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Review

A Tissue Engineering Approach to Metastatic Colon Cancer

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SUMMARY

Colon cancer remains the third most common cause of cancer in the US, and the third most common cause of cancer death. Worldwide, colon cancer is the second most common cause of cancer and cancer deaths. At least 25% of patients still present with metastatic disease, and at least 25-30% will develop metastatic colon cancer in the course of their disease. While chemotherapy and surgery remain the mainstay of treatment, understanding the fundamental cellular niche and mechanical properties that result in metastases would facilitate both prevention and cure. Advances in biomaterials, novel 3D primary human cells, modelling using microfluidics and the ability to alter the physical environment, now offers a unique opportunity to develop and test impactful treatment.

METASTATIC COLORECTAL CANCER: CLINICAL SIGNIFICANCE AND PATHOGENESIS

Colorectal cancer (CRC) continues to grow in prevalence throughout the world and now accounts for nearly one in every ten cancers worldwide, constituting the second most common malignancy worldwide and the third most common in the United States (Bray et al., 2018) (Siegel et al., 2020). For colorectal cancer, stage at diagnosis is the most important predictor of survival with 5-year relative survival rates ranging from 90% in patients with local disease (stage I, II) to 14% for those diagnosed with distant (stage IV) disease (Bray et al., 2018; Siegel et al., 2020). Despite the plethora of research and the growing number of therapeutics, more than 700,000 deaths occur from CRC annually (Lozano et al., 2012).

In 1990, Fearon and Vogelstein outlined the earliest model of colorectal tumorigenesis, the adenoma-carcinoma sequence of sporadic CRC, describing an accumulation of mutations in chromosome 5q or loss of *FAP* followed by DNA hypomethylation, *KRAS* mutation, loss of *DCC*, and loss of *TP53* (Fearon and Vogelstein, 1990) (Figure 1A). Through this sequence, normal intestinal epithelium evolves to adenoma and eventually to an invasive cancer. In the years since these initial findings, their model has been further refined with the discovery that most adenomas harbor constitutive Wnt signaling activation and accumulation of activating mutations in the EGF receptor signaling pathway and subsequent inactivating mutations in the TGF-β and *P53* pathways (Fearon, 2011). Recently, early P53 loss has been shown to promote progression of CRC (Fumagalli et al., 2017).

Despite our advancement in understanding the early stages of CRC, the medical and scientific community has been unable to decipher a genetic underpinning for the transition from localized to metastatic disease. Using an *in vivo* orthotopic organoid model utilizing CRISPR-Cas9 genome editing to generate mutations in the *Wnt, EGFR, TP53,* and *TGF-ß*, Fumagalli and colleagues determined that stem cell niche-independent growth through the accumulation of mutations in all of these pathways was necessary for tumor cells to metastasize (Fumagalli et al., 2017).

Most historical tumor models rely on the view that tumor physiology can be understood through the analysis of a single clone of cells cultured on plates or implanted *in vivo* (Ahmed et al., 2013). These reductionist models served us well in helping to understand the basic underpinnings of colorectal cancer but do not provide an accurate representation of the complex dynamics of malignancy. As a whole, they fail to recapitulate the complex tumor microenvironment composed of epithelial cells, stromal cells, immune cells, and other components of the extracellular matrix (ECM) (Egeblad et al., 2010; Holzel et al., 2013). If we

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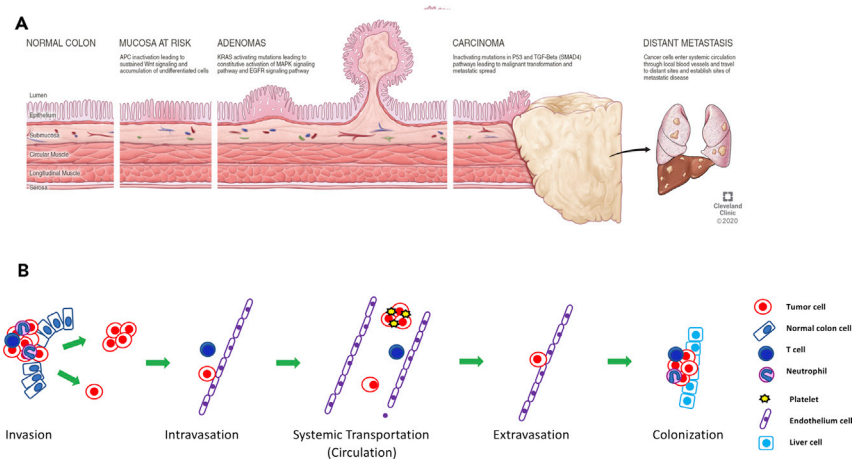

Figure 1. Colorectal Cancer Progression

(A) Normal colonic epithelium acquires an initial somatic mutation, often in the APC gene, leading to accelerated epithelial proliferation. Hyperproliferation of the epithelium leads to the formation of adenomas that eventually acquire additional mutations including those in KRAS and SMAD4. If an adenoma is not removed, it may undergo malignant transformation by acquiring further mutations, including P53 mutations, and invade through the submucosa to become an invasive cancer (fourth panel from left). Once invasion has occurred, the tumor cells have direct interaction with various elements of the microenvironment, including immune cells, blood vessels, and lymphatics. Tumor cells may then metastasize to distant sites, either hematogenously to the liver, lungs and other distant sites or via the lymphatics to local or regional lymph nodes (Right most panel). **(B)** In the metastatic process, in order to access the lymphatics, the cells must first invade through the basement membrane. The tumorigenic cells then intravasate into the blood vessel or the lymphatics, and enter the circulation. These cells then exit the circulation via extravasation, adhere, and will grow as microfoci as they colonize their new microenvironment.

are to progress in our understanding of the physiology of the complex tumor microenvironment, the field requires reductionist models that can be combined to study the interplay between various components.

METASTASIS: A COMPLICATED MULTISTEP PROCESS

Metastasis is a series of progressive steps resulting in the formation of secondary tumors in other organs (Martin et al., 2013) (Figure 1B).Primary tumors of 1 cm^3 (approximately 10^9 cells) can disperse 1 million cancer cells into the circulatory system daily with less than 0.1% of these cells developing into metastatic disease In the late nineteenth century, Stephan Paget proposed that metastatic cells of a certain type tend to distribute to themselves to specific organs (Paget, 1989). Since his initial hypothesis, the scientific community has validated these early necropsy findings and coined the term "organotropism" to define the organ-specific predisposition (Nguyen et al., 2009).. Metastatic organotropism is believed to be governed by a variety of factors including the tumor microenvironment, local cytokine and chemokine profile, tissue biomechanics, physiology, and availability of lymphatics or blood vessels (Chambers et al., 2002; Gupta and Massague, 2006; Pretzsch et al., 2019).

"Invasion" (Figure 1B), the first step in the metastatic cascade occurs via infiltration of the tumor invasive front into the local tissues. It is during this stage that the widely discussed epithelial to mesenchymal transition (EMT), which is a biological program consisting of loss of epithelial markers, increases in mesenchymal gene expression, loss of adherence junctions, and motility (Cao et al., 2015; Cheung and Ewald, 2016). Once the tumor has invaded into local tissue, they are able to gain close proximity to tissue blood vessels and lymphatic tissue (Bockhorn et al., 2007; Fares et al., 2020). For "intravasation" (Figure 1B), the transendothelial migration of cancer cells into vessels, to occur, the invasive front is drawn toward blood or lymphatic vessels via chemotactic signals at which point there is a reliance on tumor macrophages and TGF-β signaling via EMT to facilitate intravasation into vessels (Denais et al., 2016; van Zijl et al., 2011).

Once cancer cells have entered the "hematogenous or lymphatic circulation" (Figure 1B), they are generally transported in clusters supported by neutrophils. These neutrophils function to decrease the activation

of other elements of the immune system and platelet coating allowing for immune evasion and protection against physical stress (Fares et al., 2020; Gay and Felding-Habermann, 2011).

After traveling through circulation, the vessels must escape to reach a distant site through the process of "extravasation" (Figure 1B). Many studies have shown that vascular flow plays a major role in this process with the decreased flow in capillaries leading to circulating tumor cells becoming lodged and shear forces disrupting these cells leading to immune mediated extravasation (Follain et al., 2018; Goetz, 2018; Headley et al., 2016). Prior to the arrival of circulating tumor cells to distant sites, the primary tumor has already been preparing pre-metastatic niches through the secretion of exosomes and other factors, facilitating "colonization" (Figure 1B) (Hoshino et al., 2015). On arrival, tumor cells function in this niche generating favorable conditions for their outgrowth (Benedicto et al., 2017).

CURRENT APPROACHES AND CHALLENGES

Biomedical research depends on the combination of *in vitro* cell culture models and animal models to understand and predict the human response. Animal models are expensive, have ethical issues, and are poor at predicting human responses owing to cross-species discrepancies (Eastwood et al., 2010). *In vitro* culture methods are important alternatives for discerning biomolecular and disease mechanisms. The culture model choice depends upon the type of research, questions being asked, desired physiology, fidelity representation of the human condition, and expense of culture system.

IN VITRO MODELS OF THE METASTATIC PROCESS

Two-Dimensional and Three-Dimensional Cell Culture

Two-dimensional cultures are often established by growing the cells as a monolayer onto a surface (e.g., plastic, glass, membranes, or Transwells) (Figure 2A). Such 2D culture techniques are economical, convenient, and simple and provide preliminary screening, cell function, and cellular products. These 2D methods result in cultures that do not provide an adequate model of what occurs *in vivo* because they do not create complex interactions among organs and other tissues and fail to incorporate microenvironmental factors (Sensi et al., 2018). Two-dimensional cell cultures can be improved by aligning or organizing multiple cell types though differential plating, creating self-assembling monolayers, and using chemically modified surfaces resulting in more authentic phenotypes that retain functional responses of the human tissue or organs. The 2D culturing techniques can be used in a variety of assays, such as cell proliferation (kinetics of proliferation—well plates), cell clonogenicity (viabilities—well plates), wound healing (migratory capacity of cells cultured on petri dishes), permeability, migration, transendothelial migration, and invasion (Transwells) (Benedicto et al., 2017).

Some cells easily grow in suspension, whereas others can be forced to grow in suspension by using non-adherent plates producing a dispersed 3D culture (Table 1). Other organized 3D cultures can be created by plating the cells in a hydrogel (e.g., collagen gels, agar, Matrigel, PG matrix) using a hanging droplet technique to establish the cells for development into spheroids or organoids, plating cells onto scaffolds or decellularized matrices, or using tissue slices (Figure 2B) (Crapo et al., 2011; Lu et al., 2018). These tissues may self-organize (especially organoids) or the tissue construct may be "engineered" by using a scaffold to spatially organize cells in the tissue mimic. The cellular diversity of organ microenvironments has been addressed through tissue engineering; knowledge of an organ's morphology, its microbiome or both (Jalili-Firoozinezhad et al., 2019; Shin et al., 2019); and layering various cell types together by using chemical modification, differential plating, or allowing the cells to self-organize to mimic the organ's function (Jalili-Firoozinezhad et al., 2019).

The generation of epithelial organoids as a 3D culture system to quickly grow and expand the colonic epithelium obtained from individuals with colon cancer was a breakthrough in *in vitro* modeling of the colon and facilitated functional studies of human intestinal epithelial cells (VanDussen et al., 2015). However, in both health and disease, the epithelia do not act in isolation and interactively communicate with microenvironmental factors, including the stromal compartment and ECM to maintain epithelial function (D'Angelo et al., 2020a; Neal et al., 2018). In the past, the ECM had been perceived to be passive. Now, tumor microenvironment elements, including the structural proteins and mechanical properties, have been shown to be interactive and crucially impact cancer progression and treatment responses (Romero-Lopez et al., 2017). For example, Baker et al. showed matrix stiffness increases in colorectal cancer tumors owing

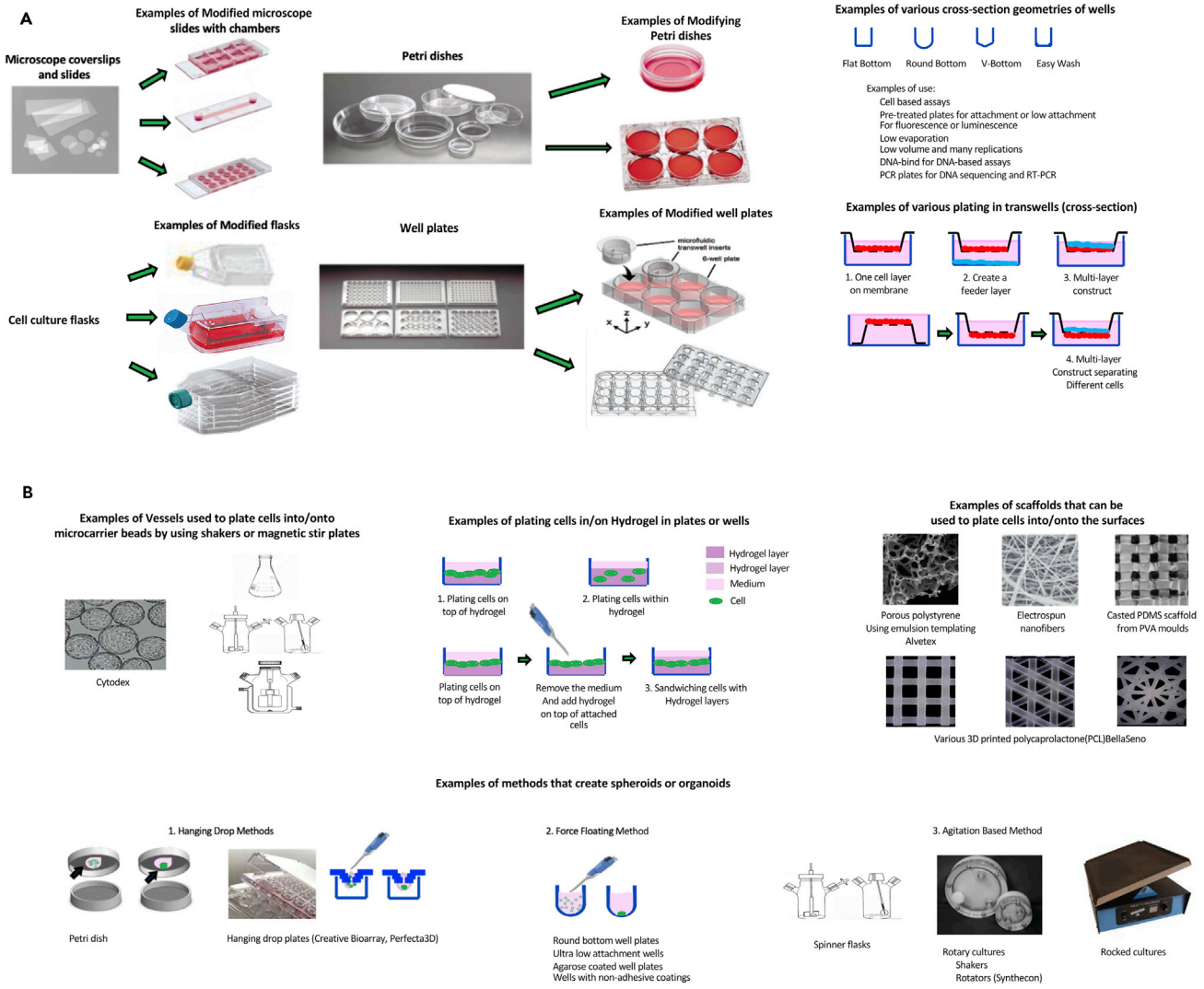

Figure 2. Propagation of 2D and 3D Cultures

(A) Multiple approaches to 2D culturing. Propagation on cells, plates, wells, and flasks are shown.

(B) Multiple approaches to 3D cultures. Alternative vessels and encapsulation into hydrogels and onto scaffolds facilitate maintenance of 3D relationships *in vitro*.

to overexpression of active LOX (Baker et al., 2013). Therefore, understanding ECM compositional changes in cancer and recapitulation of the mechanical properties of the native 3D cellular microenvironment are vital to reproduce the complexity of native tissues.

Ex Vivo Models: Biomaterials

For decades, cancer research has relied upon various *in vitro* models to investigate the mechanisms underlying development and progression of colorectal cancer. Among *in vitro* models, traditional monolayer cell cultures have been extensively used for research. Despite the advantages of these models—the cells are easily grown and maintained and biochemical assays are easily performed—traditional 2D cultures are often structurally disorganized and do not recapitulate the structural and pathophysiological complexity of the tumors. This significant inconsistency between monolayer cancer cell cultures and native tumors led to an essential need for more relevant *in vitro* tumor models to improve data for subsequent *in vivo* studies and clinical applications.

2D versus 3D	Advantages	Disadvantages
2D	• Well established • Economical, commercially available tests and media • Easy, convenient • High throughput capacity, mass screening • Repeatability and accessibility • Homogeneous growth and proliferation • Good for epithelial systems • Air-liquid interfaces • Difficult to model tumor cells • Unlimited access to oxygen, nutrients, metabolites, and signaling molecules • Changes gene expression, mRNA splicing, topology, and cell biochemistry	• Stiff substrate—plastic, glass • Lack cell-cell and cell-matrix interactions • Unable to assess tissue penetration • Unable to mimic *in vivo* microenvironment • Lack extracellular matrices • Lack natural tissue structures • Relies on adherence to flat surface • Do not control cell shape affecting bioactivity • Decrease in transcriptional genes • Morphological changes • Changes polarity • Changes in method of division
Customized 2D (e.g., microwells, micropillars, cell-adhesive islands, sandwich culture)	• Induce apical-basal polarity • Improve cell shape control • Improve morphology and function • Study uptake and efflux transport • Benefits to pharmacokinetics	• Induced polarity may alter function • Affects spreading, migrating, and sensing soluble factors and cues
3D	• Mimic the *in vivo* microenvironment • Cell-cell and cell-matrix interactions • Substrates that mimic natural extracellular matrix • Reduce overall cost and for pre-clinical *in vivo* testing • More reliable preclinical evaluation • Improve/effects protein expression, proliferation, differentiation, and metabolism • Improve architectural tissue structures • Better for studying cell migration	• More expensive (coated plates) • More complicated to establish culture models • Low throughput capacity • Overcome 2D limitations • Cell life expectancy can be short • Fewer commercially available tests

Table 1. 2D versus 3D Cultures

(Continued on next page)

2D versus 3D	Advantages	Disadvantages
	• Suspension, in a gel • Preserves morphology • Diverse phenotype and polarity • Variable access to oxygen, nutrients, metabolites, and signaling molecules • Expression of genes, splicing, topology, and cell biochemistry	

Table 1. Continued

The need for more comprehensive *in vitro* models has led to a rapid growth in disease modeling with more structured, organized, and realistic tissue and organ models. Such models use both natural and synthetic biomaterials to engineer the biological and mechanical features of the native tumors. Natural biomaterials such as type I collagen (a natural matrix), a major component of the stromal matrix in colorectal cancer (Hilska et al., 1998), have been used individually or in various combinations to provide a supportive matrix for cell-cell and cell-ECM interactions, migration, and invasion in cancer (Figure 3). As an example, co-culture of LS174T colon cancer spheroids with normal colon fibroblast cells in a 3D collagen matrix reduced the mitotic potential of LS174T cells and resulted in contraction of collagen fibers around the fibroblasts, as is observed in the human colon adenocarcinoma. On the other hand, cancer-associated fibroblasts derived from colon adenocarcinoma promoted cancer cell invasion into the collagen matrix (Dolznig et al., 2011).

However, the use of natural biomaterials in disease modeling includes some shortcomings such as inconsistent cross-linking, batch-to-batch variations, dependence on the tissue of origin, and lack of flexibility in adjustment of mechanical properties. On the other hand, synthetic biomaterials provide the biocompatibility of the natural materials along with an inert and defined structure with a wide range of mechanical properties to study the effect of each individual factor in disease progression. Chen et al. developed a thermosensitive poly (N-isopropylacrylamide)-based hydrogel as a drug delivery strategy for controlled release of doxorubicin toward CT-26 mouse colon carcinoma cells (Chen et al., 2018). In another study, a temperature-sensitive PEG-PCL-PEG triblock copolymer was synthesized to load 5-FU to improve the therapeutic effects of intraperitoneal chemotherapy (Wang et al., 2010).

Several studies have revealed that, in patient tumors, the ECM has a unique composition and in accordance with the "seed and soil hypothesis" emphasized the tumor microenvironment, including ECM composition, as a main contributor to tumor progression and drug response (Naba et al., 2014) (Table 2). Romero-López et al. highlighted the importance of recapitulating ECM composition as a vital aspect of tissue physiology by comparing ECM from normal human colon and colon tumor that had metastasized to liver; they found significant differences in protein composition, stiffness, and vascular network formation between normal and metastatic colon (Romero-Lopez et al., 2017). Recent introduction of the tissue de-cellularization technique (Ott et al., 2010) (Crapo et al., 2011), by which cells are removed while retaining the ECM structure and composition, is a substantial step forward in the field of tissue engineering and regenerative medicine. The advantage of this model compared with the other natural and synthetic 3D matrices is that the native structural proteins and soluble factors are largely preserved in the decellularized scaffold, resulting in a more comprehensive tissue recapitulation (Pinto et al., 2017). Piccoli et al. developed a technique to decellularize colorectal cancer tissue biopsies and generate a 3D "bioactive" model while maintaining the ultrastructural environment and biological activity of the native tissue (Piccoli et al., 2018). In another study, patient-derived tissue-specific decellularized ECM was introduced as a 3D culture system that recapitulated colorectal cancer liver metastasis (CRLM) to study CRLM progression and response to chemotherapy (D'Angelo et al., 2020b). Overall, the best model to recapitulate human CRC metastases remains patient-derived scaffolds. Our team has previously used decellularized human colonic tissue to model the process of intravasation and has discovered that, during intravasation, gene signatures emerge that are unique to the intravasation process (Chen et al., 2016).

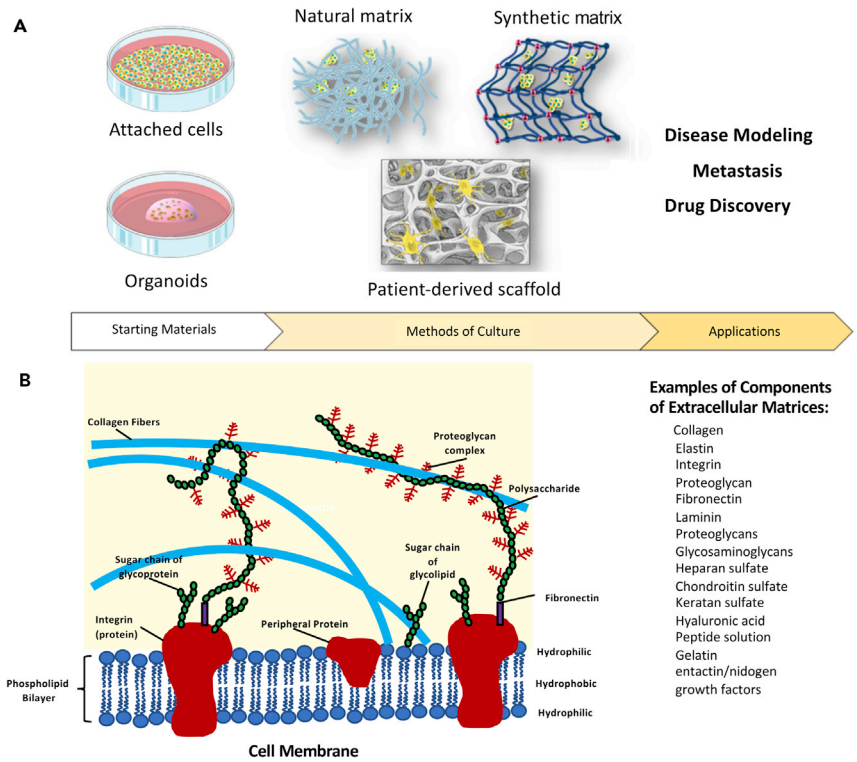

Figure 3. Schematic Representation of 3D Matrices used in Cancer Biology Research

(A) Cellular and extracellular matrices are demonstrated with ultimate applications.

(B) Cartoon of epithelial cells with surrounding cellular and extracellular matrices of the microenvironment. Components of synthetic and commercially available extracellular matrices are listed.

ADVANCES IN *IN VITRO* DEVICE TECHNIQUES

The 2D and 3D culturing techniques can be used in both single organ (Abaci et al., 2015; Esch et al., 2015; Wang et al., 2017) and multi-organ-interconnected microphysiological systems that are known as organ or body on a chip (OOC or BOC) (Abaci et al., 2015; Esch et al., 2016; Mahler et al., 2009; Maschmeyer et al., 2015; Miller and Shuler, 2016; Oleaga et al., 2016; Sin et al., 2004; Sung et al., 2010; Wagner et al., 2013; Wang et al., 2017) (Figure 4). These systems can be used to study metabolism, cell-cell and organ-organ interactions, physiological responses to chemical or biological stimuli, and drug development and toxicity, as well as for tumor metastasis. All *in vitro* 2D and 3D systems have advantages and limitations. The biological questions, desired physiology, fidelity of the representation of the human condition, and expense of the culture system are important considerations.

Advances in tissue engineering and technology have progressed so that barrier and non-barrier tissues can be incorporated into the devices (Sakolish et al., 2016; Zheng et al., 2016) (Figure 5). Many choices are available for BOC system circulation, (for example, pump devices, a pumpless device that is gravity-induced on a rocker platform (bidirectional) (Abaci et al., 2015) or without rocking (single pass), or a pumpless device on a rocker platform that allows unidirectional flow while mimicking blood recirculation (Esch et al., 2016; Wang and Shuler, 2018). Fluid shear stress and mechanical stress from the laminar flow in OOC and BOC systems provide nutrients, remove waste, promote organ polarity, physical pressure on endothelial cells, and differentiation (Kshitiz et al., 2012; Wu et al., 2020). Cell patterning on the chip is partially controlled by microfluidics, but a researcher can make surface modifications, chip templates, and 3D-printer scaffolds (e.g., hydrogels, biocompatible poly (ethylene glycol) diacrylate) to create complex channels such as vascular networks and specific microenvironments like villi (Sung et al., 2011). Methods have been developed that even combine polyvinyl acetate coating and carbon dioxide laser ablation with continuous cell seeding techniques that enable controlled epithelial-mesenchymal interactions (Li et al., 2015).

ECM versus Scaffolds	Types	Advantages	Disadvantages
ECM	• Hydrogels (e.g., Matrigel, PGmatrix) • Collagen or fibronectin • Polyacrylamide • Alginate • Soft agar	• Cell encapsulation • Tissue-like water content • Tunable biochemical and mechanical properties • Adding polyethylene glycol with photodegradable compounds to alter properties • Cells are easy to recover • Cells form tissue-like structures • Can study the aggressiveness of cells and metastatic potential	• Micron/nanometer-sized mesh too small for post-fabrication cellularization • Lack micro-topography for controlling cell shape • Gradual degradation • Time consuming • Low repeatability • Difficult to extract cells • Difficult for immunofluorescent staining • May influence structure formation
Scaffold	• Microsphere • Electrospun • Solvent-casting and particle leaching • Sphere-templated technique • Freeze drying • Gas foaming particle leaching • Solid freeform fabrication • Fiber meshes/fiber bonding • Melt molding • Solid freeform fabrication using computer-aided design (CAD), magnetic resonance imaging, computed tomography, stereolithography, fused deposition modeling, and laser sintering • Biopolymer scaffolds • Silk, collagen, laminin, alginate	• Sphere size between 1 and 1,000 μm • Applied easily with syringe • Biocompatibility, bioavailability • Porous or non-porous • Biodegradable or not • Can select polymer type • Can have ultrafine fibers • Provide cellular support • Control pore size • Porogens can help produce a uniform suspension • Can have high porosity and interconnectivity • Fine structures and complex geometries • Various sizes and/or shapes • Compatible with functional tests • Easy to prepare for immunohistochemical analysis	• Porosity control varies depending on method chosen • Leaching of particles due to method chosen • Porogens may be difficult to remove • Toxicity if organic solvents were used in production • Cells flatten and spread like 2D • Scaffold may cause various cellular behaviors • May affect adhesion and growth • Cell observation and extraction are restricted

Table 2. Strengths and Weaknesses of Extracellular Matrix versus Decellularized Scaffolds
ECM, extracellular matrix.

Improvements in monitoring cells in a 3D microfluidic setting have been made using time-lapse imaging microscopy and microsensors (Kane et al., 2019). In particular, it is possible to measure not only biomarkers, especially those in circulation but also functional responses such as electrical activity using microelectrode arrays and cellular force generation using cantilevers or by monitoring movements of labeled micro- or nanoparticles.

USE OF 3D DEVICES FOR THE STUDY OF METASTASIS

Development of *in vitro* tumor models to study the progression of cancer and metastasis depends upon knowledge of tumor microenvironment, tumor cell biology, with realistic models at the cell, tissue, and organ levels, and potential use of BOC-type devices.

Tumor cells respond to biochemical/biophysical signals from surrounding stromal cells and ECM. Stromal components (e.g., fibroblasts, endothelial cells, pericytes, various specialized cells, matrix, and extracellular proteins) help determine biochemical and biophysical properties that affect cellular growth,

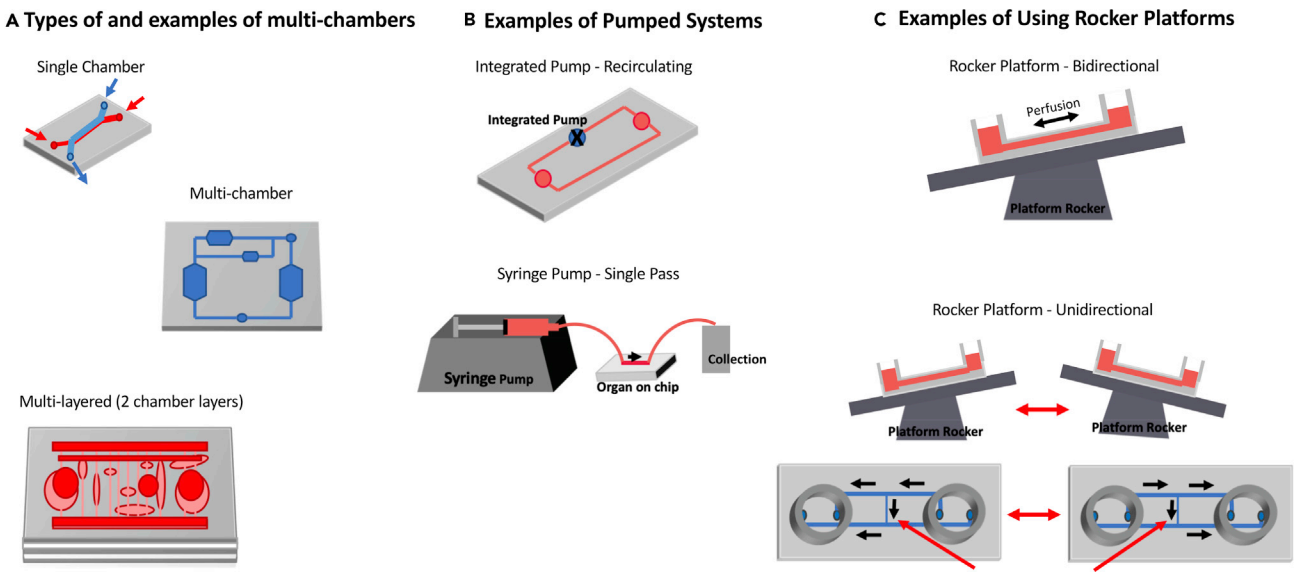

Figure 4. Microfluidic devices used to Investigate Colon Cancer Metastases (Not to Scale)

(A) Configurations of various multi-chamber devices are diagrammed.

(B) Pumped systems can be external or integrated to provide recirculating systems or create a single pass flow.

(C) Examples of microfluidic devices that use rocker platforms to provide bidirectional or unidirectional flow.

differentiation, migration, and apoptosis (Discher et al., 2005; Engler et al., 2006). Organ dysfunction can be attributed to stress conditions that signal the fibroblasts to increase plasticity and transform into myofibroblasts, remodel the ECM framework, increasing the amounts of ECM; increase inflammatory cytokines, recruit immune cells, and promote neoangiogenesis, proliferation, invasion, migration, and metastasis (Klingberg et al., 2013; Roy et al., 2020). A hypoxic tumor environment (<8–10 mm Hg) leads to a decrease in the tissue oxygen consumption rate or ATP production rate (Hockel and Vaupel, 2001). Hypoxia-inducible factors mediate adaptive responses to reduce oxygen availability and induce the expression of target genes involved in angiogenesis, cell survival, energy metabolism, differentiation, and invasion. These cellular, physiological and environmental tumor factors need to be considered when developing tumor invasion models.

In sum, numerous models mimicking tissue and organ physiology have been developed, including microfluidic and Transwell systems, organoids, spheroids, hydrogels, perfused cultures, organ- or body-on-a-chip, bioreactors (devices that produce cellular constructs at large scale), and 3D bioprinting (Krishnan et al., 2011). These complex systems provide highly interactive environments, can better mimic native tumor behaviors, can carry the tumor hypoxic or necrotic core, mimic progression, and mimic invasion (Rodrigues et al., 2018). Some of these systems can incorporate barrier and non-barrier tissues, air/liquid interfaces, vascular and/or lymphatic components, and microvascular networks. Establishment of prevascular tissues and/or seeding lymphatic endothelial cells within tissue-engineered constructs (Gibot et al., 2017) can enhance colonization and are essential for studies of normal and tumoral mechanisms in 3D systems. Generally, devices that mimic tissue/organ structure can provide a better understanding of the tumor microenvironment and cancer behavior, development, and metastasis than unstructured 2D culture systems. New improvements in technologies offer many options to enhance the capacity of 3D culture systems to better mimic metastasis.

IN VIVO MURINE MODELS

Despite advances *in vitro* modeling capabilities, studying the complex whole-body determinants of cancer metastasis still requires *in vivo* models that recapitulate the anatomical route, tumor microenvironment, or both in human patients (Figure 6). Our understanding of CRC pathogenesis has been aided by the development of genetically engineered mouse models (GEMMs), which reproduce the molecular and morphological changes that occur during the dysplastic transformation of normal colonic epithelium into carcinoma (Romano et al., 2018; Roper and Hung, 2012). Inducing metastasis in these murine models

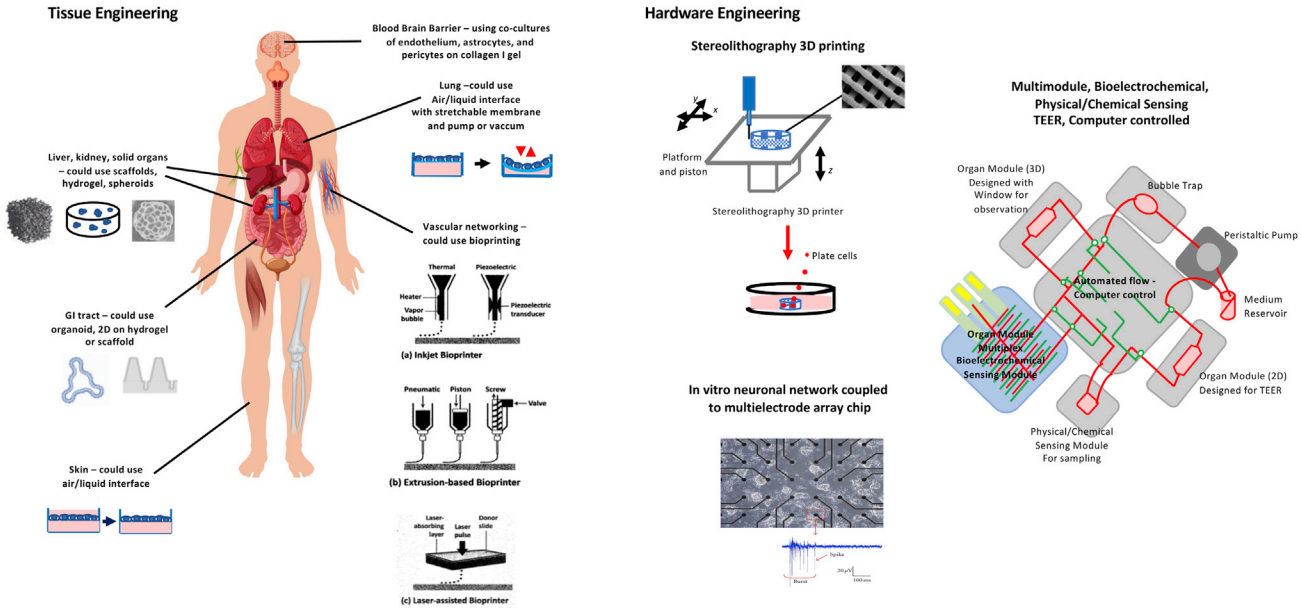

Figure 5. Advances in Microfluidic Devices Combine Current Technologies

By combining tissue engineering, hardware engineering, pharmacology, and computational modeling, one can create more advanced models that are more physiological.

has been challenging, with a few models able to reliably drive distant metastasis patterns (Burtin et al., 2020). Among these, spatial and temporal control over the expression of *Apc*, *Kras*, and *Tp53* has been used to drive primary colon tumor formation and distant metastasis, whether using 4-OH-tamoxifen-ethanol enemas in iKAP mice ($Apc^{Lox/Lox}$; $p53^{Lox/Lox}$; Tet-O-LSL-$Kras^{G12D}$; $VillinCre^{ERT2}$) or surgical adenoviral Cre administration in $Apc^{CKO/CKO}$LSL-G12D; $Kras^{tm4tyj/+}$ mice (Chanrion et al., 2014; Haigis et al., 2008; Hung et al., 2010). However, despite their usefulness in studying carcinogenesis, GEMMs tend to be costly, slow, and not necessarily representative of the diverse genetic drivers of clinical metastasis (Francia et al., 2011).

Implanting tumor cells—whether in the form of cancer cell suspensions or tumor from a patient—into mice has long been an experimental model for studying cancer biology (Bresalier et al., 1987; Fu et al., 1991). To prevent graft rejection, this approach generally requires immunodeficient mice unless murine tumor cells are injected into syngeneic hosts (e.g., CT26 cells into the BALB/c mouse). Heterotopic transplants are particularly tractable, requiring little surgical expertise as they are implanted as a tumor cell suspension injected into the mouse flank. When patient tumor cells obtained at tumor excision or biopsy are implanted into immunodeficient mice, the resulting subcutaneous tumor is called a patient-derived xenograft and has been shown to maintain the histology and genetic profile of the donor over several passages (Ben-David et al., 2017; Brown et al., 2016; Cho et al., 2014; Guenot et al., 2006). In fact, the response of the donor to chemotherapy treatment is recapitulated in the "xenopatient" mice, indicating a promising application for precision therapeutics (Gao et al., 2015; Lazzari et al., 2019; Pauli et al., 2017). However, over passages *in vivo*, the heterogeneity of the original transplants is gradually lost as the donor tissue stroma is often replaced by murine architecture and the fastest-growing clones tend to take over. These models often fail to develop metastases (Oliveira et al., 2020). The underlying biology that prevents metastasis in heterotopic transplants is likely manifold—limited vascular routes for migrating cells, a lack of colonic microenvironmental stimuli, and minimal exposure to other niche elements (Fumagalli et al., 2017). A recent study using CRC PDX samples so provided evidence for a hybrid epithelial-mesenchymal state in the CRC clusters that seeded most effectively (Mizukoshi et al., 2020). Implantation into a more highly vascularized environment, like beneath the renal capsule, has also not shown significant advantages over subcutaneous injection, suggesting vasculature is not the only factor in driving metastatic spread. In fact, CRC cells grown on decellularized liver and lung scaffolds were shown to retain their tissue-specific tropism when injected into the spleen of mice (Tian et al., 2018), whereas intrasplenic injections are otherwise known to only cause metastases to the liver (Brand et al., 1996). Contrarily, intravenous injection into the tail vein leads to the formation

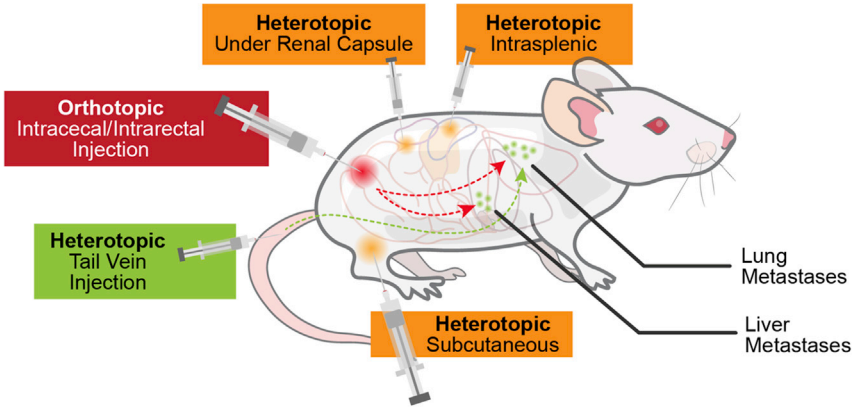

Figure 6. Murine Models of Colorectal Cancer Metastasis Rely Largely on Introduction of Cancer Cells into Circulation via Injection

Both orthotopic (red) sites and heterotopic sites (green) have been described, with different resulting patterns of metastatic spread (dotted lines).

of lung metastases, in accordance with hematogenous flow, although this approach captures only circulation, extravasation, and colonization while excluding the earlier steps occurring in primary tumors, including invasion and intravasation (Mittal et al., 2015). The predictability of these patterns indicates that these methods do not adequately capture the cellular mechanisms like matrix degradation that characterize physiological EMT.

Heterotopic tumors may be used as an intermediate *in vivo* growth platform prior to orthotopic implantation into the colon of another mouse, with the subcutaneous cell graft harvested from the flank moved to a more native tumor microenvironment. Numerous methods for orthotopic transplants have been described for inoculating tumor cells into the colon, including serosal tunneling, injection, and directly attaching the tumor pieces to the cecal wall (Lin et al., 2015; Wang et al., 2009). More recently, methods for subserosal injections into the cecal wall and intrarectal injections have been developed, both providing efficient tumor formation and metastatic behavior (Lee et al., 2014; Takahashi et al., 2004). These models have been shown to recapitulate genetic differences seen in advanced CRC in humans as well (Rajput et al., 2013; Sasaki et al., 2008). Thus, choice of orthotopic transplant method must be carefully made, particularly considering that slight variations such as injecting tumor in the mesenteric versus antimesenteric cecal wall have been shown to yield different metastatic patterns (Boni et al., 2005).

Although intracecal transplants remain a preferred model of CRC metastasis, recent evidence has suggested that intrarectal injections may provide similar metastatic capabilities, with liver and lung metastases occurring in 35%–100% of mice depending on the CRC cell line (Kishimoto et al., 2013; Moret et al., 2019; Takahashi et al., 2004). Although CRC metastasis was long thought to progress through lymph node invasion prior to liver spread, an interesting insight derived from using an intrarectal injection model showed that hepatic metastases can occur independently and in parallel with lymph node metastases (Enquist et al., 2014). Schölch et al. collected circulating tumor cells with stem cell-like features and performed a second intracecal injection, creating a model that reproducibly forms metastases in the liver and lung in a pattern recapitulating that found in humans (Kochall et al., 2017; Scholch et al., 2016). To increase the metastasis rate, many studies use CRC cell lines that are already metastatic, such as HCT116, HT29, and SW620 (Bu et al., 2018). Conversely, spontaneous models can be generated by combining GEMM with orthotopic transplantation. For example, organoids derived from the VillinCre-ER^{T2}; APC$^{fl/fl}$; KRAS$^{LSL-G12D}$; TP53$^{KO/KO}$ model, which forms spontaneous CRC tumors and reaches the endpoint of localized tumorigenic growth without metastasis, can be orthotopically transplanted again to form a single primary CRC tumor that spontaneously metastasizes to the liver and lung (Fumagalli et al., 2020).

TRACKING METASTASIS IN *IN VIVO* MODELS

Observing cellular changes in the context of a systemic and dynamic phenomenon like metastasis poses a unique challenge. In essence, most advances in this area have been derived from fluorescent labeling of tumor cells prior to either *in vitro* assays of metastatic potential like invasion assays and EMT

characterization or injection into murine models. Newer 3D culture methods, like organoid cultures and complex tumor-stroma co-culture systems, have begun to more faithfully recapitulate *in vivo* dynamics, and consequently implantation of these models into living systems has yielded metastatic features that more closely resemble those seen in patients (Devarasetty et al., 2020; Okazawa et al., 2018).

However, studying dynamic changes that occur during metastasis *in vivo* has remained challenging. Until recently, most studies tracking metastasis relied on imaging at discrete intervals, which required either sacrifice or significant disruption of the murine system at predetermined time points. These methods have generated interesting insights, like the presence of an extravascular migration pathway utilized by melanoma cells metastasizing to the brain (Bentolila et al., 2016; Kienast et al., 2010). In colon cancer models, Tanaka et al. performed intrasplenic injections of fluorescently tagged CRC cells, which were then imaged in the liver at intervals using high-resolution two photon laser scanning microscopy (Tanaka et al., 2012). Using this methodology, they were able to visualize changes in liver histology and stroma during metastatic colonization; however, this study was limited by the need to surgically exteriorize the hepatic lobes for repeated imaging.

Another popular method uses luciferase-expressing tumor cells, which emit light that can be captured by charge-coupled device cameras when luciferin is administered via intraperitoneal injection and subsequently oxidized (Thalheimer et al., 2013). However, bioluminescent imaging results are difficult to interpret because only a 10-fold change in measured intensity is possible for every added centimeter of tissue depth (Magistri et al., 2019). As such, using bioluminescent imaging in studying liver metastases, which are often deep within the peritoneal cavity, is often limited in its ability to generate new insights. Thus, beyond *in vitro* modeling systems like Transwell migration assays and tracing of characterized EMT markers, the dynamic cellular changes associated with metastasis remain largely unexplored in living experimental models.

However, intravital imaging in the recent years has provided a glimpse into the morphological and cellular changes involved in the metastatic process as it occurs *in vivo*. The development of the abdominal intravital window was a key advance in allowing visualization of peritoneal organs including the colon, liver, pancreas, and spleen for up to a month (Alieva et al., 2014; Ritsma et al., 2013). In a seminal application of this technology, Ritsma et al. imaged the metastasis of xenografted CT26 CRC cells using an abdominal intravital window in place for about 2 weeks (Ritsma et al., 2012). By visualizing the fluorescently tagged tumor cells as they metastasized to the liver, the authors showed that the seeding cells underwent clonal selection and distinct morphological changes from a "pre-micrometastatic" to a "micrometastatic" phenotype over the course of days.

Subsequent studies using similar intravital imaging methods have provided insights into the mechanistic basis underlying tumor cell invasion, generating novel hypotheses to be interrogated in future studies of CRC metastasis. In other cancer subtypes, intravital imaging has revealed novel interplay between components of the immune system and metastatic processes (Harney et al., 2015; Headley et al., 2016; Park et al., 2016). In CRC, intravital imaging of Lgr5 plasticity, a marker of CRC cancer stem cells, revealed that Lgr5-negative cells serve as the seeds of metastatic colonization before re-establishing the cellular hierarchy in the metastases (Fumagalli et al., 2020)

Although invasive intravital imaging is not feasible clinically, newer emerging technologies like microPET and SPECT have advanced our ability to image metastasis at the cellular resolution in humans as well. We expect that, as these technologies advance, we will be able to achieve resolution similar to that of the cellular imaging methods discussed here and validate that the findings in CRC murine models translate to patients with CRC.

TRANSLATIONAL APPLICATIONS

As our models of metastatic CRC expand, groups have begun to utilize these techniques to determine their feasibility to predict and model patient-specific treatment responses. In the recently published translational study, Vlachogiannis and colleagues generated a large biobank of patient-derived epithelial organoids from metastatic gastrointestinal cancers and observed that they recapitulate the chemotherapeutic response seen in the clinical setting (Vlachogiannis et al., 2018). Using *in vitro* drug screening of 16 unique colorectal primary and corresponding metastatic lesions, their group showed 100% sensitivity and 93%

specificity between *in vitro* organoid response and patient clinical response. In an effort to overcome the lack of surrounding tissue structure in the organoid model, Sensi and colleagues recellularized tumor and normal colon from resected surgical specimens and seeded these with cell lines to assess the variation in response to chemotherapeutic agents (Sensi et al., 2020). To model chemotherapeutic responses for metastatic disease, one group combined several cell types that are normally found in the lung including NL20 epithelial cells, fibroblasts, lymphatic and blood vessel endothelial cells to generate a "primitive lung in a dish." Primary colon cancer cells were then introduced into the dish leading to areas of metastatic colon carcinoma that modeled the chemotherapeutic response to that of the primary patient *in vitro* (Ramamoorthy et al., 2019). Similar to this model, another group developed a model of hepatic metastasis utilizing human hepatocytes, mesenchymal cells, and HCT116 cells in simulated microgravity rotating wall vessel bioreactors that allowed for assessment of dose-dependent cytotoxicity to 5-FU (Devarasetty et al., 2017; Skardal et al., 2015).

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AUTHOR CONTRIBUTIONS

Conceptualization, E.H.H. and M.L.S.; Writing – Original Draft, R.K.D., S.K.S., P.G.M., and S.B.; Writing – Review and Editing, M.L.S. and E.H.H.; Funding Acquisition, M.L.S., X.S., and E.H.H.; Supervision, M.L.S., X.S., and E.H.H.

DECLARATION OF INTERESTS

M.L.S. is CEO/President of Hesperos, Inc and has several patents related to "body-on-a-chip" technology: US Pat 7,288,045; 8748180 B2; 9,273,276 B2 and one application pending. X.S. is the co-founder and CEO of Xilis, Inc. The topics covered in this paper have no overlap with Xilis' operation or financial interest.

REFERENCES

Abaci, H.E., Gledhill, K., Guo, Z., Christiano, A.M., and Shuler, M.L. (2015). Pumpless microfluidic platform for drug testing on human skin equivalents. Lab Chip *15*, 882–888.

Ahmed, D., Eide, P.W., Eilertsen, I.A., Danielsen, S.A., Eknaes, M., Hektoen, M., Lind, G.E., and Lothe, R.A. (2013). Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis *2*, e71.

Alieva, M., Ritsma, L., Giedt, R.J., Weissleder, R., and van Rheenen, J. (2014). Imaging windows for long-term intravital imaging: general overview and technical insights. Intravital *3*, e29917.

Baker, A.M., Bird, D., Lang, G., Cox, T.R., and Erler, J.T. (2013). Lysyl oxidase enzymatic function increases stiffness to drive colorectal cancer progression through FAK. Oncogene *32*, 1863–1868.

Ben-David, U., Ha, G., Tseng, Y.Y., Greenwald, N.F., Oh, C., Shih, J., McFarland, J.M., Wong, B., Boehm, J.S., Beroukhim, R., et al. (2017). Patient-derived xenografts undergo mouse-specific tumor evolution. Nat. Genet. *49*, 1567–1575.

Benedicto, A., Marquez, J., Herrero, A., Olaso, E., Kolaczkowska, E., and Arteta, B. (2017). Decreased expression of the beta2 integrin on tumor cells is associated with a reduction in liver metastasis of colorectal cancer in mice. BMC Cancer *17*, 827.

Bentolila, L.A., Prakash, R., Mihic-Probst, D., Wadehra, M., Kleinman, H.K., Carmichael, T.S., Peault, B., Barnhill, R.L., and Lugassy, C. (2016). Imaging Angiotropism/vascular Co-option A Murine Model Brain Melanoma Implications Melanoma Progression Along Extravascular Pathways. Sci. Rep. *6*, 23834.

Bockhorn, M., Jain, R.K., and Munn, L.L. (2007). Active versus passive mechanisms in metastasis: do cancer cells crawl into vessels, or are they pushed? Lancet Oncol. *8*, 444–448.

Boni, L., Benevento, A., Dionigi, G., Rovera, F., Diurni, M., and Dionigi, R. (2005). Injection of colorectal cancer cells in mesenteric and antimesenteric sides of the colon results in different patterns of metastatic diffusion: an experimental study in rats. World J. Surg. Oncol. *3*, 69.

Brand, M.I., Casillas, S., Dietz, D.W., Milsom, J.W., and Vladisavljevic, A. (1996). Development of a reliable colorectal cancer liver metastasis model. J. Surg. Res. *63*, 425–432.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. *68*, 394–424.

Bresalier, R.S., Raper, S.E., Hujanen, E.S., and Kim, Y.S. (1987). A new animal model for human colon cancer metastasis. Int. J. Cancer *39*, 625–630.

Brown, K.M., Xue, A., Mittal, A., Samra, J.S., Smith, R., and Hugh, T.J. (2016). Patient-derived xenograft models of colorectal cancer in pre-clinical research: a systematic review. Oncotarget *7*, 66212–66225.

Bu, P., Chen, K.Y., Xiang, K., Johnson, C., Crown, S.B., Rakhilin, N., Ai, Y., Wang, L., Xi, R., Astapova, I., et al. (2018). Aldolase B-mediated fructose metabolism drives metabolic reprogramming of colon cancer liver metastasis. Cell Metab. *27*, 1249–1262 e1244.

Burtin, F., Mullins, C.S., and Linnebacher, M. (2020). Mouse models of colorectal cancer: past, present and future perspectives. World J. Gastroenterol *26*, 1394–1426.

Cao, H., Xu, E., Liu, H., Wan, L., and Lai, M. (2015). Epithelial-mesenchymal transition in colorectal cancer metastasis: a system review. Pathol. Res. Pract. *211*, 557–569.

Chambers, A.F., Groom, A.C., and MacDonald, I.C. (2002). Dissemination and growth of cancer cells in metastatic sites. Nat. Rev. Cancer *2*, 563–572.

Chanrion, M., Kuperstein, I., Barriere, C., El Marjou, F., Cohen, D., Vignjevic, D., Stimmer, L., Paul-Gilloteaux, P., Bieche, I., Tavares Sdos, R., et al. (2014). Concomitant Notch activation and

p53 deletion trigger epithelial-to-mesenchymal transition and metastasis in mouse gut. Nat. Commun. *5*, 5005.

Chen, C.H., Kuo, C.Y., Chen, S.H., Mao, S.H., Chang, C.Y., Shalumon, K.T., and Chen, J.P. (2018). Thermosensitive injectable hydrogel for simultaneous intraperitoneal delivery of doxorubicin and prevention of peritoneal adhesion. Int. J. Mol. Sci. *19*, 1373.

Chen, H.J., Wei, Z., Sun, J., Bhattacharya, A., Savage, D.J., Serda, R., Mackeyev, Y., Curley, S.A., Bu, P., Wang, L., et al. (2016). A recellularized human colon model identifies cancer driver genes. Nat. Biotechnol *34*, 845–851.

Cheung, K.J., and Ewald, A.J. (2016). A collective route to metastasis: seeding by tumor cell clusters. Science *352*, 167–169.

Cho, Y.B., Hong, H.K., Choi, Y.L., Oh, E., Joo, K.M., Jin, J., Nam, D.H., Ko, Y.H., and Lee, W.Y. (2014). Colorectal cancer patient-derived xenografted tumors maintain characteristic features of the original tumors. J. Surg. Res. *187*, 502–509.

Crapo, P.M., Gilbert, T.W., and Badylak, S.F. (2011). An overview of tissue and whole organ decellularization processes. Biomaterials *32*, 3233–3243.

D'Angelo, E., Lindoso, R.S., Sensi, F., Pucciarelli, S., Bussolati, B., Agostini, M., and Collino, F. (2020a). Intrinsic and extrinsic modulators of the epithelial to mesenchymal transition: driving the fate of tumor microenvironment. Front. Oncol *10*, 1122.

D'Angelo, E., Natarajan, D., Sensi, F., Ajayi, O., Fassan, M., Mammano, E., Pilati, P., Pavan, P., Bresolin, S., Preziosi, M., et al. (2020b). Patient-derived scaffolds of colorectal cancer metastases as an organotypic 3D model of the liver metastatic microenvironment. Cancers (Basel) *12*, 364.

Denais, C.M., Gilbert, R.M., Isermann, P., McGregor, A.L., Lindert, M., Weigelin, B., Davidson, P.M., Friedl, P., Wolf, K., and Lammerding, J. (2016). Nuclear envelope rupture and repair during cancer cell migration. Science *352*, 353–358.

Devarasetty, M., Dominijanni, A., Herberg, S., Shelkey, E., Skardal, A., and Soker, S. (2020). Simulating the human colorectal cancer microenvironment in 3D tumor-stroma co-cultures in vitro and in vivo. Sci. Rep. *10*, 9832.

Devarasetty, M., Wang, E., Soker, S., and Skardal, A. (2017). Mesenchymal stem cells support growth and organization of host-liver colorectal-tumor organoids and possibly resistance to chemotherapy. Biofabrication *9*, 021002.

Discher, D.E., Janmey, P., and Wang, Y.L. (2005). Tissue cells feel and respond to the stiffness of their substrate. Science *310*, 1139–1143.

Dolznig, H., Rupp, C., Puri, C., Haslinger, C., Schweifer, N., Wieser, E., Kerjaschki, D., and Garin-Chesa, P. (2011). Modeling colon adenocarcinomas in vitro a 3D co-culture system induces cancer-relevant pathways upon tumor cell and stromal fibroblast interaction. Am. J. Pathol. *179*, 487–501.

Eastwood, D., Findlay, L., Poole, S., Bird, C., Wadhwa, M., Moore, M., Burns, C., Thorpe, R., and Stebbings, R. (2010). Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4+ effector memory T-cells. Br. J. Pharmacol. *161*, 512–526.

Egeblad, M., Nakasone, E.S., and Werb, Z. (2010). Tumors as organs: complex tissues that interface with the entire organism. Dev. Cell *18*, 884–901.

Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. (2006). Matrix elasticity directs stem cell lineage specification. Cell *126*, 677–689.

Enquist, I.B., Good, Z., Jubb, A.M., Fuh, G., Wang, X., Junttila, M.R., Jackson, E.L., and Leong, K.G. (2014). Lymph node-independent liver metastasis in a model of metastatic colorectal cancer. Nat. Commun. *5*, 3530.

Esch, M.B., Prot, J.M., Wang, Y.I., Miller, P., Llamas-Vidales, J.R., Naughton, B.A., Applegate, D.R., and Shuler, M.L. (2015). Multi-cellular 3D human primary liver cell culture elevates metabolic activity under fluidic flow. Lab Chip *15*, 2269–2277.

Esch, M.B., Ueno, H., Applegate, D.R., and Shuler, M.L. (2016). Modular, pumpless body-on-a-chip platform for the co-culture of GI tract epithelium and 3D primary liver tissue. Lab Chip *16*, 2719–2729.

Fares, J., Fares, M.Y., Khachfe, H.H., Salhab, H.A., and Fares, Y. (2020). Molecular principles of metastasis: a hallmark of cancer revisited. Signal. Transduct. Target. Ther. *5*, 28.

Fearon, E.R. (2011). Molecular genetics of colorectal cancer. Annu. Rev. Pathol. *6*, 479–507.

Fearon, E.R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell *61*, 759–767.

Follain, G., Osmani, N., Azevedo, A.S., Allio, G., Mercier, L., Karreman, M.A., Solecki, G., Garcia Leon, M.J., Lefebvre, O., Fekonja, N., et al. (2018). Hemodynamic forces tune the arrest, adhesion, and extravasation of circulating tumor cells. Dev. Cell *45*, 33–52 e12.

Francia, G., Cruz-Munoz, W., Man, S., Xu, P., and Kerbel, R.S. (2011). Mouse models of advanced spontaneous metastasis for experimental therapeutics. Nat. Rev. Cancer *11*, 135–141.

Fu, X.Y., Besterman, J.M., Monosov, A., and Hoffman, R.M. (1991). Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically intact patient specimens. Proc. Natl. Acad. Sci. U S A *88*, 9345–9349.

Fumagalli, A., Drost, J., Suijkerbuijk, S.J., van Boxtel, R., de Ligt, J., Offerhaus, G.J., Begthel, H., Beerling, E., Tan, E.H., Sansom, O.J., et al. (2017). Genetic dissection of colorectal cancer progression by orthotopic transplantation of engineered cancer organoids. Proc. Natl. Acad. Sci. U S A *114*, E2357–E2364.

Fumagalli, A., Oost, K.C., Kester, L., Morgner, J., Bornes, L., Bruens, L., Spaargaren, L., Azkanaz, M., Schelfhorst, T., Beerling, E., et al. (2020). Plasticity of Lgr5-negative cancer cells drives metastasis in colorectal cancer. Cell Stem Cell *26*, 569–578 e567.

Gao, H., Korn, J.M., Ferretti, S., Monahan, J.E., Wang, Y., Singh, M., Zhang, C., Schnell, C., Yang, G., Zhang, Y., et al. (2015). High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. Nat. Med. *21*, 1318–1325.

Gay, L.J., and Felding-Habermann, B. (2011). Contribution of platelets to tumour metastasis. Nat. Rev. Cancer *11*, 123–134.

Gibot, L., Galbraith, T., Bourland, J., Rogic, A., Skobe, M., and Auger, F.A. (2017). Tissue-engineered 3D human lymphatic microvascular network for in vitro studies of lymphangiogenesis. Nat. Protoc. *12*, 1077–1088.

Goetz, J.G. (2018). Metastases go with the flow. Science *362*, 999–1000.

Guenot, D., Guerin, E., Aguillon-Romain, S., Pencreach, E., Schneider, A., Neuville, A., Chenard, M.P., Duluc, I., Du Manoir, S., Brigand, C., et al. (2006). Primary tumour genetic alterations and intra-tumoral heterogeneity are maintained in xenografts of human colon cancers showing chromosome instability. J. Pathol. *208*, 643–652.

Gupta, G.P., and Massague, J. (2006). Cancer metastasis: building a framework. Cell *127*, 679–695.

Haigis, K.M., Kendall, K.R., Wang, Y., Cheung, A., Haigis, M.C., Glickman, J.N., Niwa-Kawakita, M., Sweet-Cordero, A., Sebolt-Leopold, J., Shannon, K.M., et al. (2008). Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. Nat. Genet. *40*, 600–608.

Harney, A.S., Arwert, E.N., Entenberg, D., Wang, Y., Guo, P., Qian, B.Z., Oktay, M.H., Pollard, J.W., Jones, J.G., and Condeelis, J.S. (2015). Real-time imaging reveals local, transient vascular permeability, and tumor cell intravasation stimulated by TIE2hi macrophage-derived VEGFA. Cancer Discov. *5*, 932–943.

Headley, M.B., Bins, A., Nip, A., Roberts, E.W., Looney, M.R., Gerard, A., and Krummel, M.F. (2016). Visualization of immediate immune responses to pioneer metastatic cells in the lung. Nature *531*, 513–517.

Hilska, M., Collan, Y., Peltonen, J., Gullichsen, R., Paajanen, H., and Laato, M. (1998). The distribution of collagen types I, III, and IV in normal and malignant colorectal mucosa. Eur. J. Surg. *164*, 457–464.

Hockel, M., and Vaupel, P. (2001). Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J. Natl. Cancer Inst. *93*, 266–276.

Holzel, M., Bovier, A., and Tuting, T. (2013). Plasticity of tumour and immune cells: a source of heterogeneity and a cause for therapy resistance? Nat. Rev. Cancer *13*, 365–376.

Hoshino, A., Costa-Silva, B., Shen, T.L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., Molina, H., Kohsaka, S., Di Giannatale, A., Ceder, S., et al. (2015). Tumour exosome integrins determine organotropic metastasis. Nature *527*, 329–335.

Hung, K.E., Maricevich, M.A., Richard, L.G., Chen, W.Y., Richardson, M.P., Kunin, A., Bronson, R.T., Mahmood, U., and Kucherlapati, R. (2010). Development of a mouse model for sporadic and metastatic colon tumors and its use in assessing drug treatment. Proc. Natl. Acad. Sci. U S A *107*, 1565–1570.

Jalili-Firoozinezhad, S., Gazzaniga, F.S., Calamari, E.L., Camacho, D.M., Fadel, C.W., Bein, A., Swenor, B., Nestor, B., Cronce, M.J., Tovaglieri, A., et al. (2019). A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip. Nat. Biomed. Eng. *3*, 520–531.

Kane, K.I.W., Moreno, E.L., Hachi, S., Walter, M., Jarazo, J., Oliveira, M.A.P., Hankemeier, T., Vulto, P., Schwamborn, J.C., Thoma, M., et al. (2019). Automated microfluidic cell culture of stem cell derived dopaminergic neurons. Sci. Rep. *9*, 1796.

Kienast, Y., von Baumgarten, L., Fuhrmann, M., Klinkert, W.E., Goldbrunner, R., Herms, J., and Winkler, F. (2010). Real-time imaging reveals the single steps of brain metastasis formation. Nat. Med. *16*, 116–122.

Kishimoto, H., Momiyama, M., Aki, R., Kimura, H., Suetsugu, A., Bouvet, M., Fujiwara, T., and Hoffman, R.M. (2013). Development of a clinically-precise mouse model of rectal cancer. PLoS One *8*, e79453.

Klingberg, F., Hinz, B., and White, E.S. (2013). The myofibroblast matrix: implications for tissue repair and fibrosis. J. Pathol. *229*, 298–309.

Kochall, S., Thepkaysone, M.L., Garcia, S.A., Betzler, A.M., Weitz, J., Reissfelder, C., and Scholch, S. (2017). Isolation of circulating tumor cells in an orthotopic mouse model of colorectal cancer. J Vis Exp *125*, e55357.

Krishnan, V., Shuman, L.A., Sosnoski, D.M., Dhurjati, R., Vogler, E.A., and Mastro, A.M. (2011). Dynamic interaction between breast cancer cells and osteoblastic tissue: comparison of two- and three-dimensional cultures. J. Cell. Physiol *226*, 2150–2158.

Kshitiz, P.J., Kim, P., Helen, W., Engler, A.J., Levchenko, A., and Kim, D.H. (2012). Control of stem cell fate and function by engineering physical microenvironments. Integr. Biol. (Camb.) *4*, 1008–1018.

Lazzari, L., Corti, G., Picco, G., Isella, C., Montone, M., Arcella, P., Durinikova, E., Zanella, E.R., Novara, L., Barbosa, F., et al. (2019). Patient-derived xenografts and matched cell lines identify pharmacogenomic vulnerabilities in colorectal cancer. Clin. Cancer Res. *25*, 6243–6259.

Lee, W.Y., Hong, H.K., Ham, S.K., Kim, C.I., and Cho, Y.B. (2014). Comparison of colorectal cancer in differentially established liver metastasis models. Anticancer Res. *34*, 3321–3328.

Li, Y.C., Lin, M.W., Yen, M.H., Fan, S.M., Wu, J.T., Young, T.H., Cheng, J.Y., and Lin, S.J. (2015). Programmable laser-assisted surface microfabrication on a poly(vinyl alcohol)-coated glass chip with self-changing cell adhesivity for heterotypic cell patterning. ACS Appl. Mater. Inter. *7*, 22322–22332.

Lin, W., Zhuang, Q., Zheng, L., Cao, Z., Shen, A., Li, Q., Fu, C., Feng, J., and Peng, J. (2015). Pien Tze Huang inhibits liver metastasis by targeting TGF-beta signaling in an orthotopic model of colorectal cancer. Oncol. Rep. *33*, 1922–1928.

Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., Abraham, J., Adair, T., Aggarwal, R., Ahn, S.Y., et al. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet *380*, 2095–2128.

Lu, S., Cuzzucoli, F., Jiang, J., Liang, L.G., Wang, Y., Kong, M., Zhao, X., Cui, W., Li, J., and Wang, S. (2018). Development of a biomimetic liver tumor-on-a-chip model based on decellularized liver matrix for toxicity testing. Lab Chip *18*, 3379–3392.

Magistri, P., Battistelli, C., Toietta, G., Strippoli, R., Sagnotta, A., Forgione, A., Di Benedetto, F., Uccini, S., Vittorioso, P., D'Angelo, F., et al. (2019). In vivo bioluminescence-based monitoring of liver metastases from colorectal cancer: an experimental model. J. Microsc. Ultrastruct. *7*, 136–140.

Mahler, G.J., Esch, M.B., Glahn, R.P., and Shuler, M.L. (2009). Characterization of a gastrointestinal tract microscale cell culture analog used to predict drug toxicity. Biotechnol. Bioeng. *104*, 193–205.

Martin, T.A., Ye, L., Sanders, A.J., Lane, J., and Jiang, W.G. (2013). Cancer invasion and metastasis: molecular and cellular perspective. In Metastatic Cancer: Clinical and Biological Perspectives, R. Jandia, ed. (Austin (TX): Landes Bioscience), pp. 135–168.

Maschmeyer, I., Lorenz, A.K., Schimek, K., Hasenberg, T., Ramme, A.P., Hubner, J., Lindner, M., Drewell, C., Bauer, S., Thomas, A., et al. (2015). A four-organ-chip for interconnected long-term co-culture of human intestine, liver, skin and kidney equivalents. Lab Chip *15*, 2688–2699.

Miller, P.G., and Shuler, M.L. (2016). Design and demonstration of a pumpless 14 compartment microphysiological system. Biotechnol. Bioeng. *113*, 2213–2227.

Mittal, V.K., Bhullar, J.S., and Jayant, K. (2015). Animal models of human colorectal cancer: current status, uses and limitations. World J. Gastroenterol *21*, 11854–11861.

Mizukoshi, K., Okazawa, Y., Haeno, H., Koyama, Y., Sulidan, K., Komiyama, H., Saeki, H., Ohtsuji, N., Ito, Y., Kojima, Y., et al. (2020). Metastatic seeding of human colon cancer cell clusters expressing the hybrid epithelial/mesenchymal state. Int. J. Cancer *146*, 2547–2562.

Moret, R., Hellmers, L., Zhang, X., Gills, J., Hite, N., Klinger, A., Maresh, G.A., Canter, D., Bardot, S., Margolin, D.A., et al. (2019). Patient-derived Orthotopic Xenograft Models for Human Urothelial Cell Carcinoma and Colorectal Cancer Tumor Growth and Spontaneous Metastasis. J. Vis. Exp. *147*, e59223.

Naba, A., Clauser, K.R., Whittaker, C.A., Carr, S.A., Tanabe, K.K., and Hynes, R.O. (2014). Extracellular matrix signatures of human primary metastatic colon cancers and their metastases to liver. BMC Cancer *14*, 518.

Neal, J.T., Li, X., Zhu, J., Giangarra, V., Grzeskowiak, C.L., Ju, J., Liu, I.H., Chiou, S.H., Salahudeen, A.A., Smith, A.R., et al. (2018). Organoid modeling of the tumor immune microenvironment. Cell *175*, 1972–1988 e1916.

Nguyen, D.X., Bos, P.D., and Massague, J. (2009). Metastasis: from dissemination to organ-specific colonization. Nat. Rev. Cancer *9*, 274–284.

Okazawa, Y., Mizukoshi, K., Koyama, Y., Okubo, S., Komiyama, H., Kojima, Y., Goto, M., Habu, S., Hino, O., Sakamoto, K., et al. (2018). High-sensitivity Detection of Micrometastases Generated by GFP Lentivirus-transduced Organoids Cultured from a Patient-derived Colon Tumor. J. Vis. Exp. *136*, e57374.

Oleaga, C., Bernabini, C., Smith, A.S., Srinivasan, B., Jackson, M., McLamb, W., Platt, V., Bridges, R., Cai, Y., Santhanam, N., et al. (2016). Multi-Organ toxicity demonstration in a functional human in vitro system composed of four organs. Sci. Rep. *6*, 20030.

Oliveira, R.C., Abrantes, A.M., Tralhao, J.G., and Botelho, M.F. (2020). The role of mouse models in colorectal cancer research-The need and the importance of the orthotopic models. Anim. Model. Exp. Med. *3*, 1–8.

Ott, H.C., Clippinger, B., Conrad, C., Schuetz, C., Pomerantseva, I., Ikonomou, L., Kotton, D., and Vacanti, J.P. (2010). Regeneration and orthotopic transplantation of a bioartificial lung. Nat. Med. *16*, 927–933.

Paget, S. (1989). The distribution of secondary growths in cancer of the breast. 1889. Cancer Metastasis Rev. *8*, 98–101.

Park, J., Wysocki, R.W., Amoozgar, Z., Maiorino, L., Fein, M.R., Jorns, J., Schott, A.F., Kinugasa-Katayama, Y., Lee, Y., Won, N.H., et al. (2016). Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. Sci. Transl. Med. *8*, 361ra138.

Pauli, C., Hopkins, B.D., Prandi, D., Shaw, R., Fedrizzi, T., Sboner, A., Sailer, V., Augello, M., Puca, L., Rosati, R., et al. (2017). Personalized in vitro and in vivo cancer models to guide precision medicine. Cancer Discov. *7*, 462–477.

Piccoli, M., D'Angelo, E., Crotti, S., Sensi, F., Urbani, L., Maghin, E., Burns, A., De Coppi, P., Fassan, M., Rugge, M., et al. (2018). Decellularized colorectal cancer matrix as bioactive microenvironment for in vitro 3D cancer research. J. Cell. Physiol. *233*, 5937–5948.

Pinto, M.L., Rios, E., Silva, A.C., Neves, S.C., Caires, H.R., Pinto, A.T., Duraes, C., Carvalho, F.A., Cardoso, A.P., Santos, N.C., et al. (2017). Decellularized human colorectal cancer matrices polarize macrophages towards an anti-inflammatory phenotype promoting cancer cell invasion via CCL18. Biomaterials *124*, 211–224.

Pretzsch, E., Bosch, F., Neumann, J., Ganschow, P., Bazhin, A., Guba, M., Werner, J., and Angele, M. (2019). Mechanisms of metastasis in colorectal cancer and metastatic organotropism: hematogenous versus peritoneal spread. J. Oncol. *2019*, 7407190.

Rajput, A., Agarwal, E., Leiphrakpam, P., Brattain, M.G., and Chowdhury, S. (2013). Establishment and validation of an orthotopic metastatic mouse

model of colorectal cancer. ISRN Hepatol. *2013*, 206875.

Ramamoorthy, P., Thomas, S.M., Kaushik, G., Subramaniam, D., Chastain, K.M., Dhar, A., Tawfik, O., Kasi, A., Sun, W., Ramalingam, S., et al. (2019). Metastatic tumor-in-a-dish, a novel multicellular organoid to study lung colonization and predict therapeutic response. Cancer Res. *79*, 1681–1695.

Ritsma, L., Steller, E.J., Beerling, E., Loomans, C.J., Zomer, A., Gerlach, C., Vrisekoop, N., Seinstra, D., van Gurp, L., Schafer, R., et al. (2012). Intravital microscopy through an abdominal imaging window reveals a pre-micrometastasis stage during liver metastasis. Sci. Transl. Med. *4*, 158ra145.

Ritsma, L., Steller, E.J., Ellenbroek, S.I., Kranenburg, O., Borel Rinkes, I.H., and van Rheenen, J. (2013). Surgical implantation of an abdominal imaging window for intravital microscopy. Nat. Protoc. *8*, 583–594.

Rodrigues, T., Kundu, B., Silva-Correia, J., Kundu, S.C., Oliveira, J.M., Reis, R.L., and Correlo, V.M. (2018). Emerging tumor spheroids technologies for 3D in vitro cancer modeling. Pharmacol. Ther. *184*, 201–211.

Romano, G., Chagani, S., and Kwong, L.N. (2018). The path to metastatic mouse models of colorectal cancer. Oncogene *37*, 2481–2489.

Romero-Lopez, M., Trinh, A.L., Sobrino, A., Hatch, M.M., Keating, M.T., Fimbres, C., Lewis, D.E., Gershon, P.D., Botvinick, E.L., Digman, M., et al. (2017). Recapitulating the human tumor microenvironment: colon tumor-derived extracellular matrix promotes angiogenesis and tumor cell growth. Biomaterials *116*, 118–129.

Roper, J., and Hung, K.E. (2012). Priceless GEMMs: genetically engineered mouse models for colorectal cancer drug development. Trends Pharmacol. Sci. *33*, 449–455.

Roy, V., Magne, B., Vaillancourt-Audet, M., Blais, M., Chabaud, S., Grammond, E., Piquet, L., Fradette, J., Laverdiere, I., Moulin, V.J., et al. (2020). Human organ-specific 3D cancer models produced by the stromal self-assembly method of tissue engineering for the study of solid tumors. Biomed. Res. Int. *2020*, 6051210.

Sakolish, C.M., Esch, M.B., Hickman, J.J., Shuler, M.L., and Mahler, G.J. (2016). Modeling barrier tissues in vitro: methods, achievements, and challenges. EBioMedicine *5*, 30–39.

Sasaki, H., Miura, K., Horii, A., Kaneko, N., Fujibuchi, W., Kiseleva, L., Gu, Z., Murata, Y., Karasawa, H., Mizoi, T., et al. (2008). Orthotopic implantation mouse model and cDNA microarray analysis indicates several genes potentially involved in lymph node metastasis of colorectal cancer. Cancer Sci. *99*, 711–719.

Scholch, S., Garcia, S.A., Iwata, N., Niemietz, T., Betzler, A.M., Nanduri, L.K., Bork, U., Kahlert, C., Thepkaysone, M.L., Swiersy, A., et al. (2016). Circulating tumor cells exhibit stem cell characteristics in an orthotopic mouse model of colorectal cancer. Oncotarget *7*, 27232–27242.

Sensi, F., D'Angelo, E., D'Aronco, S., Molinaro, R., and Agostini, M. (2018). Preclinical three-dimensional colorectal cancer model: the next generation of in vitro drug efficacy evaluation. J. Cell. Physiol *234*, 181–191.

Sensi, F., D'Angelo, E., Piccoli, M., Pavan, P., Mastrotto, F., Caliceti, P., Biccari, A., Corallo, D., Urbani, L., Fassan, M., et al. (2020). Recellularized colorectal cancer patient-derived scaffolds as in vitro pre-clinical 3D model for drug screening. Cancers (Basel) *12*, 681.

Shin, W., Hinojosa, C.D., Ingber, D.E., and Kim, H.J. (2019). Human intestinal morphogenesis controlled by transepithelial morphogen gradient and flow-dependent physical cues in a microengineered gut-on-a-chip. iScience *15*, 391–406.

Siegel, R.L., Miller, K.D., Goding Sauer, A., Fedewa, S.A., Butterly, L.F., Anderson, J.C., Cercek, A., Smith, R.A., and Jemal, A. (2020). Colorectal cancer statistics, 2020. CA Cancer J. Clin *70*, 145–164.

Sin, A., Chin, K.C., Jamil, M.F., Kostov, Y., Rao, G., and Shuler, M.L. (2004). The design and fabrication of three-chamber microscale cell culture analog devices with integrated dissolved oxygen sensors. Biotechnol. Prog *20*, 338–345.

Skardal, A., Devarasetty, M., Rodman, C., Atala, A., and Soker, S. (2015). Liver-tumor hybrid organoids for modeling tumor growth and drug response in. Vitro. Ann. Biomed. Eng. *43*, 2361–2373.

Sung, J.H., Kam, C., and Shuler, M.L. (2010). A microfluidic device for a pharmacokinetic-pharmacodynamic (PK-PD) model on a chip. Lab Chip *10*, 446–455.

Sung, J.H., Yu, J., Luo, D., Shuler, M.L., and March, J.C. (2011). Microscale 3-D hydrogel scaffold for biomimetic gastrointestinal (GI) tract model. Lab Chip *11*, 389–392.

Takahashi, T., Morotomi, M., and Nomoto, K. (2004). A novel mouse model of rectal cancer established by orthotopic implantation of colon cancer cells. Cancer Sci. *95*, 514–519.

Tanaka, K., Morimoto, Y., Toiyama, Y., Okugawa, Y., Inoue, Y., Uchida, K., Kimura, K., Mizoguchi, A., and Kusunoki, M. (2012). Intravital dual-colored visualization of colorectal liver metastasis in living mice using two photon laser scanning microscopy. Microsc. Res. Tech. *75*, 307–315.

Thalheimer, A., Korb, D., Bonicke, L., Wiegering, A., Muhling, B., Schneider, M., Koch, S., Riedel, S.S., Germer, C.T., Beilhack, A., et al. (2013). Noninvasive visualization of tumor growth in a human colorectal liver metastases xenograft model using bioluminescence in vivo imaging. J. Surg. Res. *185*, 143–151.

Tian, X., Werner, M.E., Roche, K.C., Hanson, A.D., Foote, H.P., Yu, S.K., Warner, S.B., Copp, J.A., Lara, H., Wauthier, E.L., et al. (2018). Organ-specific metastases obtained by culturing colorectal cancer cells on tissue-specific decellularized scaffolds. Nat. Biomed. Eng. *2*, 443–452.

van Zijl, F., Krupitza, G., and Mikulits, W. (2011). Initial steps of metastasis: cell invasion and endothelial transmigration. Mutat. Res. *728*, 23–34.

VanDussen, K.L., Marinshaw, J.M., Shaikh, N., Miyoshi, H., Moon, C., Tarr, P.I., Ciorba, M.A., and Stappenbeck, T.S. (2015). Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays. Gut *64*, 911–920.

Vlachogiannis, G., Hedayat, S., Vatsiou, A., Jamin, Y., Fernández-Mateos, J., Khan, K., Lampis, A., Eason, K., Huntingford, I., Burke, R., et al. (2018). Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. Science *359*, 920–926.

Wagner, I., Materne, E.M., Brincker, S., Sussbier, U., Fradrich, C., Busek, M., Sonntag, F., Sakharov, D.A., Trushkin, E.V., Tonevitsky, A.G., et al. (2013). A dynamic multi-organ-chip for long-term cultivation and substance testing proven by 3D human liver and skin tissue co-culture. Lab Chip *13*, 3538–3547.

Wang, J., Rajput, A., Kan, J.L., Rose, R., Liu, X.Q., Kuropatwinski, K., Hauser, J., Beko, A., Dominquez, I., Sharratt, E.A., et al. (2009). Knockdown of Ron kinase inhibits mutant phosphatidylinositol 3-kinase and reduces metastasis in human colon carcinoma. J. Biol. Chem. *284*, 10912–10922.

Wang, Y., Gong, C., Yang, L., Wu, Q., Shi, S., Shi, H., Qian, Z., and Wei, Y. (2010). 5-FU-hydrogel inhibits colorectal peritoneal carcinomatosis and tumor growth in mice. BMC Cancer *10*, 402.

Wang, Y.I., Abaci, H.E., and Shuler, M.L. (2017). Microfluidic blood-brain barrier model provides in vivo-like barrier properties for drug permeability screening. Biotechnol. Bioeng. *114*, 184–194.

Wang, Y.I., and Shuler, M.L. (2018). UniChip enables long-term recirculating unidirectional perfusion with gravity-driven flow for microphysiological systems. Lab Chip *18*, 2563–2574.

Wu, Q., Liu, J., Wang, X., Feng, L., Wu, J., Zhu, X., Wen, W., and Gong, X. (2020). Organ-on-a-chip: recent breakthroughs and future prospects. Biomed. Eng. Online *19*, 9.

Zheng, F., Fu, F., Cheng, Y., Wang, C., Zhao, Y., and Gu, Z. (2016). Organ-on-a-Chip systems: microengineering to biomimic living systems. Small *12*, 2253–2282.