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Designer Biomaterials to Model Cancer Cell Invasion In Vitro: Predictive Tools or Just Pretty Pictures?

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Metastasis is the leading cause of mortality in cancer patients. Underlying this process is the invasion and colonization of cancer cells into healthy tissues. Engineered hydrogel models of tumor microenvironments present an opportunity to understand the microenvironmental determinants of cellular invasion. The biochemical and mechanical cues, presented in the form of adhesion sites, degradable cues, matrix stiffness, and architecture, have significant effects on the extent of cancer cell migration, and the mechanisms employed by these cells to move through their matrix. Coculture with stromal cells such as cancer associated fibroblasts, endothelial cells, and immune cells that are associated with poor prognosis demonstrate that these cells exacerbate cancer cell invasion. With these models, researchers aim not only to recapitulate known cancer cell behaviors in a dish, but also to uncover new insights into mechanisms underlying these phenomena, paving the way for novel treatment strategies. In this perspective, the design of engineered models that are used to study cancer cell invasion and metastasis in vitro is discussed. To this end, the authors seek to understand and put into perspective: do these models reveal relevant mechanisms of cancer cell migration, or are they simply pretty pictures with little biological translatability?

1. Introduction

1.1. Cancer Cell Invasion Is a Hallmark of Disease Progression

Cancer cell infiltration into healthy tissue marks the transformation of a locally growing tumor into a systemic and life-threatening disease.[1–3] Following local invasion, the process of tumor metastasis can begin, where cancer cells spread from the primary tumor and disseminate into distant tissues through lymphatic and vascular networks. Advancements in early cancer detection and treatment have rendered many solid tumors manageable and even curable. However, once cancers metastasize beyond the primary site, they are often fatal; metastatic disease is responsible for ≈90% of cancer patient deaths.[1] Moreover, the signaling cascades involved in cancer invasion are poorly understood, and subsequently, therapies targeting cancer invasion and metastases have been met with limited success.[2]

Cellular invasion is a complex process orchestrated by reciprocal signaling between cancer cells and their physical, biochemical, and cellular microenvironment. The tumor extracellular matrix (ECM) is a key regulator of cancer invasion. Changes to the extracellular matrix are a prognostic marker of tumor progression as malignant cells produce and remodel their ECM to facilitate their colonization of healthy adjacent tissues.[3] Moreover, crosstalk with stromal cells is also a key regulator of cancer cells. The presence of cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), the emergence of new blood vessels, and ECM remodeling are all associated with cell invasion and metastasis.[3] For decades, researchers have been modeling cancer cell migration to better understand mechanisms of metastasis to treat disease; however, recapitulation of the tumor niche is a complex, iterative process that requires strategic design to capture relevant biomolecular interactions and cell dynamics. Complex hydrogel models still aim to mimic only a simplified tumor microenvironment, yet, despite their simplicity, these models can elucidate relevant mechanisms of cancer cell invasion (**Figure 1**).

1.2. Experimental Models of Cancer Invasion

The biological complexity involved in tumor metastasis necessitates the use of multicomponent experimental systems for studying this process. The simplest and highest-throughput methods of studying cell invasion and migration utilize wound-healing assays and transwell assays.[4] While these methods are simple and thus easily scalable, they lack 3D extracellular matrix

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cues and thus are limited to studying cell-autonomous mechanisms of motility, restricting mechanistic studies of tumor invasion that rely on microenvironmental interactions. Nonetheless, the scalability of these types of experiments has made them popular for use in drug and genetic screens, and has been comprehensively reviewed elsewhere.[5] Due to the biological complexity involved in metastasis, in vivo models remain the gold standard. For this reason, several tumor transplantation and genetically engineered mouse models of tumor metastasis have been developed.[6,7] However, in vivo methodologies are limited in that they recapitulate the mouse microenvironment rather than the human, often require immunocompromised hosts (for transplantation models), require extensive breeding programs (for genetically engineered models), and are accompanied by ethical concerns. Thus, in vivo metastasis models are accompanied by significant time and cost implications. Notably, no single metastasis model is sufficient, and the proper selection of the optimal model is necessary for each biological question.

As cellular migration and invasion processes are associated with cell–ECM dynamic interactions, researchers seeking to study tumor cell invasion in vitro have incorporated ECM components into in vitro assays to create a more biomimetic environment. The simplest of these systems involves tissue culture polystyrene or transwell membranes with ECM components. In order to model 3D tumor cell migration, many have used naturally derived hydrogel scaffolds such as collagen or Matrigel. Collagen is highly expressed in many tumor tissues, and its fibrillar structure is thought to provide architectural cues for cell migration.[8] Matrigel, a laminin-rich material derived from the Engelbreth-Holm-Swarm mouse sarcoma, is another popular hydrogel. While these materials offer the advantages of supporting cancer cell growth and migration, they lack structural stability, tunability, and reproducibility, due to their xenogeneic sources. To address these limitations, a wide range of chemically defined hydrogels have been designed with features to allow for precise control of the cell's physicochemical environment. Using these sophisticated engineered hydrogel scaffolds, discrete aspects of the cell's microenvironment can be recapitulated to understand and decouple their effects on cell behavior.

Hydrogel-based engineered platforms have the potential to bridge the gap between current in vitro and in vivo methods of studying cellular invasion by mimicking aspects of the complex tumor stroma, while bringing the advantages of tunability, scalability, and reproducibility of in vitro assays. This perspective discusses the bioengineering strategies employed to mimic these tumor-microenvironment interactions in the context of cancer invasion.

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2. Invasion Is Directed by the Physicochemical Environment

The migration process of a single cell within its microenvironment can be thought of as a series of cellular processes.[9] The cell must remodel the surrounding matrix in order to move into it. The leading edge of the cell will extend protrusions into the matrix and attach to adjacent ECM through cell-surface receptors. This leads to the propagation of intracellular signals resulting in cytoskeletal contractions, which propels the cell forward with consequent detachment of its trailing edge. Accordingly, in order to model the process of cellular invasion in vitro, hydrogel platforms must incorporate the biochemical cues to enable this cascade to occur. Toward this end, biomaterial platforms have been formulated with adhesive sites, degradable components, and chemoattractants, to mimic the dynamic remodeling, attachment, and detachment required for cell motility in vivo.

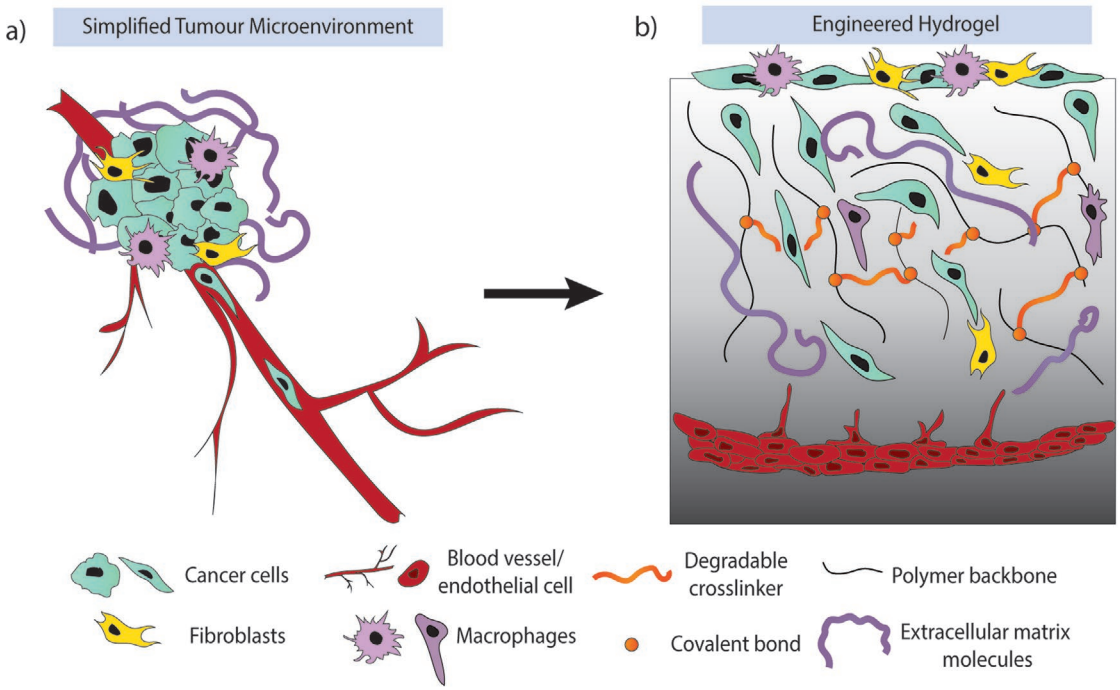

Figure 1. a,b) Hydrogel models of cancer cell invasion aim to recapitulate determinants of invasive behavior in vivo.

There are several molecular interactions that mediate cell–matrix attachment, with the most common being the interactions between the surrounding matrix with cell surface integrins.[9] Integrins are heterodimeric transmembrane receptors that bind to proteins on adjacent cells or in the ECM including fibronectin, laminin, and collagens. Integrins are composed of alpha and beta subunits, where the combination of these subunits dictates the specificity for its ligand. The arginine-glycine-glutamic acid, or RGD, motif is a common integrin-binding amino acid sequence present in ECM proteins including fibronectin, vitronectin, fibrinogen, osteopontin, and bone sialoprotein, in addition to some laminins and collagens.[10,11] Activation of these cell surface integrins via matrix interaction can lead to downstream activation of proliferation and invasion pathways, for instance by regulating proteolytic matrix degradation.[9] Incorporation of the RGD peptide sequence into biomaterials has been effective in directing cell attachment and enabling the remodeling of many different biomaterials.[12,13]

Matrix remodeling often occurs through proteolytic degradation of the ECM. The matrix metalloprotease (MMP) group of enzymes has been most frequently associated with the context of tumor invasion and metastasis. MMPs play a role in modulating tumor cell motility, invadopoedia or protrusion formation, the activation of downstream proinvasive pathways, as well as the epithelial to mesenchymal transition (EMT).[14] As such, this group of enzymes is an important biomarker of cancer progression: substantial evidence links MMP expression with poor patient prognosis.[15] The substrates of MMPs have been well characterized,[16] providing a blueprint for engineering matrices capable of cellular remodeling.

2.1. Matrix Adhesion and Degradation Facilitate Cell Invasion in Engineered Hydrogels

Poly(ethylene glycol) (PEG) is a bioinert hydrophilic polymer commonly used in hydrogel fabrication. PEG-based hydrogels provide a blank slate with which to test the influence of biomolecules on cell behavior. In their seminal work, Lutolf et al. demonstrated that fully synthetic hydrogels could facilitate fibroblast cell invasion by the incorporation of cell-responsive MMP-degradable peptide crosslinkers.[17] They found that the rate of fibroblast invasion into the hydrogel correlated to the rate of proteolytic degradation of the incorporated peptide substrate. They also immobilized RGD sequences into the hydrogel to facilitate cell attachment. Notably, invasion was influenced by RGD concentration in a bimodal fashion, with invasion being reduced in substrates with either very low or very high concentrations of RGD (**Figure 2**). A median concentration of RGD was necessary for cellular invasion, even in the most MMP-degradable substrates, illustrating the interplay between cell adhesion and matrix degradation required for cell invasion.[9] Importantly, this work showed that cell invasion into a completely artificial matrix is possible with the incorporation of bioactive peptide sequences.

Although this research focused on engineering cell–biomaterial interactions for regenerative medicine applications,[18–20] similar principles were later adapted for applications in studying cancer cell migration. Schwartz et al. used a similar PEG-based hydrogel incorporating an MMP-degradable crosslinker and an RGD adhesive peptide to study proteolytically driven mechanisms of HT-1080 fibrosarcoma cell invasion.[21] This study similarly reported that a median concentration of RGD

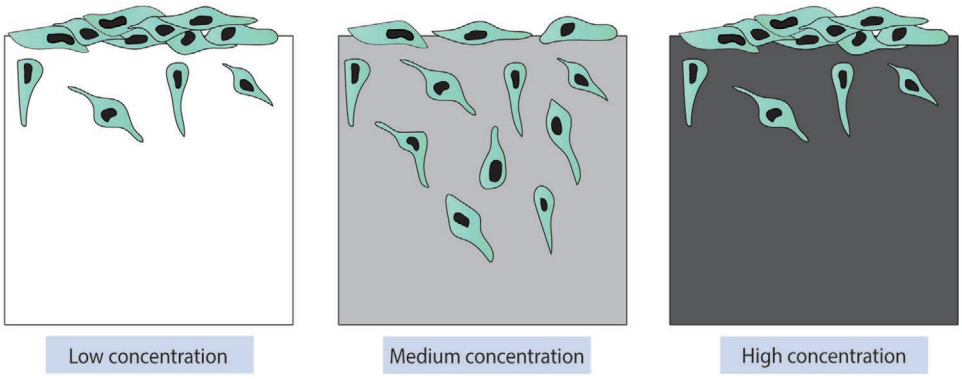

Figure 2. Effects of adhesive peptide ligand immobilization on cell invasion. A median concentration of immobilized adhesive ligand is associated with maximum cell invasion.[17]

resulted in maximal cell migration, though the dependence was less striking than with the original fibroblast study. The fibrosarcoma cells invaded with a rounded ameboid morphology, in a highly Rho Kinase (ROCK)-dependent manner with only weak integrin-dependence.

While PEG-based hydrogels can provide a blank slate for functionalization with biologically responsive elements, another approach to hydrogel engineering utilizes a bioactive polymer as the backbone. The glycosaminoglycan hyaluronan (HA) is a popular choice for this due to its prevalence in the tumor microenvironment as well as its ease of chemical functionalization. HA is a polymer of disaccharides comprising of D-glucuronic acid and *N*-acetyl-D-glucosamine with the D-glucuronic acid sugar moiety providing a carboxylic acid handle for facile chemical modification of the HA into a multifunctionalized polymer backbone for hydrogel fabrication. HA is prevalent in the ECM throughout the body and is overexpressed in the tumor microenvironment of many cancers.[22,23] HA is known to activate intracellular signaling pathways via cell surface receptors CD44 and the receptor for hyaluronan-mediated motility (RHAMM), which are both known to drive invasion.[24] In breast cancer, HA accumulation has been correlated with enhanced cancer cell invasiveness and poor patient outcomes,[25] thereby making it a promising substrate to mimic the tumor microenvironment. Fisher et al. used an HA-based hydrogel with MMP-degradable and RGD adhesive sequences in their work studying breast cancer invasion.[26] Interestingly, in this case, changing the RGD concentration in the gel had no effect on cell invasion. Instead, increased RGD correlated to increased cell numbers, suggesting that integrin engagement may activate proliferation in this system. In this study, MDA-MB-231 breast cancer cells invaded the hydrogel when crosslinked with an MMP-degradable sequence, but not a non-degradable sequence, demonstrating active cellular remodeling. Moreover, addition of the pan-MMP inhibitor GM6001 reduced cell invasion into the hydrogel, though not completely, suggesting that the cells were invading into the hydrogel by a combination of protease dependent and independent mechanisms.

HA-containing hydrogels have been used for in vitro studies of glioblastoma (GBM) invasion due to the prevalence of HA in the brain tumor microenvironment.[24,27,28] Accordingly, Cha et al. reported that the addition of HA to a collagen network resulted in increased invasion of patient-derived glioblastoma cells.[29] This was consistent with a previous report which showed that HA addition into a gelatin matrix increased the expression of malignancy-associated genes including MMP2, fibronectin, VEGF, and HIF-1.[30] In contrast, others reported a decrease in invasion of glioblastoma cells with the addition of HA to collagen matrices.[31–33] Chen et al. studied the effects of matrix bound HA on GBM migration.[33–35] Interestingly, these studies found that the molecular weight of HA greatly influenced the migration of glioblastoma cells, with low molecular weight HA being proinvasive and high molecular weight HA reducing cell invasion. This result highlights the importance of HA-mediated signaling in GBM invasiveness, and provides insight into how HA may influence cell invasion.

Notably, in addition to cell interactions with ECM scaffolds directing cell behavior, the ECM also plays a role in creating a diffusive barrier for growth factors and chemoattractants. One of the most common cancer-associated growth factors is epidermal growth factor (EGF). EGF and its receptors are commonly overexpressed in many solid tumors where it is associated with increased proliferation and tumor metastasis.[36] Using a hydrogel in a microfluidic system, Truong et al. demonstrated significantly increased breast cancer cell migration speed and persistence in response to a transient EGF gradient.[37] In order to study the effects of spatially defined EGF gradients on breast cancer invasion, Fisher et al. used two-photon confocal microscopy to photopattern discrete EGF gradients within an HA-based hydrogel and found increased invasion of MDA-MB-231 cells in the presence of EGF gradients (**Figure 3**).[38] In order to study the effects of spatiotemporally controlled EGF presentation, Meng et al. utilized stimuli-responsive EGF microcapsules, 3D-printed into lung cancer A549 tumor cell embedded fibrin hydrogels, noting increased cellular migration toward higher EGF concentrations.[39] These studies highlight the importance of the chemotactic cues in cellular microenvironments as drivers of cellular invasion.

2.2. Mechanical Cues from the Microenvironment Influences Cellular Invasion

In addition to providing biochemical signals, the extracellular matrix also presents itself as a physical barrier through which

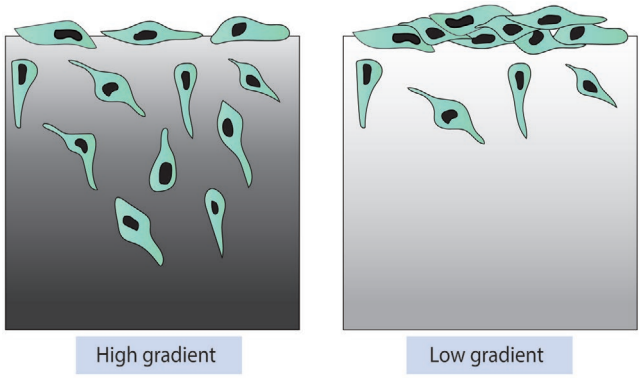

Figure 3. Effect of growth factor gradients on cancer cell migration. Cancer cells migrate toward regions of higher growth factor concentration.[38]

cancer cells must migrate for tumor expansion and invasion. During the progression of many solid tumors, the tumor ECM becomes stiffer as a result of increased deposition of ECM components.[40] An increase in matrix stiffness corresponds to a denser, more crosslinked barrier through which cancer cells must invade, requiring greater protease secretion and cellular traction forces to maneuver through this dense matrix. Notably, when cancer cells are seeded on top of stiffer matrices, greater motility, spreading, and cell proliferation have been reported.[41,42] However, cancer cells embedded within a stiff matrix have been shown to invade less than those in softer substrates,[26,41] reflecting how the ECM can provide a physical barrier to inhibit invasion (**Figure 4**). Peela et al. reported a micropatterned breast tumor model which enabled precise localization of a tumor spheroid within a stiff matrix and adjacent to a soft matrix.[43] Here, MDA-MB-231 cells from the stiff matrix invaded into the softer surrounding matrix, recapitulating the migration of tumor cells from a stiff tumor microenvironment to softer healthy tissue.

Notably, cancer cells have been reported to adapt invasion mechanisms based on mechanosensing. For example, U87 glioblastoma cells cultured on stiffer substrates were shown

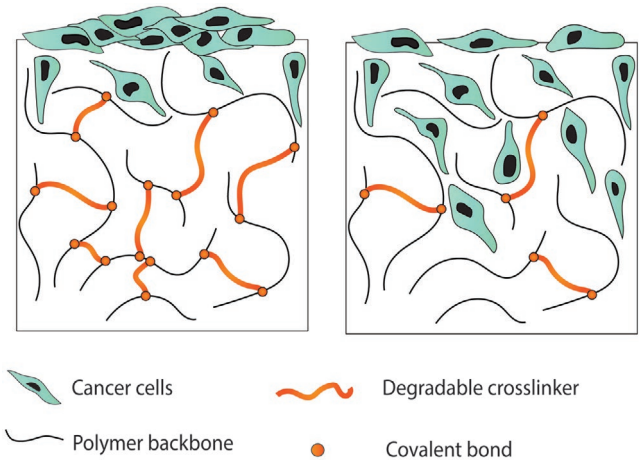

Figure 4. Effects of crosslink density on cancer cell migration. Stiffer, highly crosslinked matrices are less permissible to cellular invasion.[26]

to increase HA synthetase 1 (HAS-1) and MMP1 expression and reduce HAS-2 and MMP9 expression, suggesting a change in matrix remodeling strategy based on substrate stiffness.[31] MMP9 was shown to be upregulated in softer hydrogels. This protease cleaves type IV collagen, which is abundant in GBM tissue, suggesting enhanced matrix remodeling in softer matrices and consequently facilitated cell proliferation and tumor expansion. In their work, Aung et al. demonstrated that MDA-MB-231 breast cancer cells switch to protease-independent invasion mechanisms in Matrigel matrices of low mechanical resistances.[44]

Tumor cells can navigate through their ECM to create channels through which other cells can follow. As such, researchers have sought to uncover how confined ECM topography can influence cell invasion. Yang et al. showed that matrix porosity might be a greater determinant of glioblastoma invasion than stiffness; collagen matrices of equivalent collagen concentration and bulk stiffness were prepared with different pore sizes by temperature dependent gelation and showed that cells invaded into matrices with larger pore sizes.[45] Fisher et al. showed that in collagen gels, leading HT1080 fibrosarcoma cells create single cells invasion tunnels via MT1-MMP mediated proteolysis for other cells to follow.[46]

It is important to note the use of collagen as the base material in these examples, likely due to its self-assembled fibrillar nanostructure. Sophisticated engineering techniques have been used to systematically introduce nanofibrillar structures often present in the tumor ECM. In order to decouple matrix confinement and stiffness, Pathak and Kumar used microfabricated channels of defined wall stiffness and geometry to study glioblastoma cell migration in an inert polyacrylamide hydrogel.[47] They found that for a given ECM stiffness, cells confined to narrower channels migrate faster than cells in wider channels, or unconfined cells, attributing these effects to increased polarization of cellular traction forces in confined environments. Another strategy for fabricating complex yet precisely controlled microenvironmental mechanics is two-photon lithography. In their work, Lemma et al. utilized this technique to create stiffness gradients in 3D scaffolds, showing increased rates of cell invasion in softer architectures.[48]

These findings demonstrate the advantage of engineered cellular microenvironments in which matrix mechanical properties can be individually tuned to understand how cellular mechanosensing drives invasion behaviors.

3. Coculture Effects on Cell Movement through Hydrogels

Although monoculture invasion models enable mechanistic studies of migration modes and the role of ECM properties, tumors are heterogeneous and complex cell–cell interactions are known to promote tumorigenesis and enable metastasis.[49] Coculture disease models using engineered hydrogels have begun to emerge as a means of studying advanced biological interactions between cell types that are associated with cancer progression. The majority of these models focus on the coculture of cancer cells with CAFs, TAMs, and human vascular

endothelial cells. Hydrogels represent simplified extracellular matrices of cancer microenvironments and enable more representative studies of cell migration compared to 2D culture or transwell assays. Coculture adds complexity to these studies by revealing how other cell types found in the ECM can influence the extent and mode of cell migration—experiments which are difficult to execute and quantify in vivo.

3.1. Effects of CAFs on Cancer Cell Migration in Engineered Hydrogels

CAFs are activated fibroblasts, meaning that they exhibit a myofibroblast phenotype with high contractility.[50] Present in the tumor microenvironment and rich in vimentin and α-smooth muscle actin (αSMA), CAFs support tumor growth, EMT, and invasion through MMP secretion.[50–52] CAFs are associated with worse patient prognosis and are promising drug targets; however, it is difficult to find efficacious drugs using basic monoculture in vitro models, which exclude tumor-CAF interactions.[51] Researchers have recognized the importance of studying these cell–cell interactions to elucidate novel drug targets and basic biology that can lead to new therapies for patients. The association of CAFs in tumor microenvironments with metastasis is well established; however, the dynamics of cell–cell communication leading to cell invasion is difficult to study in vivo. Using engineered hydrogel systems, researchers have been able to elucidate mechanisms of crosstalk between CAFs and cancer cells.[37,51–55]

While it is broadly accepted that both paracrine signaling and direct physical contact between cancer cells and CAFs promote invasion, it has been suggested that physical contact enhances cancer cell invasion beyond the effects of paracrine communication.[52,55] Gaggioli et al. used a blend of Matrigel and collagen I to study the physical interactions between squamous cell carcinoma (SCC) and CAFs during SCC migration.[54] Interestingly, fibroblasts were the leading cells in each migratory pathway as they created tracks for the invasion of SCCs, which were unable to migrate independently (**Figure 5**a). In a related study, Yamaguchi et al. observed that the migration of scirrhous gastric carcinoma cells and CaF37 fibroblasts through Matrigel was enhanced by coculture.[53] In microfluidic model, Sugimoto et al. observed that A549 lung cancer cells migrated through a synthetic vessel seeded with fibroblasts. In this case, a core–shell alginate hydrogel strategy was used where a softer alginate hydrogel formed a micropassage from the core and shell to enable directed cell migration.[56] Through the addition of RGD peptide-conjugated alginate, cancer cell invasion was significantly increased after 7 days in culture compared to unconjugated alginate. The effect of anticancer drugs (cisplatin, paclitaxel, and 5-fluorouracil) on invasion and gene expression were studied in both monoculture and coculture conditions. While drug treatment decreased invasion, coculture mitigated these effects, supporting the theory that the presence of CAFs promotes drug resistance. Moreover, coculture enhanced the expression of cancer invasion associated genes HIF-1α, VEGF, and ABCB1 following cisplatin treatment, suggesting that fibroblast coculture induces drug resistance in A549 cells. Some studies have compared the direct versus indirect effects of CAF coculture on migration.[51,55] Young et al. used their tissue roll for the analysis of cellular environment and response (TRACER) platform to study the migration of squamous carcinoma cells of the hypopharynx (FaDu) in the presence of primary CAFs from head and neck cancer patients.[51] In these studies, collagen-embedded FaDu and CAFs were separated by a collagen hydrogel and, by 48 h, both coculture and FaDu monoculture supplemented with coculture conditioned media increased FaDu migration into the collagen hydrogel compared to FaDu monoculture alone (Figure 5b). In a comparable study by Attieh et al., conditioned media from colon tumor-derived CAFs did not enhance the invasion of CT26 colon cancer cells; however, direct coculture did (Figure 5c).[55]

Together, these studies demonstrate the heterogeneity of CAF-cancer cell interactions and the dependence on CAFs for not only extent of migration, but initiation of migration for some cell types. Furthermore, this body of research eludes to the complexity of coculture studies involving fibroblasts. The requirements for paracrine signaling versus direct contact may be different for each cancer variety, or even cell type, studied in hydrogel systems, mirroring the complexity of cancer biology. Future studies that compare multiple cancer types in relevant ECM niches could help reveal the degree of influence of CAFs on cancer-cell migration more generally. Recognizing that CAFs are a heterogeneous group of cells that have the potential to influence the immune population,[50] the use of primary CAFs from the tumors of interest would enhance the relevance of these studies.

3.2. Macrophage-Cancer Cell Crosstalk in Engineered Hydrogels

TAMs are drivers of immune suppression and cancer cell migration. TAMs migrate to the cancer microenvironment as monocytes and are differentiated and polarized at the tumor site, typically to an alternatively activated, or M2, phenotype with angiogenic and ECM remodeling characteristics.[57–59] The presence of TAMs are associated with poor prognosis as confirmed for a wide range of solid tumors using histology.[59] However, histology alone is unable to capture the dynamic processes of TAM recruitment to the microenvironment and cancer cell invasion following TAM accumulation. Thus, hydrogel disease models incorporating TAMs aim to recapitulate aggressive tumor microenvironments.[59–62]

It is well established that the presence of macrophages in a tumor is associated with metastasis;[63] however, Guiet et al. showed that coculturing human macrophages with breast cancer cells influenced the mode of tumor cell invasion into Matrigel.[60] In the absence of macrophages, SUM159PT cells migrated via a mesenchymal, MMP-dependent migration mode whereas when macrophages were present, SUM159PT cells switched to an amoeboid mode (**Figure 6**a). Inhibitors of MMPs (GM6001), lysosomal proteases (LyPI), and ROCK were used to elucidate these modes of migration. They also showed that tumor cell migration was dependent on macrophage contact; direct coculture increased cell migration over indirect coculture in a transwell setup with macrophages seeded in the bottom chamber (Figure 6b). The effect of macrophage phenotype on migration and the effect of coculture on macrophage polarization were excluded from the study.

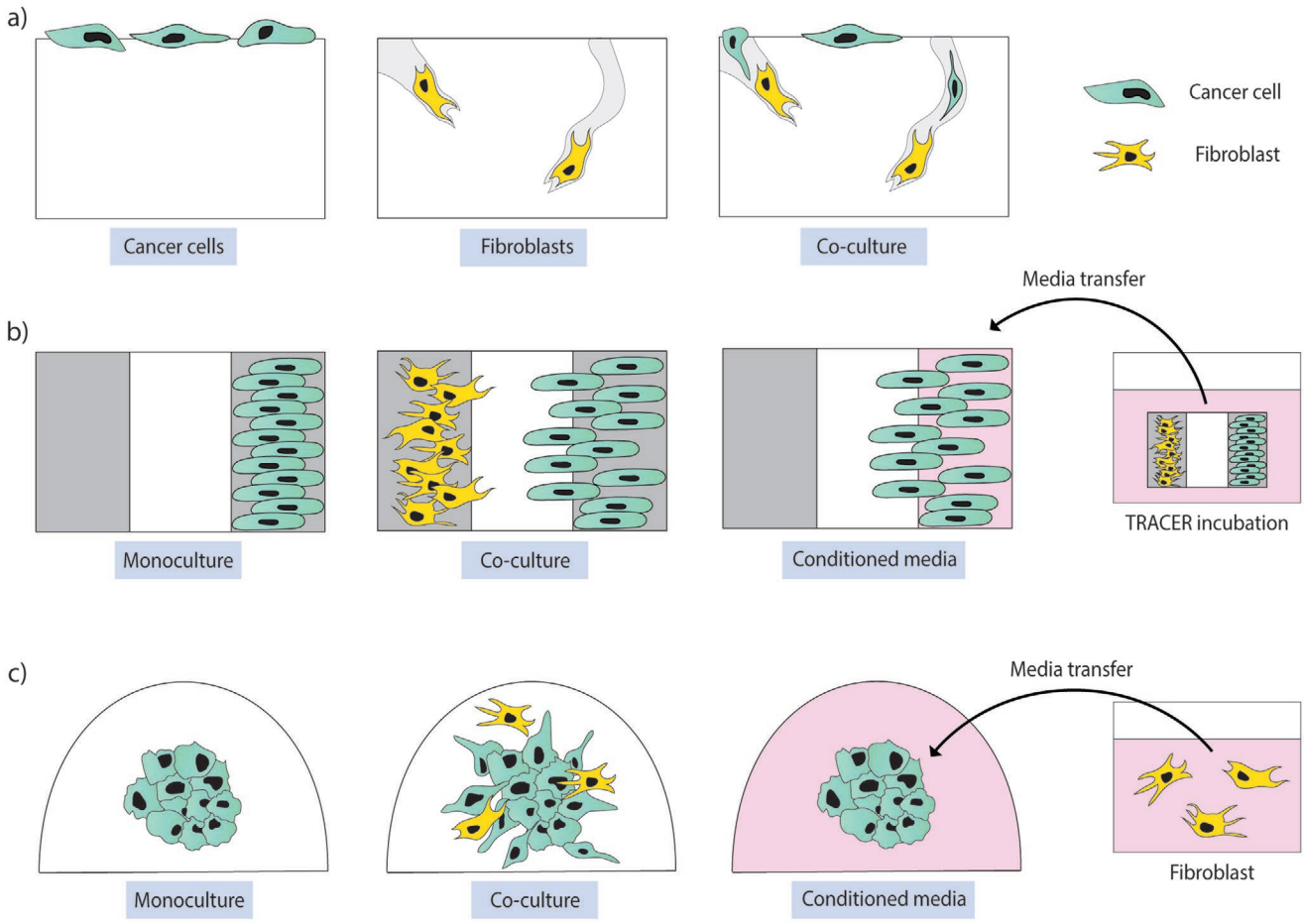

Figure 5. Cancer associated fibroblasts influence cancer cell invasion through hydrogels. a) Squamous cell carcinoma (SCC) cells invade into a Matrigel/collagen I hydrogel following fibroblast migration but do not invade alone.[54] b) Squamous carcinoma cells (FuDu) cultured on collagen I hydrogels within a TRACER device invade with primary CAFs coculture (from head and neck cancer patients) or treatment with CAF conditioned media.[51] c) Colon cancer cells (CT26) invade from cancer spheroids in response to coculture with colon tumor-derived CAFs but do not invade following treatment with CAF conditioned media.[51,54,55]

Although it is becoming increasingly accepted that macrophage polarization states exist on a spectrum and are unique to the microenvironment,[64,65] M1 and M2 polarized macrophages are used to model proinflammatory and tissue remodeling, respectively. Some studies have polarized macrophages to an M2 state using interleukin-4 (IL-4) and IL-13 to model advanced stage tumors where CD163+ cells make up the majority of this cell population.[65] Karnevi et al. studied the influence of M2 macrophages on BxPC-3 pancreatic cancer cell invasion into a blend of Matrigel and collagen I.[65] The depth of BxPC-3 migration increased with M2 cell coculture after 14 days in culture (Figure 6c). Notably, there were no differences after 7 days in culture, emphasizing the importance of culture duration.

The aforementioned studies focused on the interaction between two or more cell types, treating the hydrogel as an inert scaffold. In contrast, Lee and Cha studied how two factors influenced spheroid growth of MCF-7 breast cancer cells and M2 marker CD206 expression:[62] stiffness of gelatin-methacrylate hydrogels and coculture with murine macrophage cell line RAW264.7. While increasing macrophage content from 30% to 50–70% enhanced spheroid growth, increasing stiffness from <2.5 to ≈30 kPa decreased both spheroid growth and CD206 expression. This work did not focus on cell invasion; however, these studies demonstrate the importance of both scaffold properties and coculture on macrophage polarization and cell proliferation. Furthermore, this study demonstrated the importance of studying the effects of cell–cell and cell–scaffold interactions, which can both influence cancer cell behavior.

With the increasing number of immunotherapies that are progressing through clinical trials and those that are failing,[4] it is crucial that researchers begin to incorporate relevant components of the immune system into 3D coculture models to better predict clinical outcomes. Biomaterials have been shown to affect immune cells[64] and may require tuning to support the culture of specific immune cell states. Notwithstanding the importance of the immune system, additional cell types should be introduced in a systematic fashion in a platform which enables cell separation for subsequent analyses including, but not limited to, gene profiling, proteomics, immunohistochemistry, and flow cytometry.

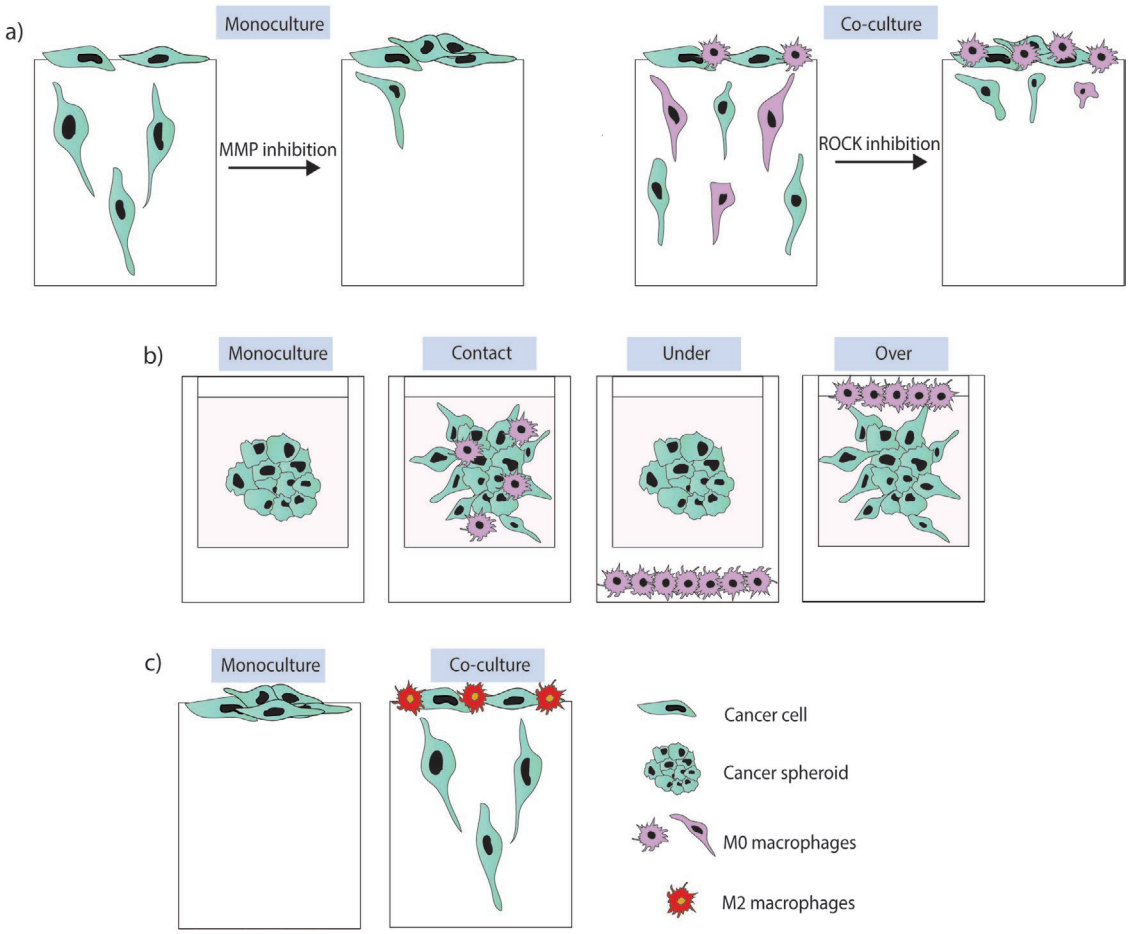

Figure 6. Macrophage coculture affects cell invasion through hydrogels. a) In a monoculture setup in Matrigel, breast cancer cells (SUM159PT) migrate using an MMP-dependent mechanism; with macrophage coculture, cells migrate using a ROCK-dependent mechanism.[60] b) In a transwell setup, SUM159PT cells are cultured as spheroids within Matrigel with or without macrophages. SUM159PT cells invade away from cancer spheroids toward the surrounding matrix when coculture spheroids are used or when macrophages are cultured on top of hydrogels. Cells do not migrate in a monoculture setup or when macrophages are cultured underneath transwells.[60] c) BxPC-3 pancreatic cancer cells migrate into a Matrigel/collagen I hydrogel blend when cultured with M2 macrophages.[60,65]

3.3. Modeling Metastasis with Endothelial Cell Coculture

During cancer metastasis, cells intravasate and then extravasate through blood vessels, leading to the formation of a secondary tumors at distal sites. This process requires the co-operation of multiple cell types.[49] Recent coculture models that incorporate endothelial cells aim to capture early stages of cancer metastasis. In these models, common readouts of metastasis include cancer cell migration and endothelial cell spouting as a proxy for tumor-associated angiogenesis.

There are few in vitro mechanistic studies of metastasis involving interactions among tumor associated macrophages and cancer cells. Recently, Cui et al. used a microfluidic model to probe the influence of macrophage polarization on angiogenesis in the context of glioblastoma.[66] In this study, RAW264.7 cells were treated with conditioned media from mouse glioma cell lines GL261 or CT-2A. It was found that unlike M0/M1 macrophages, conditioned macrophages ("TAMs"), which had an M2-like signature in terms of cytokine production, promoted endothelial cell (EC) sprouting (**Figure 7**a). Mechanistic studies revealed that TAM-mediated EC sprouting was dependent on both TGF beta receptor 1 (TGFβ-R1) and alpha-v beta-3 ($\alpha v\beta 3$). Interestingly, no effect on ECs was observed following inhibition of the angiogenic-associated gene vascular endothelial growth factor receptor (VEGFR) in TAMs.

The influence of endothelial cell coculture on cancer cell migration has been studied in hydrogels. Across many models, researchers have found that EC coculture enhances migration toward ECs when cells are cultured in close proximity within hydrogels.[39,67,68] For example, Truong et al. modeled the invasion of GB3 glioblastoma cancer cells in the presence of ECs using a microfluidic device and found that GB3 migration through Matrigel was enhanced with HUVEC coculture.[68] In this model, HUVECs formed blood vessel-like structures that surrounded GB3 spheroids within fibrin gels. Researchers established a dependence of this effect on the chemokine-receptor interaction between CXCL12 and CXCR4 using AMD3100, an inhibitor of CXCR4 (Figure 7b). In a similar model by Meng et al., who also used a microfluidic device and a fibrin hydrogel, A549 lung cancer cell migration from

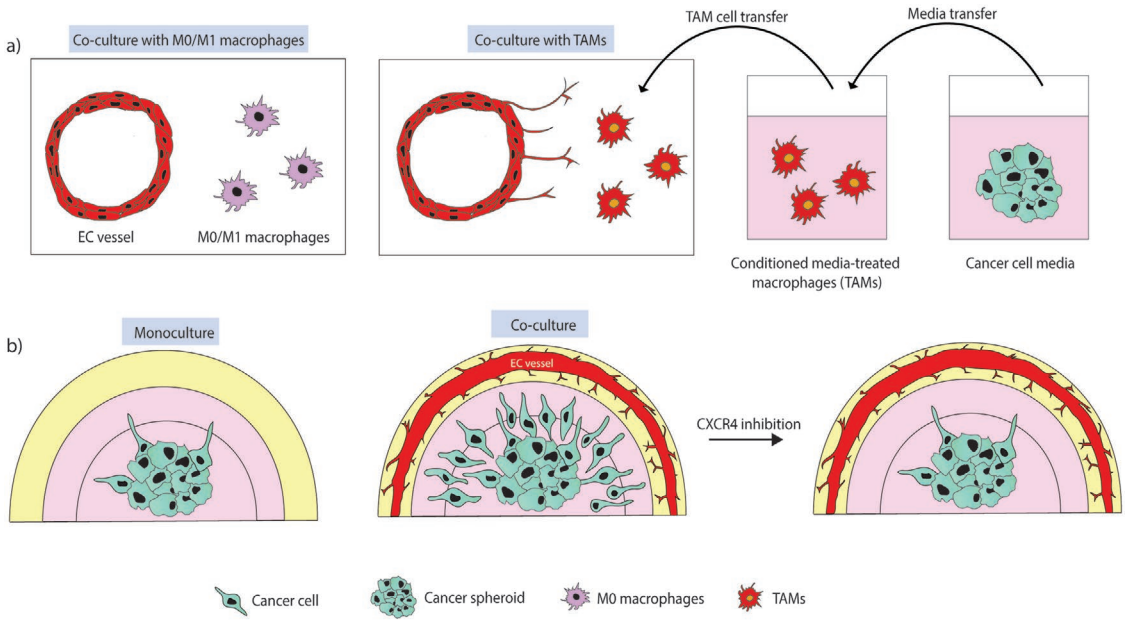

Figure 7. Invasion influenced by the vasculature in engineered hydrogels. a) In RGD-modified collagen I, macrophages treated with glioma cell (GL261 or CT-2A) conditioned media promote endothelial cell (EC) sprouting. Sprouting is not promoted with M0/M1 coculture.[66] b) HUVEC vessel coculture promotes glioblastoma cell (GB3) invasion from spheroids and is dependent on CXCR4.[66,68]

a primary spheroid toward HUVEC vessels was observed in addition to HUVEC sprouting toward the spheroid.[39] Notably, the influence of HUVEC coculture on A549 migration was not studied directly. Rather, the observed effects were attributed to EGF that was introduced into the device through microcapsules.

Together, these models aim to capture early stages of metastasis. More recently, recapitulating cancer cell interactions with the vasculature and the bone microenvironment together are of increasing interest to researchers as bone metastasis is a common site of metastasis (for cancers including breast, lung, and prostate) and a hallmark of late stage disease leading to palliative care.[69]

With the aim of modeling bone metastasis, researchers have made use of microfluidic devices to accurately image and quantify cell migration through endothelial structures toward bone microenvironments.[70,71] Sano et al. designed a microfluidic device where spheroids (containing ECs, fibroblasts, and mesenchymal stromal cells (MSCs)) were embedded in a fibrin-collagen I hydrogel bordered by functional blood vessels (verified through cell perfusion experiments).[70] Through confocal microscopy, researchers observed that osteo-differentiated MSCs enhanced MDA-MB-231 breast cancer cell migration through vessels compared to undifferentiated MSCs. Mei et al. recently published a similar model of breast to bone metastasis through endothelial vessels formed in a hydrogel blend of collagen I and Matrigel. Using this model, they aimed to study the effects of mechanostimulation of osteocytes on cancer cell extravasation.[71] Interestingly, MDA-MB-231 breast cancer cell extravasation through HUVEC vessels decreased with MLO-Y4 osteocyte mechanical stimulation (oscillatory fluid flow). This work demonstrates that microfluidic devices allow complex cell setups and enable studies of how fluid flow affects metastatic readouts.

4. Challenges and Opportunities for Cancer Invasion Models

Hydrogel models of cancer invasion have proven to be useful tools in rebuilding the metastatic tumor niche, and establishing the importance of the physical, biochemical, and cellular microenvironment in cancer cell migration. Efforts of recapitulating invasive cancer cell behavior rely on a simplification of the tumor ECM to include the basic components required by a cell to migrate. Most examples of monoculture systems rely on similar criteria for the cellular microenvironment where cells can adhere and remodel. Using these systems, researchers have been able to show different strategies of cell invasion in vitro, and even dynamic switches between proteolytic and nonproteolytic ECM remodeling. The migration modes of cancer cells depend on hydrogel composition, stiffness, and biochemical gradients of growth factors that can be designed for each cell type. Accordingly, hydrogel-based microenvironments have been engineered to mimic these behaviors in vitro. Multilevel analysis of the evolving "matrisome" of the metastatic microenvironment has resulted in the identification of common matrix responses across different diseases.[71] Such rich data sets have the potential to guide and improve biomaterial design of relevant tumor microenvironments.

Using hydrogel models, researchers have established the importance of biochemical and mechanical properties on cell migration. Recapitulating the native extracellular matrix in which cells reside in vivo is prioritized when designing these experiments to elicit relevant mechanisms of cell invasion. In contrast, emerging coculture models that offer cellular diversity often ignore the influence of the hydrogel itself on cellular outputs. These models use commercially available materials, mainly Matrigel and collagen I, which have significantly

advanced the fields of organoid culture and clinical 3D drug screening;[72] however, their biological significance in mechanistic studies of cell migration, tumor growth, and drug response need to be validated for each cancer type. Appreciating that hydrogel mechanics and biochemical cues can alter invasion phenotypes,[73] mimicking the ECM to support the tumor niche while supporting relevant invasion mechanisms is critical to achieve representative models.

In the field of organoid culture, organogenesis and hydrogel design inform one another; spatiotemporally controlled mechanical and biochemical cues within hydrogels can reveal developmental phenomenon that would be difficult to study in conventional systems.[74] Often, in vitro cancer models ignore the effects of matrix properties on cell behavior and the heterogeneity of tumors. Learning from organoid culture, primary cancer cell culture on hydrogels with unique properties can inform researchers of interesting cancer biology while enhancing the impact of studies. The heterogeneity of tumors combined with the complexity of extracellular matrices in which they reside makes it difficult to recapitulate the stages of metastasis in vitro. Recognizing the heterogeneity of the tumor microenvironment from patient to patient, the optimization of hydrogel design using primary cells is expected to enhance the predictive power of 3D models. Although resection and subsequent 3D culture is not a straightforward process in the cases of inaccessible or small tumors and has additional hurdles compared to cell line use (including ethics board applications and risk to researchers), primary cell use has the benefit of patient variability, tumor heterogeneity, and enables personal medicine to identify the best drug for the individual.

It is difficult to determine the correlation between in vitro cell behavior and in vivo phenomenon, which are often represented as outcomes of gene knockout studies in animals, histological snapshots of tumors, and patient outcomes. Model validation is a major subject of debate in the field of 3D cell-culture and the chosen method depends on the purpose of the hydrogel. Some proposed methods include benchmarking drug response with healthy control cells,[75] and profiling cell genetics and correlating expression with animal or patient samples.[76] For the former, Tam et al. used an HA-based hydrogel, which supported MMP-dependent and MMP-independent cell invasion to screen inhibitors of cell invasion and viability in tandem, arguing that this dual-readout system is critical during drug screening of highly invasive cells such as those in lymphangioleiomyomatosis (LAM) and lung cancer.[75] Gene profiling has been adopted by the 3D culture community as a method of validating hydrogel models.[76] Recently, Baker et al. validated an HA-crosslinked hydrogel for breast cancer spheroid culture through comparison with Matrigel, the standard 3D culture model, against xenografts using a pancancer gene expression panel.[76] It was found that spheroids grown in the crosslinked gel better mimicked xenograft gene expression profiles compared to Matrigel, and supported the growth of patient-derived cells in vitro. The validation process is not linear and must be defined for each biological question.

It is critical that academic researchers communicate with clinicians and pathologists to produce models that 1) represent the native ECM, 2) include relevant cell types in their models, and 3) use readouts that can benefit patients through improved drug screening and personalized medicine. We advocate against solely targeted gene or protein expression analyses in coculture studies and instead propose dynamic readouts of cell migration and unbiased approaches to gene profiling when possible, as biomarkers of patient survival are not always effective drug targets.[77] While Matrigel and collagen I are commercially available and easy to use, they do not mimic the tumor niches sufficiently, may lead to false negatives and false positives in drug screening, and could limit the usefulness of invasion models. In contrast, added complexity will not necessarily improve their usefulness. To ensure utility and predictive power of tailored hydrogel design, the following should be utilized: relevant cell sources, dynamic readouts, quality control measures to confirm reproducibility, benchmarking against commercial hydrogels (Matrigel and Collagen I), approved drugs, and/or xenograft models.

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Conflict of Interest

The authors declare no conflict of interest.

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