophages also can facilitate tumor development and progression by supporting tumor proliferation, survival, angiogenesis and invasive behavior [139]. For example, co-culture of breast cancer cell lines with promonocytes in 3D reconstituted basement membrane increased the monocytic expression of proteases MMP 1 and 9 as well as inflammatory factor cyclooxygenase 2, illustrating how the tumor cell can modulate the gene expression profile of immune cells in the tumor microenvironment [140].

While it is important to study the bidirectional interaction between tumor and immune cells, it is also critical to consider and understand the role of immune regulators in the tumor microenvironment. As an example, CAF recruitment and the initiation of fibrosis typically are events associated with the inflammatory response [139]. Being a major component of the tumor stroma, CAFs not only physically impede the penetration of antitumor T cell into the tumor but also modulates immune function via the secretion of numerous cytokines and chemokines [139, 141]. Taking one step further in complexity, Phan-Lai et al. developed a tri-culture system to investigate the effect of CAFs on T lymphocyte function in the presence of breast cancer cells [141]. Using 3D chitosan-alginate scaffolds for the tri-culture and using TNF-α as a measure of helper T cell activation, it was found that the presence of CAFs impaired the ability of the T cells to secrete TNF-α, highlighting the immunosuppressive role of CAFs in this system. Further investigations suggest that this immunosuppression is partly mediated by TGF-β and IL-10. In the broader context, the

studies described above illustrate the immense challenge of studying tumor-immune system interactions given that multiple players in the immune system and tumor stroma are involved in sculpting the eventual outcome on cancer cells. Nevertheless, continuous efforts in developing 3D tumor-immune system platforms are needed to support the promising recent advancements in immunotherapy [142].

2.2.3. With Perfusion—While bioreactors are commonly used in tissue engineering to perfuse and support the exchange of nutrients and wastes in large tissue constructs, 3D perfused tumor models aimed at recapitulating biomechanical cues in the tumor microenvironment are few in the literature. In a recent study, Santoro et al. cultured Ewing sarcoma cells on an electrospun polymeric scaffold within a flow perfusion bioreactor and showed that flow-derived shear stress significantly enhanced the production of insulin-like growth factor-1 (IGF-1) over static conditions [143]; the IGF1/IGF-1 receptor pathway is a current clinical target for this pediatric disease. It was further demonstrated that drug response to an IGF-1 receptor inhibitor, dalotuzumab, was dependent on flow rate, underscoring the importance of mechanical stimulation on the cancer cell phenotype and drug sensitivity.

As microfluidic systems enable greater control over fluid dynamics at the cellular level, most research groups have adopted the microfluidic approach to incorporate fluid flow in 3D tumor models. The Kamm group in particular, has developed microfluidic platforms that enable the study of interstitial flow and cancer cell migration [144, 145]. Encapsulating single breast cancer cells in collagen type 1 gels placed between two channels and applying a hydrostatic pressure gradient across the gel region, Polacheck et al. demonstrated that the directional bias of cell migration is interstitial flow rate and cell density dependent. Specifically, cells seeded at high concentration or subjected to CCR7 signaling (involved in autologous chemotaxis) blockade migrated against the flow. In seeking to understand the cellular mechanisms governing cell migration under interstitial flow, it was further demonstrated that to balance fluid stresses and maintain static equilibrium, a transcellular gradient in matrix adhesion tension is generated; this tension activates β1-integrin adhesion complexes and the corresponding localization and activation of focal adhesion proteins near the upstream membrane, where matrix adhesion tension is highest [145]. In another study building upon the findings by the Kamm group, Huang et al. found that interstitial flow favors amoeboid motility over mesenchymal motility and that the fastest moving cells were the amoeboid cells, consistent with *in vivo* findings. Together, these studies underscore the importance of biophysical forces in modulating cancer cell migration and further studies are warranted to increase our understanding of this understudied aspect in the tumor microenvironment.

3. Modeling Tumor Heterogeneity and Evolution in 3D

3.1. Tumors are Heterogeneous, Multi-Clonal Tissues

Every tumor is unique. In fact, it has been shown for breast cancer that no two tumor cells are the same [146]. Between tumors (intertumoral heterogeneity) and within tumors (intratumoral heterogeneity), genetic and phenotypic variations are pervasive [147]. Despite

the development of numerous non-targeted and targeted approaches in the past decades, inherent or acquired drug resistance remains as the main contributor to patient mortality [148]. Multiple biochemical processes such as induction of drug transporters and changes in survival/apoptotic pathways were thought to underlie drug resistance [148]. However, this knowledge has not resulted in significant improvements in patient outcomes [148]. Instead, it is currently believed that partial treatment responses, drug resistance and disease relapse observed in patients are manifestations of a more fundamental tumor characteristic – intratumoral heterogeneity [149]. In this section, the causes of intratumoral heterogeneity will be described. By understanding how cancer cells acquire genetic and phenotypic variations within individual tumors, we can then appreciate the extensive heterogeneity between individual tumors, as seen through large-scale sequencing analyses [150, 151].

Within tumors, different subclonal populations can intermingle or be spatially distinct, and the clonal architecture they form changes with time [147]. By performing multiregion genetic analysis from tumors derived from patients with renal carcinoma, Gerlinger et al. reported the presence of spatially separated heterogeneous somatic mutations and chromosomal imbalances [152]. In another similar study, Sottoriva et al. also reported heterogeneous copy number events between different regions of the same tumor for glioblastoma [153]. Furthermore, by single nucleus genome sequencing, it has been shown that no two tumor cells are the same [146]. While it is still unknown how spatially separated genetically distinct clones arise in tumors [152], it has been postulated to be a result of different microenvironmental niches within the tumor that uniquely shapes the clonal architecture in each niche [147]. These studies indicate that conventional single tumor-biopsy unlikely represents the tumor as a whole and hence, presents major hurdles to personalized medicine [152]. Besides having spatial distinction, intratumoral heterogeneity varies over time. In order to understand the temporal development of subclonal variation in breast cancer, Nik-Zainal et al. reconstructed the genomic history of 21 breast cancers. By examining copy number events and point mutation spectrums over time, significant differences in early and late point mutation signatures were found. Specifically, C>T transitions contributed significantly to the early acquisition of mutations but the proportion of C>T mutations decreased over time.

3.2. Causes of Tumor Heterogeneity

Intratumoral heterogeneity has largely been explained by various mechanisms including genomic heterogeneity [154], cancer cell plasticity [155], and microenvironmental factors including tumor-stromal interactions, tumor-tumor interactions, and therapy-induced heterogeneity [4, 156]. Both the cancer stem cell model and clonal evolution model have been postulated to account for genomic heterogeneity [154, 157]. In the former, cancer stem cells are proposed to be small subpopulations of tumorigenic cells that drive the growth and progression of cancers; akin to the hierarchical organization in normal tissues, cancer stem cells then give rise to diverse nontumorigenic cells that constitute the bulk of tumors via epigenetic changes [154]. Supporting this model are studies of human leukemias such as acute myeloid leukemia (AML), where it was shown that patient-derived $CD34^{+}CD38^{-}$ cells implanted in severe combined immune-deficient (SCID) mice could initiate leukemia [158, 159]. The identification of a specific AML-initiating cell population suggests the presence of

a hierarchy in AML, supported by leukemic stem cells. Apart from AML, cancer stem-like cells have also been identified in other tumor types such as head and neck cancer, breast cancer, colon cancer and pancreatic cancer [160–163]. However, the rarity of such tumorigenic cancer stem-like cells, an inherent feature of the cancer stem cell model, has been questioned by studies in melanoma and leukemias [164, 165]. These studies suggest that the poor permissibility of the foreign murine environment to enable growth of human cancer cells is responsible for the rare frequency of tumorigenic cancer stem cells observed in studies of xenotransplantation [164]. Because a robust measure to define what constitutes a cancer stem-like cell is lacking, the extent to which the cancer stem cell model contributes to intratumoral heterogeneity is still unclear. To account for the possibility that tumorigenicity may not be restricted to rare cancer stem-like cells, the clonal evolution model has been proposed as another mechanism that leads to intratumoral heterogeneity. The clonal evolution model posits that genetic and epigenetic alterations occur over time in individual cancer cells and cells that acquire advantageous characteristics under selection pressure will then thrive and out-compete other clones [154, 166]. Quintana et al. reported that at least 25% of unselected melanoma cells from different patients were capable of forming tumors *in vivo*, suggesting that many cells in melanoma have similar tumorigenic capacity [165]. That heterogeneity arises from clonal evolution would imply that most or all cells in a tumor would have to be targeted, as opposed to the selective targeting of rare tumorigenic stem-like cells [154]. To complicate matters, the notion that non-tumorigenic cells unidirectionally arise specifically from tumorigenic stem cells in a hierarchical cell-lineage structure has been challenged by studies that propose instead, that cancer cells can bidirectionally interconvert between tumorigenic and non-tumorigenic states [155, 167]. As an example, Mani et al. showed that cells that have undergone EMT, a process that enables tumor metastasis, acquired stem-cell traits [168]. While to be proven, the reversible plasticity of cancer cells may serve as a bridge to unify the cancer stem cell model with the clonal evolution model which would account for genetic heterogeneity in cancers [148].

As previously described, tumors do not comprise solely of genetically aberrant cells but co-exist within a dynamic microenvironment that also accommodates other cell types such as fibroblasts, immune and vascular cells. In accordance with the clonal evolution model, intratumoral heterogeneity arises from the presence of distinct microenvironments within a tumor that exert unique selection pressures [4]. The outgrowth of unique clonal populations able to survive and expand in the different niches results in intratumoral heterogeneity. Regional differences in selective pressures occur because the tumor stroma is not static and co-evolves with the cancer cells. As an example of stromal co-evolution with cancer cells, CAFs have been shown to harbor increased tumor-promoting potential as compared to normal fibroblasts, providing cues that support cancer cell proliferation, survival, invasiveness and tumor-initiation [169]. Furthermore, the observation that these cells do not originate from a single progenitor but trans-differentiate form multiple types of fibroblastic cells including resident fibroblasts, endothelial cells, preadipocytes, stellate cells, pericytes and bone-marrow derived cells may generate even greater heterogeneity in the tumor-CAF interaction [169]. Besides CAFs, the poorly structured tumor-associated vasculature also contributes to intratumoral heterogeneity. The dysfunctional vasculature results in regional variations in hypoxia, interstitial pressure, nutrient and waste exchange and, drug transport,

creating distinct microenvironments (and hence, selection pressures) within the tumor [4]. Moreover, the tumor vasculature may also influence tumor-immune interactions, given that endothelial cells has a regulatory role in T cell migration into tumors [170]. Lastly, the recruitment and spatial localization of immune cells, which can include multiple cell types with different functions, varies within tumors and has been shown to associate with clinical outcome [4, 171].

Intratumoral heterogeneity contributes to therapeutic resistance through the presence of pre-existing drug-resistant sub-clones prior to treatment [172]. Using longitudinal sampling, several studies have investigated the changes in clonal populations that occur during cancer therapy, an external selection pressure that shapes the clonal architecture [172]. By performing genome-wide copy number analyses on matched diagnosis and relapse samples from patients with acute lymphoblastic leukemia, Mullinghan et al. showed that the relapse clonal population often was present as minor subpopulations during diagnosis prior to the initiation of therapy, suggesting that cancer treatment selects for genomic abnormalities that contribute to the disease [173]. Drug treatment also can generate drug resistant cells and change the course of genome evolution. Low-grade gliomas are often treated with mutagenic alkylating agent temozolomide as adjuvant therapy after surgical resection. Johnson et al. compared the genomic landscape of the initial low-grade glioma to their post-treatment recurrence and found that these tumors reflected characteristics of temozolomide-induced mutagenesis and followed an alternative evolutionary path towards high-grade glioma [174].

4. Recent Advances in Preclinical Tumor Modeling to Address Tumor Heterogeneity

4.1. Patient-Derived Xenograft Tumor Models

The dynamism of intratumoral heterogeneity as influenced by various factors described in the previous section underscores cancer as a highly complex, patient-specific disease. However, for decades, cancer biologists have relied on homogeneous cancer cell lines grown as monolayers *in vitro* and cell line-derived xenografts to represent patient tumors [175]. Selective pressure exerted by the artificial *in vitro* environment favors the most undifferentiated cells to proliferate and leads to the loss of important biological characteristics such as tumor heterogeneity [11]. Moreover, cell lines do not contain the supporting tumor stroma [176]. Indeed, data suggests that cell lines have adapted to *in vitro* growth and consequently acquired irreversible gene expression changes [177]. In recognition that the high failure rate of new therapeutic agents in oncological drug development is likely attributed to the use of these cancer cell line-based models with poor clinical predictive power [175, 178], the NCI-60 panel of human cancer cell lines from the US NCI drug screening program was recently retired in favor of PDX models [13].

There are several excellent reviews on PDX models and their role in translational cancer research [14, 175, 178]. Though not new – the first ones were generated in the 1980s [179] – PDX models have gained increasing traction as an alternative tumor model that can overcome the limitations of cell-line based models. PDX models are generated by grafting primary tumor fragments directly from the patient into an immunocompromised mouse [11].

Following engraftment, the PDX models are then maintained by passing the expanded tumors from mouse to mouse. Studies have demonstrated that PDX models retain key characteristics of the parental tumors, including histology, gene expression profiles and copy number variants [175, 177, 180–183]. As such, PDX models have found broad utility in cancer research, including identification of predictive biomarkers for treatment response, mechanism of resistance, and cancer stem cells (as summarized by Sia et al. [11]). As an example, Gao et al. recently demonstrated the feasibility of using PDX models as an *in vivo* screen to model inter-patient response heterogeneity and predict potential clinical trial response at the population level, identifying responsive subpopulations and mechanisms of resistance [184]. In another example of use in personalized medicine, PDX models have also been proposed to have utility as 'mouse avatars', where each patient is allowed to have their own tumor grown as a PDX to enable the identification of a personalized therapeutic regimen [185].

While PDX models are improved platforms for cancer research, there are still several limitations to this model that need to be addressed for it to be truly useful. First, not every tumor type engrafts, and engraftment time in mice (varying from 2 to 12 months) may be longer than the survival time that patients have [14, 175]. This precludes certain patient populations from the personalized drug screening approach as described above. Moreover, studies have suggested that the process of tumor engraftment and propagation in mice exerts selective pressures that result in the biased selection for more aggressive phenotypes [14, 180]. Another critical limitation of PDX models is the replacement of human tumor stroma by murine stroma through expansion in mice [14]. It is unclear whether murine stroma supports tumor growth and progression in the same way that human stroma does [186]. Additionally, the need for severely immunodeficient host animals precludes the use of PDX models for the evaluation of therapeutics targeting tumor-immune interactions [186]. Lastly, the number of drugs and combinations that can be tested, or the throughput, is currently still limited by the high costs and intense labor associated with the PDX model [187].

4.2. Patient-Derived Organoid Cultures

Given the limitations of cell lines and PDX models, in the past few years there has been an exponential interest in PDO models for cancer research. The conditions developed to grow patient-derived tumors as organoids were derived from conditions that were initially developed to grow benign tissues as organoids. The landmark study in 2009 by the Clevers group established the minimal requirements for sustainable growth of murine crypt-villus structures without mesenchyme [188]. This includes Wnt agonist R-spondin 1 to enhance Wnt signaling, epidermal growth factor (EGF) to stimulate proliferation, Noggin to inhibit bone morphogenetic protein (BMP) signaling and laminin-rich Matrigel® as an ECM matrix to support intestinal epithelial growth. Since then, subsequent studies have demonstrated the feasibility of using these basal organoid growth conditions with tissue-specific adaptations to generate organoids from other epithelial organs such as colon, stomach, liver, prostate and pancreas [15, 189–192]. Based on these conditions that enable the indefinite propagation of multiple benign epithelial lineages, recent studies have demonstrated the applicability of similar growth conditions for the development of organoid cultures derived from prostate, colorectal and pancreatic cancers [15–17]. As an example, the Clevers group recently

reported the development of a ‘living biobank’ consisting of tumor organoid cultures from colorectal carcinoma (CRC) patients that captures the major molecular subtypes in CRC [17]. Using this platform, the authors demonstrated the feasibility of using PDO for high throughput screening of gene-drug associations. As an example, the screen was able to identify a previously reported association between mutated TP53 and resistance to nutlin-3a, an inhibitor of MDM2. Furthermore, it was also shown that the organoid approach is also applicable in the metastatic setting, where only a limited amount of tissue can be obtained via biopsies [193]. In another similar study, Gao et al. demonstrated the feasibility of growing patient-derived prostate cancer cells which are notoriously difficult to propagate *in vitro* [16]. Using the organoid system, the authors reported the establishment of patient-derived prostate cancer organoid lines that recapitulate the genomic landscape of the disease, including *TMPRSS2-ERG* fusion, *SPOP* mutation, *SPINK1* overexpression and *CHD1* loss. As opposed to the top-down approach, Matano et al. recently demonstrated the feasibility of the bottom-up approach in recreating colon cancer by introducing colorectal cancer driver mutations into primary human colon organoids cultures using clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 genome editing [194]. While organoids engineered to harbor key driver mutations exhibited limited tumorigenicity *in vivo*, organoids that were derived from human adenomas with a chromosomal instability phenotype were able to form macrometastatic colonies, suggesting that additional genetic events besides driver mutations are necessary to unleash invasive behavior.

While the potential of the PDO model in enabling high throughput drug screening as well as mechanistic studies into tumor biology has been shown, there are several limitations to this model that have to be addressed for it to be truly useful. The use of a poorly-defined matrix (Matrigel®) may present the problem of batch-to-batch variation and hence, generate inconsistency between experiments. It remains to be explored whether engineered matrices that mimic the properties of Matrigel® can be used as substitutes to overcome this problem. More significantly, organoid cultures are largely epithelial and lack tumor stroma such as immune or vascular cells. In the absence of any native stromal components, it is unlikely this model can be leveraged for any meaningful studies on tumor-microenvironment interactions or clonal evolution. Lastly, it remains to be demonstrated whether the high culture success rates obtained with colorectal, pancreatic and prostate cancers will hold the same for other epithelial cancers.

5. New Opportunities and Challenges in 3D Tumor Modeling

These recent advances in tumor modeling – the replacement of cancer cell lines with PDX models and the rise of PDO models – mandates a paradigm shift in tumor modeling. The ability to engineer the tumor microenvironment in 3D enables us to address some of the limitations associated with PDX models and PDO models. For example, a common drawback of these models is the lack of human stroma. With the 3D technologies as described above, it now is possible to overcome this limitation by growing these primary cells in defined co-culture with human stromal cells, such as immune cells. Additionally, with 3D systems, we are better able to manipulate the tumor microenvironment *in vitro* to investigate how various microenvironmental cues promote the selection of specific cancer clonal populations. It will be highly informative to understand how changes in the

microenvironment translate into changes in the tumor clonal architecture. However, we note that there are inherent limitations with current 3D models, a major shortcoming being that 3D models typically only model a single snapshot or at most a few stages in the complex process of tumorigenesis and tumor progression. Moreover, it is yet possible to incorporate the entire tumor ecosystem in 3D models. *In vivo* models, such as genetically induced mouse models of cancer, as well as companion animals with spontaneous neoplasms, will still be needed to complement 3D *in vitro* models to understand the natural history of cancer [195]. For example, in mouse models engineered with genetic aberrations, tumors arise *de novo* in the presence of a normal immune system and coevolve with an intact stroma; such a system enables the study of causative changes in tumor development and growth [196].

As PDX and PDO models become more commonplace, multidisciplinary networks of researchers comprising clinicians, pathologists, tumor biologists, tumor engineers, biostatisticians and bioinformaticians must be established to maximize the use of patient tumor samples and data. Multiple tumor sampling will be necessary to avoid sampling bias and to address intratumor heterogeneity. Longitudinal tumor sampling will be required to monitor clonal evolution over time. Pathologists will play a critical role in annotating tumor samples, whether resected or biopsied, for normal versus malignant regions, tumor versus stromal content, and viable versus necrotic regions. These details, together with 'omics'-based data processed by bioinformaticians, are especially critical for the proper analysis of datasets from tumor samples for both *in vivo* and *in vitro* modeling. Several questions then arise once the tumor tissue is available to the tumor biologist and tumor engineer:

1. Tumor versus Stroma – Is there a need to isolate the cancer cells from the surrounding stromal tissue? Such an approach may be necessary to study specific tumor-stromal interactions in a bottom-up approach. To understand the prostate cancer-osteoblast crosstalk, we encapsulated prostate cancer PDX cells enriched for cancer cells together with osteoblastic cells in an engineered hyaluronan-based matrix and demonstrated the feasibility of using 3D PDX models to dissect and define complex *in vivo* interactions *in vitro* (Figure 2) [87]. If stromal cells were warranted for studies of tumor-stromal interactions, how relevant is animal-derived stroma for tumor-microenvironment studies? Species specificity of ligand-receptor interactions may affect the relevance of human tumor-animal stroma interactions [197]. Moreover, inherent differences exist between mouse and human immunology [198]. Given this potential incompatibility, should immortalized human stromal cell lines be used? As an example, HUVEC endothelial cells are commonly used as a vascular cell source *in vitro* – to what extent do these large vessel HUVEC cells truly represent tumor-associated endothelial cells? The ideal case would be to use patient-specific stromal cells but obtaining these cells may be a challenge. Methods also need to be developed to expand patient-specific stromal cells with retention of the original tumor-reprogrammed phenotype. On a similar note, the matrix requirements for enabling stromal cell growth and culture likely differ from that of cancer cells. While cancer cells can depend on cell-cell interactions for survival, stromal cells typically require integrin-based attachments to the surrounding matrix for survival. Beyond the current tri-culture limit, what are the 3D matrix

requirements necessary to support multiple cell types? With several cell types, how can tumor-specific and stromal-specific measurements be made?

2. Tumor Heterogeneity – How can tumor heterogeneity be monitored and characterized *in vitro*? Perhaps it is time to shift away from global/bulk population averaging measurements and analyses made in typical *in vitro* assays towards more specific methodologies (such as the use of single-cell omics technologies [199], mass cytometry [200, 201] and high content imaging [202]) that enable analyses of heterogeneous cell populations. For example, drug efficacy *in vitro* is typically an averaged response evaluated using standard assays such as those based on tetrazolium or resazurin reduction, or by measuring the amount of cellular ATP. However, with heterogeneous PDX and patient-derived samples, it might be more useful to identify specifically the clonal populations that are responsive or resistant to drug treatment. More comprehensive methods of assessing heterogeneity in drug response *in vitro* will be needed. Lastly, given the prevalence of intratumor heterogeneity, can we construct stable tumor hybrids to study the codependency of different clonal populations on drug resistance and tumor progression?

3. Tissue Expansion – What is the best approach to expand often limited tumor samples for subsequent studies such as drug testing? At present, available strategies include the use of PDX models and organoid cultures [16, 203] but these may not be optimal methods. A greater understanding of the underlying selection pressures during tumor adaptation (to the new environment) and propagation, if any, is needed. Are we selecting for specific clonal populations with these expansion strategies? In the presence of animal stroma (as in the case of PDX models) or absence of stroma (as in the case of organoid cultures) during tumor propagation, changes to the tumor phenotype may occur. For example, Pearson et al. recently showed that PDX tumors of primary human head and neck squamous cell carcinoma and salivary gland adenoid cystic carcinoma increase in growth rate with *in vivo* passaging [204]. Significant correlations between passage number and histopathological features of higher tumor grade were also observed. Analogous to 2D passaging of cells, is it possible to design and engineer 3D platforms to propagate primary PDX and patient-derived cells in the presence of human stroma?

To conclude, the present is a tremendously exciting era in cancer research. We have begun to recognize that tumors are multifaceted and address head-on the need to reflect their heterogeneity and dynamism in our preclinical models to understand how they evolve and evade the immune system and therapeutics. With NCI's recent moonshot for better preclinical tumor models [13], cancer cell lines that have served us for decades are now being replaced by new cell sources that enable us to study cancer as close as possible to its original form. However, many challenges lie ahead, some of which are illustrated by the questions above. While various approaches have been developed thus far to mimic the complexity of cancer in 3D as described in this review, steps now are being made to capture simultaneously both tumor complexity and heterogeneity *in vitro* (Figure 1 and 2), an endeavor which will bring the field much closer in being able to accurately model cancer. It

must be borne in mind though, that no preclinical model will be perfect and models are truly clinically relevant only if they are used properly. Figure 3 illustrates the spectrum of preclinical models with their associated advantages and limitations. As we make further headway in developing better preclinical models, efforts must also be made to ensure different subsets of patients are matched with and represented by similarly stratified tumor models. Emphasis needs to be placed on model-based biomarker development that ensures the right patients are identified to receive and likely benefit from the treatment. Lastly, significant advances are also being made in cancer 'omics'; the recent collection and public availability of multi-'omics' databases (with accompanying clinical data) such as The Cancer Genome Atlas and more recently, the Cancer RNA-Seq Nexus [205], have and will enable cancer discoveries such as that of new oncogenic drivers and biomarkers. These headways in understanding the complex molecular landscape in patients, coupled with advances in tumor modeling and biomarker development, will pave the way for increased success in understanding, treating and preventing cancer.

Acknowledgments

M.C. Farach-Carson's and D.A. Harrington's contributions were supported by the National Institutes of Health Grant P01 CA098912. H. Yu was supported by funding from the Mechanobiology Institute (National University of Singapore), Institute of Bioengineering and Nanotechnology (A*STAR, Singapore), and Singapore-MIT Alliance for Research and Technology. H. Yu and E.L.S. Fong were both supported by funding from the National Medical Research Council (Ministry of Health, Singapore, Grant Number: R-185-000-294-511).

References

1. Toniatti C, Jones P, Graham H, Pagliara B, Draetta G. Oncology drug discovery: planning a turnaround. Cancer discovery. 2014; 4:397–404. [PubMed: 24706659]
2. Kamb A. What's wrong with our cancer models? Nature reviews Drug discovery. 2005; 4:161–5. [PubMed: 15688078]
3. Rubin EH, Gilliland DG. Drug development and clinical trials–the path to an approved cancer drug. Nature reviews Clinical oncology. 2012; 9:215–22.
4. Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. Nature. 2013; 501:346–54. [PubMed: 24048067]
5. Fischbach C, Chen R, Matsumoto T, Schmelzle T, Brugge JS, Polverini PJ, et al. Engineering tumors with 3D scaffolds. Nature methods. 2007; 4:855–60. [PubMed: 17767164]
6. Fong EL, Lamhamedi-Cherradi SE, Burdett E, Ramamoorthy V, Lazar AJ, Kasper FK, et al. Modeling Ewing sarcoma tumors in vitro with 3D scaffolds. Proc Natl Acad Sci U S A. 2013; 110:6500–5. [PubMed: 23576741]
7. Petersen OW, Ronnov-Jessen L, Howlett AR, Bissell MJ. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc Natl Acad Sci U S A. 1992; 89:9064–8. [PubMed: 1384042]
8. Shoemaker RH. The NCI60 human tumour cell line anticancer drug screen. Nat Rev Cancer. 2006; 6:813–23. [PubMed: 16990858]
9. Gillet JP, Calcagno AM, Varma S, Marino M, Green LJ, Vora MI, et al. Redefining the relevance of established cancer cell lines to the study of mechanisms of clinical anti-cancer drug resistance. Proc Natl Acad Sci U S A. 2011; 108:18708–13. [PubMed: 22068913]
10. Consortium F. Clst RP, Forrest AR, Kawaji H, Rehli M, et al. A promoter-level mammalian expression atlas. Nature. 2014; 507:462–70. [PubMed: 24670764]
11. Sia D, Moeini A, Labgaa I, Villanueva A. The future of patient-derived tumor xenografts in cancer treatment. Pharmacogenomics. 2015; 16:1671–83. [PubMed: 26402657]

12. Yap TA, Gerlinger M, Futreal PA, Pusztai L, Swanton C. Intratumor heterogeneity: seeing the wood for the trees. Science translational medicine. 2012; 4:127ps10.
13. Ledford H. US cancer institute to overhaul tumour cell lines. Nature. 2016; 530:391. [PubMed: 26911756]
14. Hidalgo M, Amant F, Biankin AV, Budinska E, Byrne AT, Caldas C, et al. Patient-derived xenograft models: an emerging platform for translational cancer research. Cancer discovery. 2014; 4:998–1013. [PubMed: 25185190]
15. Boj SF, Hwang CI, Baker LA, Chio II, Engle DD, Corbo V, et al. Organoid models of human and mouse ductal pancreatic cancer. Cell. 2015; 160:324–38. [PubMed: 25557080]
16. Gao D, Vela I, Sboner A, Iaquinta PJ, Karthaus WR, Gopalan A, et al. Organoid cultures derived from patients with advanced prostate cancer. Cell. 2014; 159:176–87. [PubMed: 25201530]
17. van de Wetering M, Francies HE, Francis JM, Bounova G, Iorio F, Pronk A, et al. Prospective derivation of a living organoid biobank of colorectal cancer patients. Cell. 2015; 161:933–45. [PubMed: 25957691]
18. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000; 100:57–70. [PubMed: 10647931]
19. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144:646–74. [PubMed: 21376230]
20. McMillin DW, Negri JM, Mitsiades CS. The role of tumour-stromal interactions in modifying drug response: challenges and opportunities. Nature reviews Drug discovery. 2013; 12:217–28. [PubMed: 23449307]
21. Park JH, Richards CH, McMillan DC, Horgan PG, Roxburgh CS. The relationship between tumour stroma percentage, the tumour microenvironment and survival in patients with primary operable colorectal cancer. Annals of oncology: official journal of the European Society for Medical Oncology/ESMO. 2014; 25:644–51.
22. Erkan M, Michalski CW, Rieder S, Reiser-Erkan C, Abiatari I, Kolb A, et al. The activated stroma index is a novel and independent prognostic marker in pancreatic ductal adenocarcinoma. Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association. 2008; 6:1155–61. [PubMed: 18639493]
23. Yanagisawa N, Li R, Rowley D, Liu H, Kadmon D, Miles BJ, et al. Stromogenic prostatic carcinoma pattern (carcinomas with reactive stromal grade 3) in needle biopsies predicts biochemical recurrence-free survival in patients after radical prostatectomy. Human pathology. 2007; 38:1611–20. [PubMed: 17868773]
24. Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. The Journal of cell biology. 2012; 196:395–406. [PubMed: 22351925]
25. Theocharis AD, Skandalis SS, Gialeli C, Karamanos NK. Extracellular matrix structure. Advanced drug delivery reviews. 2016; 97:4–27. [PubMed: 26562801]
26. Farach-Carson MC, Warren CR, Harrington DA, Carson DD. Border patrol: insights into the unique role of perlecan/heparan sulfate proteoglycan 2 at cell and tissue borders. Matrix biology: journal of the International Society for Matrix Biology. 2014; 34:64–79. [PubMed: 24001398]
27. Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. Nature reviews Molecular cell biology. 2014; 15:786–801. [PubMed: 25415508]
28. Schafer M, Werner S. Cancer as an overhealing wound: an old hypothesis revisited. Nature reviews Molecular cell biology. 2008; 9:628–38. [PubMed: 18628784]
29. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. The New England journal of medicine. 1986; 315:1650–9. [PubMed: 3537791]
30. Desmouliere A, Guyot C, Gabbiani G. The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. The International journal of developmental biology. 2004; 48:509–17. [PubMed: 15349825]
31. Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. Clinical cancer research: an official journal of the American Association for Cancer Research. 2002; 8:2912–23. [PubMed: 12231536]
32. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. Journal of cell science. 2010; 123:4195–200. [PubMed: 21123617]

33. Grindel BJ, Martinez JR, Pennington CL, Muldoon M, Stave J, Chung LW, et al. Matrilysin/matrix metalloproteinase-7(MMP7) cleavage of perlecan/HSPG2 creates a molecular switch to alter prostate cancer cell behavior. Matrix biology: journal of the International Society for Matrix Biology. 2014; 36:64–76. [PubMed: 24833109]
34. Eke I, Cordes N. Focal adhesion signaling and therapy resistance in cancer. Seminars in cancer biology. 2015; 31:65–75. [PubMed: 25117005]
35. Holle AW, Young JL, Spatz JP. In vitro cancer cell-ECM interactions inform in vivo cancer treatment. Advanced drug delivery reviews. 2016; 97:270–9. [PubMed: 26485156]
36. Singha NC, Nekoroski T, Zhao C, Symons R, Jiang P, Frost GI, et al. Tumor-associated hyaluronan limits efficacy of monoclonal antibody therapy. Molecular cancer therapeutics. 2015; 14:523–32. [PubMed: 25512619]
37. Bourguignon LY, Peyrollier K, Xia W, Gilad E. Hyaluronan-CD44 interaction activates stem cell marker Nanog, Stat-3-mediated MDR1 gene expression, and ankyrin-regulated multidrug efflux in breast and ovarian tumor cells. The Journal of biological chemistry. 2008; 283:17635–51. [PubMed: 18441325]
38. Bourguignon LY, Shiina M, Li JJ. Hyaluronan-CD44 interaction promotes oncogenic signaling, microRNA functions, chemoresistance, and radiation resistance in cancer stem cells leading to tumor progression. Advances in cancer research. 2014; 123:255–75. [PubMed: 25081533]
39. Damiano JS. Integrins as novel drug targets for overcoming innate drug resistance. Current cancer drug targets. 2002; 2:37–43. [PubMed: 12188919]
40. Shiga K, Hara M, Nagasaki T, Sato T, Takahashi H, Takeyama H. Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. Cancers. 2015; 7:2443–58. [PubMed: 26690480]
41. Mehner C, Radisky DC. Triggering the landslide: The tumor-promotional effects of myofibroblasts. Experimental cell research. 2013; 319:1657–62. [PubMed: 23528452]
42. Radisky DC, Kenny PA, Bissell MJ. Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? Journal of cellular biochemistry. 2007; 101:830–9. [PubMed: 17211838]
43. Orimo A, Weinberg RA. Heterogeneity of stromal fibroblasts in tumors. Cancer biology & therapy. 2007; 6:618–9. [PubMed: 18027438]
44. Ishii G, Ochiai A, Neri S. Phenotypic and functional heterogeneity of cancer-associated fibroblast within the tumor microenvironment. Advanced drug delivery reviews. 2015
45. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005; 121:335–48. [PubMed: 15882617]
46. Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, et al. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nature cell biology. 2007; 9:1392–400. [PubMed: 18037882]
47. Mertens JC, Fingas CD, Christensen JD, Smoot RL, Bronk SF, Werneburg NW, et al. Therapeutic effects of deleting cancer-associated fibroblasts in cholangiocarcinoma. Cancer Res. 2013; 73:897–907. [PubMed: 23221385]
48. Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nature reviews Drug discovery. 2011; 10:417–27. [PubMed: 21629292]
49. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science. 2005; 307:58–62. [PubMed: 15637262]
50. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer cell. 2003; 3:347–61. [PubMed: 12726861]
51. Bottaro DP, Liotta LA. Cancer: Out of air is not out of action. Nature. 2003; 423:593–5. [PubMed: 12789320]
52. Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. Nat Rev Cancer. 2011; 11:393–410. [PubMed: 21606941]
53. Arjaans M, Schroder CP, Oosting SF, Dafni U, Kleibeuker JE, de Vries EG. VEGF pathway targeting agents, vessel normalization and tumor drug uptake: from bench to bedside. Oncotarget. 2016

54. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer. 2008; 8:592–603. [PubMed: 18650835]
55. Meng MB, Zaorsky NG, Deng L, Wang HH, Chao J, Zhao LJ, et al. Pericytes: a double-edged sword in cancer therapy. Future oncology. 2015; 11:169–79. [PubMed: 25143028]
56. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nature immunology. 2002; 3:991–8. [PubMed: 12407406]
57. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annual review of immunology. 2004; 22:329–60.
58. Sharma P, Wagner K, Wolchok JD, Allison JP. Novel cancer immunotherapy agents with survival benefit: recent successes and next steps. Nat Rev Cancer. 2011; 11:805–12. [PubMed: 22020206]
59. Sharma P, Allison JP. The future of immune checkpoint therapy. Science. 2015; 348:56–61. [PubMed: 25838373]
60. Nieman KM, Romero IL, Van Houten B, Lengyel E. Adipose tissue and adipocytes support tumorigenesis and metastasis. Biochimica et biophysica acta. 2013; 1831:1533–41. [PubMed: 23500888]
61. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nature reviews Immunology. 2011; 11:85–97.
62. Bochet L, Lehuede C, Dauvillier S, Wang YY, Dirat B, Laurent V, et al. Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer. Cancer Res. 2013; 73:5657–68. [PubMed: 23903958]
63. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. Cancer Res. 2011; 71:2455–65. [PubMed: 21459803]
64. Gazi E, Gardner P, Lockyer NP, Hart CA, Brown MD, Clarke NW. Direct evidence of lipid translocation between adipocytes and prostate cancer cells with imaging FTIR microspectroscopy. Journal of lipid research. 2007; 48:1846–56. [PubMed: 17496269]
65. Munson JM, Shieh AC. Interstitial fluid flow in cancer: implications for disease progression and treatment. Cancer management and research. 2014; 6:317–28. [PubMed: 25170280]
66. Chang SF, Chang CA, Lee DY, Lee PL, Yeh YM, Yeh CR, et al. Tumor cell cycle arrest induced by shear stress: Roles of integrins and Smad. Proc Natl Acad Sci U S A. 2008; 105:3927–32. [PubMed: 18310319]
67. Fleury ME, Boardman KC, Swartz MA. Autologous morphogen gradients by subtle interstitial flow and matrix interactions. Biophysical journal. 2006; 91:113–21. [PubMed: 16603487]
68. Munson JM, Bellamkonda RV, Swartz MA. Interstitial flow in a 3D microenvironment increases glioma invasion by a CXCR4-dependent mechanism. Cancer Res. 2013; 73:1536–46. [PubMed: 23271726]
69. Rutkowski JM, Swartz MA. A driving force for change: interstitial flow as a morphoregulator. Trends in cell biology. 2007; 17:44–50. [PubMed: 17141502]
70. Shields JD, Fleury ME, Yong C, Tomei AA, Randolph GJ, Swartz MA. Autologous chemotaxis as a mechanism of tumor cell homing to lymphatics via interstitial flow and autocrine CCR7 signaling. Cancer cell. 2007; 11:526–38. [PubMed: 17560334]
71. Wang F, Weaver VM, Petersen OW, Larabell CA, Dedhar S, Briand P, et al. Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. Proc Natl Acad Sci U S A. 1998; 95:14821–6. [PubMed: 9843973]
72. Benton G, Arnaoutova I, George J, Kleinman HK, Koblinski J. Matrigel: from discovery and ECM mimicry to assays and models for cancer research. Advanced drug delivery reviews. 2014; 79–80:3–18.
73. Yeung TM, Gandhi SC, Wilding JL, Muschel R, Bodmer WF. Cancer stem cells from colorectal cancer-derived cell lines. Proc Natl Acad Sci U S A. 2010; 107:3722–7. [PubMed: 20133591]
74. Conklin MW, Keely PJ. Why the stroma matters in breast cancer: insights into breast cancer patient outcomes through the examination of stromal biomarkers. Cell adhesion & migration. 2012; 6:249–60. [PubMed: 22568982]

75. Barron DA, Rowley DR. The reactive stroma microenvironment and prostate cancer progression. Endocrine-related cancer. 2012; 19:R187–204. [PubMed: 22930558]
76. Cheung KJ, Gabrielson E, Werb Z, Ewald AJ. Collective invasion in breast cancer requires a conserved basal epithelial program. Cell. 2013; 155:1639–51. [PubMed: 24332913]
77. Riching KM, Cox BL, Salick MR, Pehlke C, Riching AS, Ponik SM, et al. 3D collagen alignment limits protrusions to enhance breast cancer cell persistence. Biophysical journal. 2014; 107:2546–58. [PubMed: 25468334]
78. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell. 2009; 139:891–906. [PubMed: 19931152]
79. Lang NR, Skodzek K, Hurst S, Mainka A, Steinwachs J, Schneider J, et al. Biphasic response of cell invasion to matrix stiffness in three-dimensional biopolymer networks. Acta biomaterialia. 2015; 13:61–7. [PubMed: 25462839]
80. Cassereau L, Miroshnikova YA, Ou G, Lakins J, Weaver VM. A 3D tension bioreactor platform to study the interplay between ECM stiffness and tumor phenotype. Journal of biotechnology. 2015; 193:66–9. [PubMed: 25435379]
81. Dunne LW, Huang Z, Meng W, Fan X, Zhang N, Zhang Q, et al. Human decellularized adipose tissue scaffold as a model for breast cancer cell growth and drug treatments. Biomaterials. 2014; 35:4940–9. [PubMed: 24661550]
82. Lu WD, Zhang L, Wu CL, Liu ZG, Lei GY, Liu J, et al. Development of an acellular tumor extracellular matrix as a three-dimensional scaffold for tumor engineering. PLoS One. 2014; 9:e103672. [PubMed: 25072252]
83. Mishra DK, Thrall MJ, Baird BN, Ott HC, Blackmon SH, Kurie JM, et al. Human lung cancer cells grown on acellular rat lung matrix create perfusable tumor nodules. The Annals of thoracic surgery. 2012; 93:1075–81. [PubMed: 22385822]
84. Moll C, Reboredo J, Schwarz T, Appelt A, Schurlein S, Walles H, et al. Tissue engineering of a human 3D in vitro tumor test system. Journal of visualized experiments: JoVE. 2013
85. Engel BJ, Constantinou PE, Sablatura LK, Doty NJ, Carson DD, Farach-Carson MC, et al. Multilayered, Hyaluronic Acid-Based Hydrogel Formulations Suitable for Automated 3D High Throughput Drug Screening of Cancer-Stromal Cell Cocultures. Adv Healthc Mater. 2015; 4:1664–74. [PubMed: 26059746]
86. Fong EL, Martinez M, Yang J, Mikos AG, Navone NM, Harrington DA, et al. Hydrogel-based 3D model of patient-derived prostate xenograft tumors suitable for drug screening. Molecular pharmaceutics. 2014; 11:2040–50. [PubMed: 24779589]
87. Fong EL, Wan X, Yang J, Morgado M, Mikos AG, Harrington DA, et al. A 3D in vitro model of patient-derived prostate cancer xenograft for controlled interrogation of in vivo tumor-stromal interactions. Biomaterials. 2016; 77:164–72. [PubMed: 26599623]
88. Gurski LA, Xu X, Labrada LN, Nguyen NT, Xiao L, van Golen KL, et al. Hyaluronan (HA) interacting proteins RHAMM and hyaluronidase impact prostate cancer cell behavior and invadopodia formation in 3D HA-based hydrogels. PLoS One. 2012; 7:e50075. [PubMed: 23166824]
89. Gurski LA, Jha AK, Zhang C, Jia X, Farach-Carson MC. Hyaluronic acid-based hydrogels as 3D matrices for in vitro evaluation of chemotherapeutic drugs using poorly adherent prostate cancer cells. Biomaterials. 2009; 30:6076–85. [PubMed: 19695694]
90. Xu X, Farach-Carson MC, Jia X. Three-dimensional in vitro tumor models for cancer research and drug evaluation. Biotechnology advances. 2014; 32:1256–68. [PubMed: 25116894]
91. Toole BP, Hascall VC. Hyaluronan and tumor growth. The American journal of pathology. 2002; 161:745–7. [PubMed: 12213700]
92. Xu X, Gurski LA, Zhang C, Harrington DA, Farach-Carson MC, Jia X. Recreating the tumor microenvironment in a bilayer, hyaluronic acid hydrogel construct for the growth of prostate cancer spheroids. Biomaterials. 2012; 33:9049–60. [PubMed: 22999468]
93. Pan T, Fong EL, Martinez M, Harrington DA, Lin SH, Farach-Carson MC, et al. Three-dimensional (3D) culture of bone-derived human 786-O renal cell carcinoma retains relevant clinical characteristics of bone metastases. Cancer letters. 2015; 365:89–95. [PubMed: 26004343]

94. Blehm BH, Jiang N, Kotobuki Y, Tanner K. Deconstructing the role of the ECM microenvironment on drug efficacy targeting MAPK signaling in a pre-clinical platform for cutaneous melanoma. Biomaterials. 2015; 56:129–39. [PubMed: 25934286]

95. Gombotz WR, Wang GH, Horbett TA, Hoffman AS. Protein adsorption to poly(ethylene oxide) surfaces. Journal of biomedical materials research. 1991; 25:1547–62. [PubMed: 1839026]

96. Gill BJ, West JL. Modeling the tumor extracellular matrix: Tissue engineering tools repurposed towards new frontiers in cancer biology. Journal of biomechanics. 2014; 47:1969–78. [PubMed: 24300038]

97. Loessner D, Stok KS, Lutolf MP, Hutmacher DW, Clements JA, Rizzi SC. Bioengineered 3D platform to explore cell-ECM interactions and drug resistance of epithelial ovarian cancer cells. Biomaterials. 2010; 31:8494–506. [PubMed: 20709389]

98. Wang C, Tong X, Yang F. Bioengineered 3D brain tumor model to elucidate the effects of matrix stiffness on glioblastoma cell behavior using PEG-based hydrogels. Molecular pharmaceutics. 2014; 11:2115–25. [PubMed: 24712441]

99. Raza A, Ki CS, Lin CC. The influence of matrix properties on growth and morphogenesis of human pancreatic ductal epithelial cells in 3D. Biomaterials. 2013; 34:5117–27. [PubMed: 23602364]

100. Schwartz MP, Rogers RE, Singh SP, Lee JY, Loveland SG, Koepsel JT, et al. A quantitative comparison of human HT-1080 fibrosarcoma cells and primary human dermal fibroblasts identifies a 3D migration mechanism with properties unique to the transformed phenotype. PLoS One. 2013; 8:e81689. [PubMed: 24349113]

101. Singh SP, Schwartz MP, Lee JY, Fairbanks BD, Anseth KS. A peptide functionalized poly(ethylene glycol) (PEG) hydrogel for investigating the influence of biochemical and biophysical matrix properties on tumor cell migration. Biomaterials science. 2014; 2:1024–34. [PubMed: 25105013]

102. Lutolf MP, Lauer-Fields JL, Schmoekel HG, Metters AT, Weber FE, Fields GB, et al. Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: engineering cell-invasion characteristics. Proc Natl Acad Sci U S A. 2003; 100:5413–8. [PubMed: 12686696]

103. Pradhan S, Farach-Carson MC. Mining the extracellular matrix for tissue engineering applications. Regenerative medicine. 2010; 5:961–70. [PubMed: 21082894]

104. Grindel B, Li Q, Arnold R, Petros J, Zayzafoon M, Muldoon M, et al. Perlecan/HSPG2 and matrilysin/MMP-7 as indices of tissue invasion: tissue localization and circulating perlecan fragments in a cohort of 288 radical prostatectomy patients. Oncotarget. 2016; 7:10433–47. [PubMed: 26862737]

105. Xia L, Sakban RB, Qu Y, Hong X, Zhang W, Nugraha B, et al. Tethered spheroids as an in vitro hepatocyte model for drug safety screening. Biomaterials. 2012; 33:2165–76. [PubMed: 22189144]

106. Nugraha B, Hong X, Mo X, Tan L, Zhang W, Chan PM, et al. Galactosylated cellulosic sponge for multi-well drug safety testing. Biomaterials. 2011; 32:6982–94. [PubMed: 21741702]

107. Burdett E, Kasper FK, Mikos AG, Ludwig JA. Engineering tumors: a tissue engineering perspective in cancer biology. Tissue engineering Part B, Reviews. 2010; 16:351–9. [PubMed: 20092396]

108. Majety M, Pradel LP, Gies M, Ries CH. Fibroblasts Influence Survival and Therapeutic Response in a 3D Co-Culture Model. PLoS One. 2015; 10:e0127948. [PubMed: 26053043]

109. Jaganathan H, Gage J, Leonard F, Srinivasan S, Souza GR, Dave B, et al. Three-dimensional in vitro co-culture model of breast tumor using magnetic levitation. Scientific reports. 2014; 4:6468. [PubMed: 25270048]

110. Yip D, Cho CH. A multicellular 3D heterospheroid model of liver tumor and stromal cells in collagen gel for anti-cancer drug testing. Biochemical and biophysical research communications. 2013; 433:327–32. [PubMed: 23501105]

111. Dolznig H, Rupp C, Puri C, Haslinger C, Schweifer N, Wieser E, et al. Modeling colon adenocarcinomas in vitro a 3D co-culture system induces cancer-relevant pathways upon tumor

cell and stromal fibroblast interaction. The American journal of pathology. 2011; 179:487–501. [PubMed: 21703426]

112. Sieh S, Lubik AA, Clements JA, Nelson CC, Hutmacher DW. Interactions between human osteoblasts and prostate cancer cells in a novel 3D in vitro model. Organogenesis. 2010; 6:181–8. [PubMed: 21197221]
113. Sieh S, Taubenberger AV, Lehman ML, Clements JA, Nelson CC, Hutmacher DW. Paracrine interactions between LNCaP prostate cancer cells and bioengineered bone in 3D in vitro culture reflect molecular changes during bone metastasis. Bone. 2014; 63:121–31. [PubMed: 24530694]
114. Subia B, Dey T, Sharma S, Kundu SC. Target specific delivery of anticancer drug in silk fibroin based 3D distribution model of bone-breast cancer cells. ACS applied materials & interfaces. 2015; 7:2269–79. [PubMed: 25557227]
115. Zhang W, Lee WY, Siegel DS, Tolias P, Zilberberg J. Patient-specific 3D microfluidic tissue model for multiple myeloma. Tissue engineering Part C, Methods. 2014; 20:663–70. [PubMed: 24294886]
116. Sung KE, Yang N, Pehlke C, Keely PJ, Eliceiri KW, Friedl A, et al. Transition to invasion in breast cancer: a microfluidic in vitro model enables examination of spatial and temporal effects. Integr Biol (Camb). 2011; 3:439–50. [PubMed: 21135965]
117. Choi Y, Hyun E, Seo J, Blundell C, Kim HC, Lee E, et al. A microengineered pathophysiological model of early-stage breast cancer. Lab Chip. 2015; 15:3350–7. [PubMed: 26158500]
118. Bischel LL, Beebe DJ, Sung KE. Microfluidic model of ductal carcinoma in situ with 3D, organotypic structure. BMC cancer. 2015; 15:12. [PubMed: 25605670]
119. Chwalek K, Bray LJ, Werner C. Tissue-engineered 3D tumor angiogenesis models: potential technologies for anti-cancer drug discovery. Advanced drug delivery reviews. 2014; 79–80:30–9.
120. Marcola M, Rodrigues CE. Endothelial progenitor cells in tumor angiogenesis: another brick in the wall. Stem cells international. 2015; 2015:832649. [PubMed: 26000021]
121. Seano G, Chiaverina G, Gagliardi PA, di Blasio L, Sessa R, Bussolino F, et al. Modeling human tumor angiogenesis in a three-dimensional culture system. Blood. 2013; 121:e129–37. [PubMed: 23471306]
122. Benton G, DeGray G, Kleinman HK, George J, Arnaoutova I. In vitro microtumors provide a physiologically predictive tool for breast cancer therapeutic screening. PLoS One. 2015; 10:e0123312. [PubMed: 25856378]
123. Correa de Sampaio P, Auslaender D, Krubasik D, Failla AV, Skepper JN, Murphy G, et al. A heterogeneous in vitro three dimensional model of tumour-stroma interactions regulating sprouting angiogenesis. PLoS One. 2012; 7:e30753. [PubMed: 22363483]
124. Ingthorsson S, Sigurdsson V, Fridriksdottir A Jr, Jonasson JG, Kjartansson J, Magnusson MK, et al. Endothelial cells stimulate growth of normal and cancerous breast epithelial cells in 3D culture. BMC research notes. 2010; 3:184. [PubMed: 20609224]
125. Nyga A, Loizidou M, Emberton M, Cheema U. A novel tissue engineered three-dimensional in vitro colorectal cancer model. Acta biomaterialia. 2013; 9:7917–26. [PubMed: 23624217]
126. Szot CS, Buchanan CF, Freeman JW, Rylander MN. In vitro angiogenesis induced by tumor-endothelial cell co-culture in bilayered, collagen I hydrogel bioengineered tumors. Tissue engineering Part C, Methods. 2013; 19:864–74. [PubMed: 23516987]
127. Chwalek K, Tsurkan MV, Freudenberg U, Werner C. Glycosaminoglycan-based hydrogels to modulate heterocellular communication in in vitro angiogenesis models. Scientific reports. 2014; 4:4414. [PubMed: 24643064]
128. Bray LJ, Binner M, Holzheu A, Friedrichs J, Freudenberg U, Hutmacher DW, et al. Multi-parametric hydrogels support 3D in vitro bioengineered microenvironment models of tumour angiogenesis. Biomaterials. 2015; 53:609–20. [PubMed: 25890757]
129. Chung S, Sudo R, Mack PJ, Wan CR, Vickerman V, Kamm RD. Cell migration into scaffolds under co-culture conditions in a microfluidic platform. Lab Chip. 2009; 9:269–75. [PubMed: 19107284]
130. Shin Y, Han S, Jeon JS, Yamamoto K, Zervantonakis IK, Sudo R, et al. Microfluidic assay for simultaneous culture of multiple cell types on surfaces or within hydrogels. Nature protocols. 2012; 7:1247–59. [PubMed: 22678430]

131. Bai J, Tu TY, Kim C, Thiery JP, Kamm RD. Identification of drugs as single agents or in combination to prevent carcinoma dissemination in a microfluidic 3D environment. Oncotarget. 2015; 6:36603–14. [PubMed: 26474384]

132. Jeon JS, Zervantonakis IK, Chung S, Kamm RD, Charest JL. In vitro model of tumor cell extravasation. PLoS One. 2013; 8:e56910. [PubMed: 23437268]

133. Zervantonakis IK, Hughes-Alford SK, Charest JL, Condeelis JS, Gertler FB, Kamm RD. Three-dimensional microfluidic model for tumor cell intravasation and endothelial barrier function. Proc Natl Acad Sci U S A. 2012; 109:13515–20. [PubMed: 22869695]

134. Chan JM, Zervantonakis IK, Rimchala T, Polacheck WJ, Whisler J, Kamm RD. Engineering of in vitro 3D capillary beds by self-directed angiogenic sprouting. PLoS One. 2012; 7:e50582. [PubMed: 23226527]

135. Kim S, Lee H, Chung M, Jeon NL. Engineering of functional, perfusable 3D microvascular networks on a chip. Lab Chip. 2013; 13:1489–500. [PubMed: 23440068]

136. Harris TJ, Drake CG. Primer on tumor immunology and cancer immunotherapy. Journal for immunotherapy of cancer. 2013; 1:12. [PubMed: 24829749]

137. Crusz SM, Balkwill FR. Inflammation and cancer: advances and new agents. Nature reviews Clinical oncology. 2015; 12:584–96.

138. Alonso-Nocelo M, Abuin C, Lopez-Lopez R, de la Fuente M. Development and characterization of a three-dimensional co-culture model of tumor T cell infiltration. Biofabrication. 2016; 8:025002. [PubMed: 27078888]

139. Shalapour S, Karin M. Immunity, inflammation, and cancer: an eternal fight between good and evil. J Clin Invest. 2015; 125:3347–55. [PubMed: 26325032]

140. Chimal-Ramirez GK, Espinoza-Sanchez NA, Utrera-Barillas D, Benitez-Bribiesca L, Velazquez JR, Arriaga-Pizano LA, et al. MMP1, MMP9, and COX2 expressions in promonocytes are induced by breast cancer cells and correlate with collagen degradation, transformation-like morphological changes in MCF-10A acini, and tumor aggressiveness. BioMed research international. 2013; 2013:279505. [PubMed: 23762835]

141. Phan-Lai V, Florczyk SJ, Kievit FM, Wang K, Gad E, Disis ML, et al. Three-dimensional scaffolds to evaluate tumor associated fibroblast-mediated suppression of breast tumor specific T cells. Biomacromolecules. 2013; 14:1330–7. [PubMed: 23517456]

142. Khalil DN, Smith EL, Brentjens RJ, Wolchok JD. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. Nature reviews Clinical oncology. 2016; 13:273–90.

143. Santoro M, Lamhamedi-Cherradi SE, Menegaz BA, Ludwig JA, Mikos AG. Flow perfusion effects on three-dimensional culture and drug sensitivity of Ewing sarcoma. Proc Natl Acad Sci U S A. 2015; 112:10304–9. [PubMed: 26240353]

144. Polacheck WJ, Charest JL, Kamm RD. Interstitial flow influences direction of tumor cell migration through competing mechanisms. Proc Natl Acad Sci U S A. 2011; 108:11115–20. [PubMed: 21690404]

145. Polacheck WJ, German AE, Mammoto A, Ingber DE, Kamm RD. Mechanotransduction of fluid stresses governs 3D cell migration. Proc Natl Acad Sci U S A. 2014; 111:2447–52. [PubMed: 24550267]

146. Wang Y, Waters J, Leung ML, Unruh A, Roh W, Shi X, et al. Clonal evolution in breast cancer revealed by single nucleus genome sequencing. Nature. 2014; 512:155–60. [PubMed: 25079324]

147. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. Nature. 2013; 501:338–45. [PubMed: 24048066]

148. Saunders NA, Simpson F, Thompson EW, Hill MM, Endo-Munoz L, Leggatt G, et al. Role of intratumoural heterogeneity in cancer drug resistance: molecular and clinical perspectives. EMBO molecular medicine. 2012; 4:675–84. [PubMed: 22733553]

149. Pribluda A, de la Cruz CC, Jackson EL. Intratumoral Heterogeneity: From Diversity Comes Resistance. Clinical cancer research: an official journal of the American Association for Cancer Research. 2015; 21:2916–23. [PubMed: 25838394]

150. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science. 2008; 321:1801–6. [PubMed: 18772397]

151. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008; 321:1807–12. [PubMed: 18772396]

152. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. The New England journal of medicine. 2012; 366:883–92. [PubMed: 22397650]

153. Sottoriva A, Spiteri I, Piccirillo SG, Touloumis A, Collins VP, Marioni JC, et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. Proc Natl Acad Sci U S A. 2013; 110:4009–14. [PubMed: 23412337]

154. Shackleton M, Quintana E, Fearon ER, Morrison SJ. Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell. 2009; 138:822–9. [PubMed: 19737509]

155. Gupta PB, Fillmore CM, Jiang G, Shapira SD, Tao K, Kuperwasser C, et al. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. Cell. 2011; 146:633–44. [PubMed: 21854987]

156. Ding L, Ley TJ, Larson DE, Miller CA, Koboldt DC, Welch JS, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012; 481:506–10. [PubMed: 22237025]

157. Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. Nature. 2013; 501:328–37. [PubMed: 24048065]

158. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nature medicine. 1997; 3:730–7.

159. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994; 367:645–8. [PubMed: 7509044]

160. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. Cancer Res. 2007; 67:1030–7. [PubMed: 17283135]

161. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci U S A. 2007; 104:973–8. [PubMed: 17210912]

162. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003; 100:3983–8. [PubMed: 12629218]

163. Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, et al. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A. 2007; 104:10158–63. [PubMed: 17548814]

164. Kelly PN, Dakic A, Adams JM, Nutt SL, Strasser A. Tumor growth need not be driven by rare cancer stem cells. Science. 2007; 317:337. [PubMed: 17641192]

165. Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM, Morrison SJ. Efficient tumour formation by single human melanoma cells. Nature. 2008; 456:593–8. [PubMed: 19052619]

166. Nowell PC. The clonal evolution of tumor cell populations. Science. 1976; 194:23–8. [PubMed: 959840]

167. Chaffer CL, Brueckmann I, Scheel C, Kaestli AJ, Wiggins PA, Rodrigues LO, et al. Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. Proc Natl Acad Sci U S A. 2011; 108:7950–5. [PubMed: 21498687]

168. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008; 133:704–15. [PubMed: 18485877]

169. Polanska UM, Orimo A. Carcinoma-associated fibroblasts: non-neoplastic tumour-promoting mesenchymal cells. Journal of cellular physiology. 2013; 228:1651–7. [PubMed: 23460038]

170. Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, et al. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nature medicine. 2008; 14:28–36.

171. Nawaz S, Heindl A, Koelble K, Yuan Y. Beyond immune density: critical role of spatial heterogeneity in estrogen receptor-negative breast cancer. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc. 2015; 28:1621.

172. Burrell RA, Swanton C. Tumour heterogeneity and the evolution of polyclonal drug resistance. Molecular oncology. 2014; 8:1095–111. [PubMed: 25087573]

173. Mullighan CG, Phillips LA, Su X, Ma J, Miller CB, Shurtleff SA, et al. Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. Science. 2008; 322:1377–80. [PubMed: 19039135]

174. Johnson BE, Mazor T, Hong C, Barnes M, Aihara K, McLean CY, et al. Mutational analysis reveals the origin and therapy-driven evolution of recurrent glioma. Science. 2014; 343:189–93. [PubMed: 24336570]

175. Siolas D, Hannon GJ. Patient-derived tumor xenografts: transforming clinical samples into mouse models. Cancer Res. 2013; 73:5315–9. [PubMed: 23733750]

176. Wilding JL, Bodmer WF. Cancer cell lines for drug discovery and development. Cancer Res. 2014; 74:2377–84. [PubMed: 24717177]

177. Daniel VC, Marchionni L, Hierman JS, Rhodes JT, Devereux WL, Rudin CM, et al. A primary xenograft model of small-cell lung cancer reveals irreversible changes in gene expression imposed by culture in vitro. Cancer Res. 2009; 69:3364–73. [PubMed: 19351829]

178. Tentler JJ, Tan AC, Weekes CD, Jimeno A, Leong S, Pitts TM, et al. Patient-derived tumour xenografts as models for oncology drug development. Nature reviews Clinical oncology. 2012; 9:338–50.

179. Kameya T, Shimosato Y, Tumuraya M, Ohsawa N, Nomura T. Human gastric choriocarcinoma serially transplanted in nude mice. Journal of the National Cancer Institute. 1976; 56:325–32. [PubMed: 943558]

180. DeRose YS, Wang G, Lin YC, Bernard PS, Buys SS, Ebbert MT, et al. Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes. Nature medicine. 2011; 17:1514–20.

181. Loukopoulos P, Kanetaka K, Takamura M, Shibata T, Sakamoto M, Hirohashi S. Orthotopic transplantation models of pancreatic adenocarcinoma derived from cell lines and primary tumors and displaying varying metastatic activity. Pancreas. 2004; 29:193–203. [PubMed: 15367885]

182. Zhao X, Liu Z, Yu L, Zhang Y, Baxter P, Voicu H, et al. Global gene expression profiling confirms the molecular fidelity of primary tumor-based orthotopic xenograft mouse models of medulloblastoma. Neuro-oncology. 2012; 14:574–83. [PubMed: 22459127]

183. Bertotti A, Migliardi G, Galimi F, Sassi F, Torti D, Isella C, et al. A molecularly annotated platform of patient-derived xenografts ("xenopatients") identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. Cancer discovery. 2011; 1:508–23. [PubMed: 22586653]

184. Gao H, Korn JM, Ferretti S, Monahan JE, Wang Y, Singh M, et al. High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. Nature medicine. 2015; 21:1318–25.

185. Malaney P, Nicosia SV, Dave V. One mouse, one patient paradigm: New avatars of personalized cancer therapy. Cancer letters. 2014; 344:1–12. [PubMed: 24157811]

186. Cassidy JW, Caldas C, Bruna A. Maintaining Tumor Heterogeneity in Patient-Derived Tumor Xenografts. Cancer Res. 2015; 75:2963–8. [PubMed: 26180079]

187. Aparicio S, Hidalgo M, Kung AL. Examining the utility of patient-derived xenograft mouse models. Nat Rev Cancer. 2015; 15:311–6. [PubMed: 25907221]

188. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature. 2009; 459:262–5. [PubMed: 19329995]

189. Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, et al. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. Cell stem cell. 2010; 6:25–36. [PubMed: 20085740]

190. Huch M, Dorrell C, Boj SF, van Es JH, Li VS, van de Wetering M, et al. In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. Nature. 2013; 494:247–50. [PubMed: 23354049]
191. Karthaus WR, Iaquinta PJ, Drost J, Gracanin A, van Boxtel R, Wongvipat J, et al. Identification of multipotent luminal progenitor cells in human prostate organoid cultures. Cell. 2014; 159:163–75. [PubMed: 25201529]
192. Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology. 2011; 141:1762–72. [PubMed: 21889923]
193. Weeber F, van de Wetering M, Hoogstraat M, Dijkstra KK, Krijgsman O, Kuilman T, et al. Preserved genetic diversity in organoids cultured from biopsies of human colorectal cancer metastases. Proc Natl Acad Sci U S A. 2015; 112:13308–11. [PubMed: 26460009]
194. Matano M, Date S, Shimokawa M, Takano A, Fujii M, Ohta Y, et al. Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. Nature medicine. 2015; 21:256–62.
195. Cekanova M, Rathore K. Animal models and therapeutic molecular targets of cancer: utility and limitations. Drug design, development and therapy. 2014; 8:1911–21.
196. Walrath JC, Hawes JJ, Van Dyke T, Reilly KM. Genetically engineered mouse models in cancer research. Advances in cancer research. 2010; 106:113–64. [PubMed: 20399958]
197. Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, et al. Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. The Journal of biological chemistry. 2006; 281:13964–71. [PubMed: 16547002]
198. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. Journal of immunology. 2004; 172:2731–8.
199. Navin NE. The first five years of single-cell cancer genomics and beyond. Genome research. 2015; 25:1499–507. [PubMed: 26430160]
200. Di Palma S, Bodenmiller B. Unraveling cell populations in tumors by single-cell mass cytometry. Current opinion in biotechnology. 2015; 31:122–9. [PubMed: 25123841]
201. Giesen C, Wang HA, Schapiro D, Zivanovic N, Jacobs A, Hattendorf B, et al. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. Nature methods. 2014; 11:417–22. [PubMed: 24584193]
202. Boutros M, Heigwer F, Laufer C. Microscopy-Based High-Content Screening. Cell. 2015; 163:1314–25. [PubMed: 26638068]
203. Valta MP, Zhao H, Saar M, Tuomela J, Nolley R, Linxweiler J, et al. Spheroid culture of LuCaP 136 patient-derived xenograft enables versatile preclinical models of prostate cancer. Clinical & experimental metastasis. 2016; 33:325–37. [PubMed: 26873136]
204. Pearson AT, Finkel KA, Warner KA, Nor F, Tice D, Martins MD, et al. Patient-derived xenograft (PDX) tumors increase growth rate with time. Oncotarget. 2016; 7:7993–8005. [PubMed: 26783960]
205. Li JR, Sun CH, Li W, Chao RF, Huang CC, Zhou XJ, et al. Cancer RNA-Seq Nexus: a database of phenotype-specific transcriptome profiling in cancer cells. Nucleic acids research. 2016; 44:D944–51. [PubMed: 26602695]

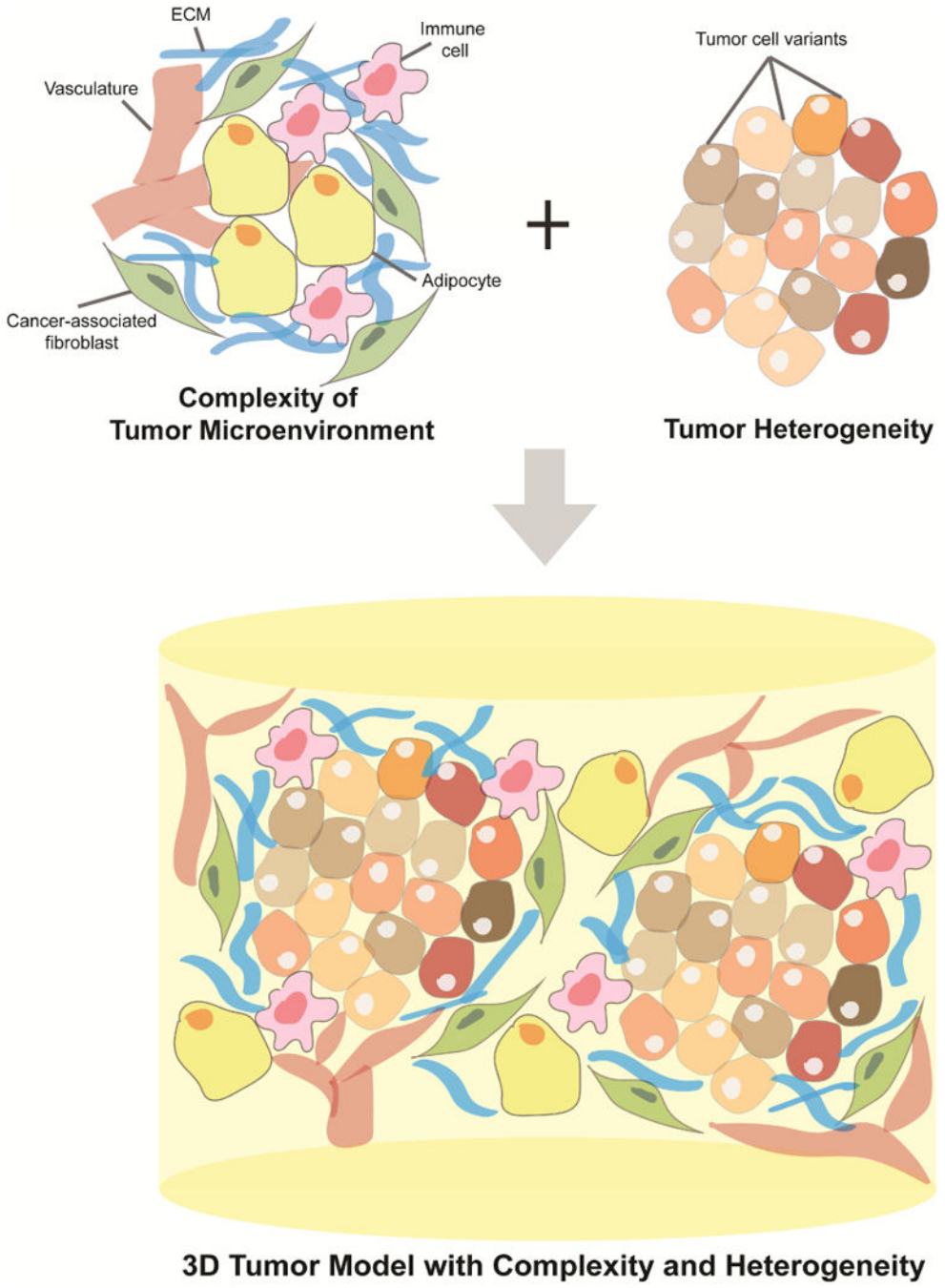

Figure 1.

The next chapter in 3D modeling requires the incorporation of both tumor microenvironment complexity and tumor heterogeneity. Such an approach is likely to enable us to better recapitulate cancer *in vitro* for both therapeutic drug development and mechanistic studies of cancer biology.

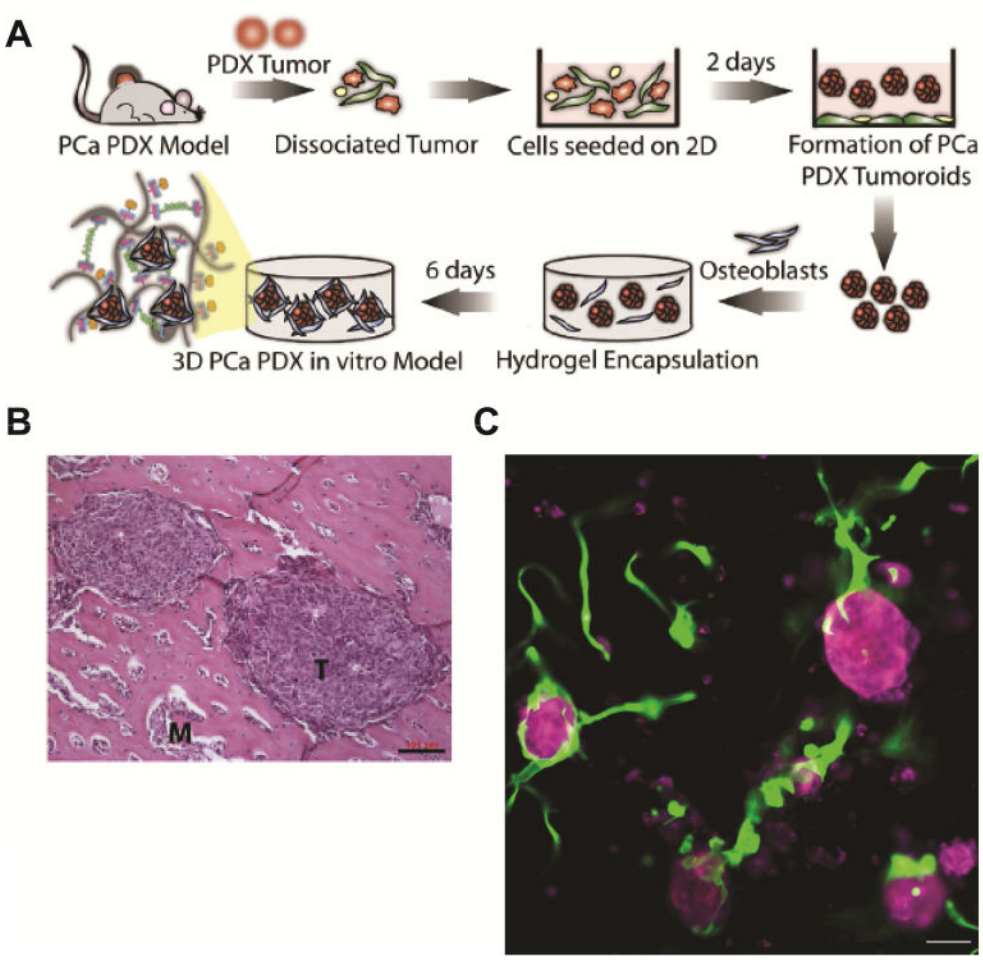

Figure 2.

Combining complexity and heterogeneity in a 3D PDX model of bone metastatic prostate cancer (PCa) [87]. (A) Schematic of experimental procedure. Bone metastatic PCa PDX cells were co-cultured with pre-osteoblasts in a 3D hyaluronan-based hydrogel matrix engineered with adhesive and protease-sensitive motifs to support cell remodeling and migration. (B) Hematoxylin-eosin-stained section of intrafemorally-grown PCa PDX cells. Shown in the image are PCa cells (T) nested within the bone matrix (M). Note that osteoblasts line the periphery of the tumor nest. Scale bar = 100 μm. (C) 3D reconstructed image of a confocal Z-stack taken of the co-cultured cells within the engineered matrix. Osteoblasts (green) were observed to ‘wrap’ around the PCa PDX cells (magenta), mimicking their interaction *in vivo*. Scale bar = 50 μm.

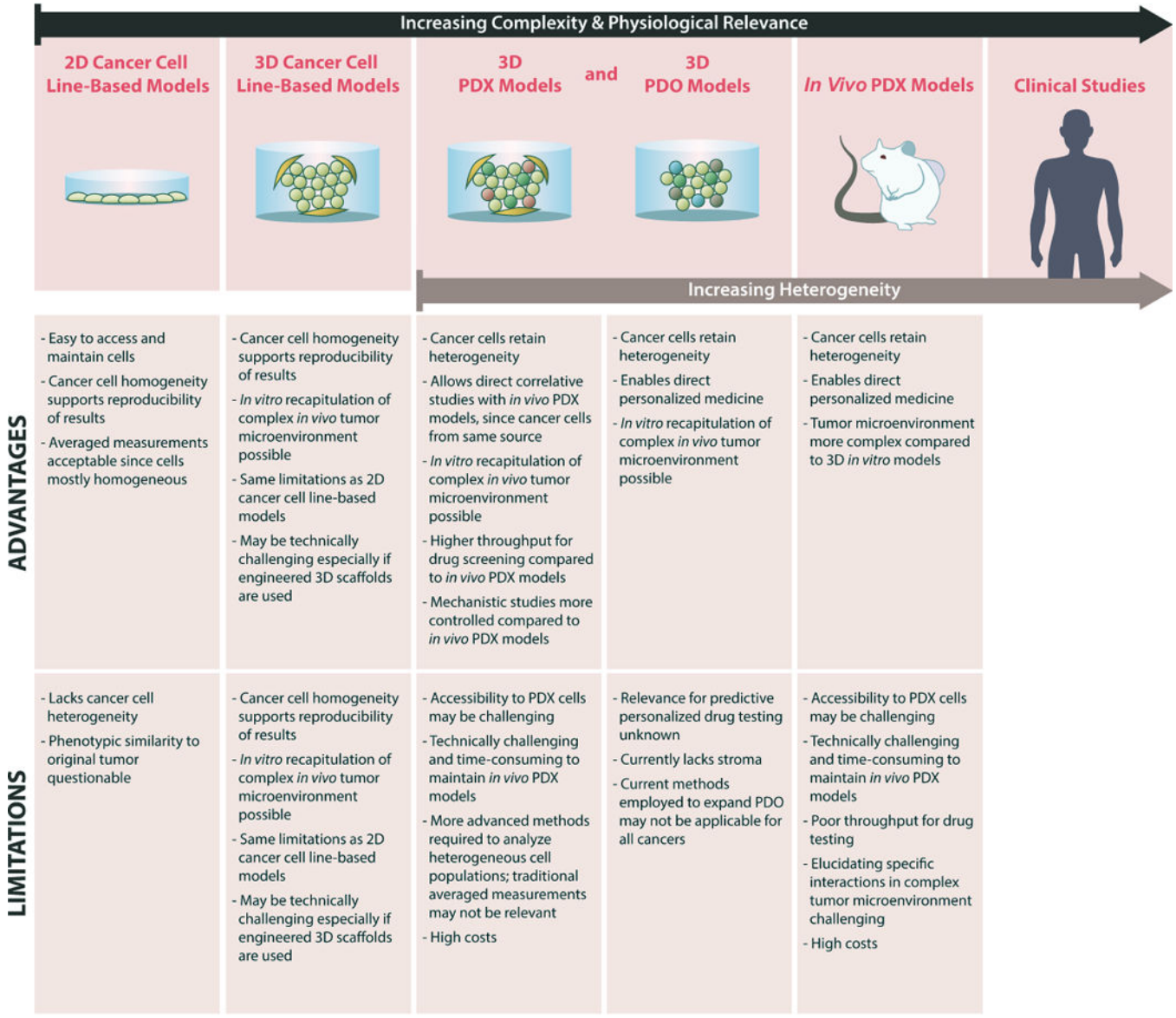

Figure 3.

Spectrum of preclinical tumor models. Table highlights the advantages and limitations associated with each model category.