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Review

Engineering the Extracellular Matrix to Model the Evolving Tumor Microenvironment

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SUMMARY

Clinical evidence supports a role for the extracellular matrix (ECM) in cancer risk and prognosis across multiple tumor types, and numerous studies have demonstrated that individual ECM components impact key hallmarks of tumor progression (e.g., proliferation, migration, angiogenesis). However, the ECM is a complex network of fibrillar proteins, glycoproteins, and proteoglycans that undergoes dramatic changes in composition and organization during tumor development. In this review, we will highlight how engineering approaches can be used to examine the impact of changes in tissue architecture, ECM composition (i.e., identity and levels of individual ECM components), and cellular- and tissue-level mechanics on tumor progression. In addition, we will discuss recently developed methods to model the ECM that have not yet been applied to the study of cancer.

INTRODUCTION

The extracellular matrix (ECM) was one of the first elements of the microenvironment shown to be altered in tumors relative to normal tissue. Composed of fibrillar proteins, glycoproteins, and proteoglycans, the ECM is an organizational structure for tissues, provides cues to direct cellular signaling, and serves as a reservoir for growth factors (Eble and Niland, 2019). Early studies demonstrated that the ECM could direct tumor cell behavior, with increases in proliferation for cells cultured on ECM compared with tissue culture plastic (Vlodavsky et al., 1980). Clinical evidence also supports a role for ECM in cancer risk and prognosis across multiple tumor types. For example, women with elevated levels of collagen in their breasts have a greater than 4-fold increased risk of developing breast cancer (McCormack and dos Santos Silva, 2006). In ovarian, lung, breast, and colorectal cancer, patterns of ECM gene expression have been identified that correlate with poor prognosis (Cheon et al., 2014; Lim et al., 2017; Pearce et al., 2018; Yuzhalin et al., 2018).

Although tumors initiate in the native ECM of the primary or metastatic site, this matrix will change throughout tumor progression. Cancer cells frequently have altered production of ECM proteins or matrix remodeling enzymes (e.g., matrix metalloproteinases [MMPs], lysyl oxidases) (Eble and Niland, 2019). In addition, proliferation and activation of fibroblasts to a cancer-associated fibroblast (CAF) phenotype results in substantial ECM deposition (Sahai et al., 2020). Utilizing human tumor cells in a mouse xenograft host, it was recently demonstrated that tumor cells produce most of the ECM modulating enzymes, stromal cells produce most of the glycoproteins, and both components produce collagens (Hebert et al., 2020). Characterization of the tumor ECM by immunohistochemical staining or RNA-based analysis has demonstrated that ECM composition around the primary tumor and metastatic sites undergoes radical changes during tumor progression. For example, many studies have documented upregulation of collagen I in primary breast, liver, lung, and esophageal tumors and in metastatic ovarian cancer (Fang et al., 2019; Fogg et al., 2020; van 't Veer et al., 2002; Zhang et al., 2018). Hyaluronic acid (HA) is also significantly elevated in a wide range of cancers (e.g., breast, prostate, bladder, colon) and is associated with an increased likelihood of metastasis and poor prognosis (Lokeshwar et al., 2014). In a tissue microarray of patients with breast cancer, higher epithelial fibronectin expression was observed in patients with overall worse survival (Bae et al., 2013). Finally, alterations in laminin composition are common in many cancers, although these findings vary significantly with laminin isoform. For example, laminin-111 is decreased in breast tumors compared with normal breast tissue (Gudjonsson et al., 2002), whereas laminin-332 is upregulated in many cancers (including breast) and is associated with poor prognosis (Guess and Quaranta, 2009).

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More recent studies have begun to address the limitations of identifying individual protein differences through immunostaining by using proteomics to describe the tissue and tumor matrisome. For instance, comparison of primary colon cancer and liver metastases revealed 23 proteins that were shared between tumor sites but were not detected in normal colon or liver (Naba et al., 2014b). Proteomic studies have also revealed differences in the matrisome of tumors with different metastatic potential. For example, ECM differences were determined from the primary tumors generated by xenografts of MBA-MB-231 breast cancer cells compared with LM2 breast cancer cells, which have higher metastatic potential (Naba et al., 2014a). In a similar manner, biopsies from more advanced omental metastases in ovarian cancer showed higher expression of glycoproteins (fibrinogen and fibronectin), along with increased proteoglycans, secreted factors, and affiliated proteins relative to smaller metastases (Pearce et al., 2018). More recent studies using proteomic analysis of the ECM have identified tenascin-C as a prognostic marker in lung cancer (Gocheva et al., 2017) and SERPINB1 as a component of brain tropism in breast cancer metastasis (Hebert et al., 2020). In a similar manner, in-depth characterization of changes in the matrisome of other tumor types may identify new therapeutic targets.

As the cellular and matrix components change with an evolving tumor microenvironment, the mechanics at the tissue and cellular levels also evolve. At the tissue level, the change in mechanics is characterized by an increase of bulk stiffness due to increased deposition of fibrillar matrix proteins, such as collagen I, as a part of a fibrotic response (Lopez et al., 2011; Pearce et al., 2018). In mouse breast cancer models, *in situ* atomic force microscopy (AFM) measurements showed that stiffness increased with tumor progression and ECM and tumor-associated vasculature were the drivers of increasing stiffness (Lopez et al., 2011). In patients with colorectal cancer, metastases to the liver were stiffer than primary tumors in the same patient owing to activation of highly contractile fibroblasts in the metastatic microenvironment and their deposition of fibrillar matrix (Shen et al., 2020). Stiffness can reinforce fibroblast activation, creating a pro-tumorigenic, positive-feedback loop. These findings support that a mature tumor microenvironment continually enhances fibrosis, stiffening the mechanics of the environment. When the tumor microenvironment is examined at a cellular level, it becomes apparent that these mechanical changes also result from increased matrix cross-linking and reorganization of fiber architecture, alterations that can physically guide cells and influence their behaviors. For example, in breast cancer, it has been seen that collagen cross-linking by lysyl oxidase promotes integrin clustering and focal adhesion formation, which, in turn, promote tumor progression via increased cell invasion (Levental et al., 2009).

Examining the impact of changes in ECM composition and mechanics *in vivo* remains challenging owing to the complexity and cost of animal models, as well as the need to induce these matrix changes in a tumor-localized manner. In contrast to genetically engineered models of oncogenic drivers of cancer, most models of altered ECM remain systemic rather than subject to site-specific (e.g., Cre/Lox) or temporal (e.g., TetR) activation. The potential to use a site-specific Cre/Lox system has been demonstrated with subcutaneous versican depletion in mice; however, to our knowledge this is the only published report of these techniques to study the role of ECM in cancer progression (Fanhchaksai et al., 2016). In this review, we will describe recent advances in engineering methodologies that are being used to examine the effects of tumor-associated alterations in the ECM, including changes in tissue-level architecture, ECM composition, and tissue- and cellular-level mechanics. These methods can be used to reconstruct the microenvironment of different tumor types to determine how the altered ECM drives tumor progression and subsequently identify mechanisms to slow or stop these effects.

ENGINEERED ECM MODELS MIMIC CANCER-ASSOCIATED CHANGES IN TISSUE-LEVEL ARCHITECTURE

Normal tissues have multi-cellular organizations that are key to their function and are disrupted in cancer. Pioneering studies from the group of Mina Bissell demonstrated that questions about tissue architecture could be examined *in vitro*, as cells had different morphologies when cultured on top versus embedded in basement membrane (Figure 1). This was extended to cancer cells, demonstrating that blocking interactions with the ECM could revert a malignant phenotype (Weaver et al., 1997) or make tumor cells susceptible to apoptosis (Wang et al., 2002). Building from this approach, networks of endothelial cells have been formed in fibrin gels to examine the influence of different stimuli on tumor cell intravasation (Zervantonakis et al., 2012) and extravasation (Escribano et al., 2019; Song et al., 2018). In recent work, this platform was extended to incorporate an embedded "tumor," an endothelial-lined vessel, and patterned growth-factor

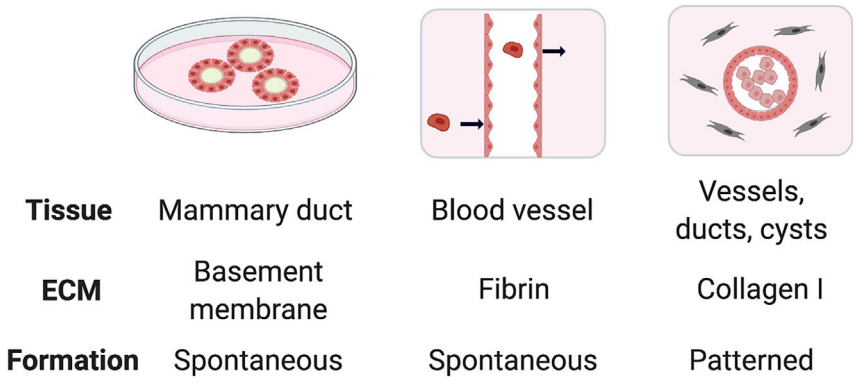

Figure 1. Examples of *In Vivo* Architectures Reproduced *In Vitro* Using ECM

Structures resembling mammary ducts form when mammary epithelial cells are cultured in 3D gels of basement membrane. Endothelial cells embedded in fibrin can generate vessel structures; when coupled with tumor cells embedded in the matrix or flown through the vessel, the processes of intravasation or extravasation can be examined. Finally, when collagen I is gelled around molds, structures with different architectures can be formed and lined with cells, such as the model of DCIS shown here. Created with BioRender.com.

releasing particles to generate chemotactic gradients (Meng et al., 2019). Such multi-component models are an important step to faithfully recreate tumors *in vitro*.

Of course, many tumors become fibrotic as they progress, such that culture in collagen gels may be more physiologically relevant than basement membrane. Collagen I gels also have greater mechanical integrity than basement membrane or fibrin, which has enabled engineers to "pre-pattern" the tissue structure through microfabricated molds inserted into gelling matrix and examine the effects of different macro-structures on cell behavior (Nelson et al., 2006). Using a method developed to generate lumens to mimic blood or lymph vessels (Jimenez-Torres et al., 2016), a recent model of ductal carcinoma *in situ* (DCIS, the earliest stage of breast cancer) has been described (Figure 1). In this model, a lumen with a diameter of approximately 300 μm is lined with normal mammary epithelial cells and then left empty in the center (normal) or filled with breast cancer cells (DCIS) (Ayuso et al., 2018). This lumen-generating method has been modified to generate models of ovarian cortical inclusion cysts, demonstrating that, of the two proposed cells of origin for ovarian cancer, only fallopian tube epithelial cells are able to invade into the surrounding matrix (Fleszar et al., 2018). As cortical inclusion cysts range in size and are believed to resolve over time, a recent paper modeled the range of curvatures observed in human cysts and determined that as curvature increased (i.e., the cyst diameter decreased) the fallopian tube epithelial monolayer transitioned from stable to invasive (Fleszar et al., 2019).

BIOMATERIAL APPROACHES ENABLE MODELING OF TUMOR-ASSOCIATED CHANGES IN ECM COMPOSITION

Although the studies highlighted above utilized ECM to generate physiologically and pathophysiologically relevant structures *in vitro*, they did not ask how *changes* in the ECM influence tumor progression. To better understand the role of ECM composition changes in cancer progression, biomaterials-based approaches have been used to build *in vitro* models of the tumor ECM microenvironment. Here, we will discuss the design and use of 3D scaffolds derived from decellularized ECM, intact ECM molecules, and functional fragments of ECM molecules such as minimal adhesion sequences (Figure 2). To construct a scaffold, these materials are either directly gelled or incorporated into materials that are modified to enable cross-linking (e.g., crosslinker-modified poly(ethylene glycol) [PEG], gelatin, and HA).

Decellularized Matrix

Decellularized matrix from animal models, patient samples, and cell cultures allows one to examine the full diversity of matrix components present in the native tissue (Hoshiba, 2019). Although early studies relied on decellularized matrix isolated in individual laboratories and were subject to protocol variation, reconstituted decellularized matrix is now available from commercial suppliers for use as a surface coating or to generate a 3D hydrogel (e.g., Xylyx Bio). These matrices are typically isolated from healthy tissues and

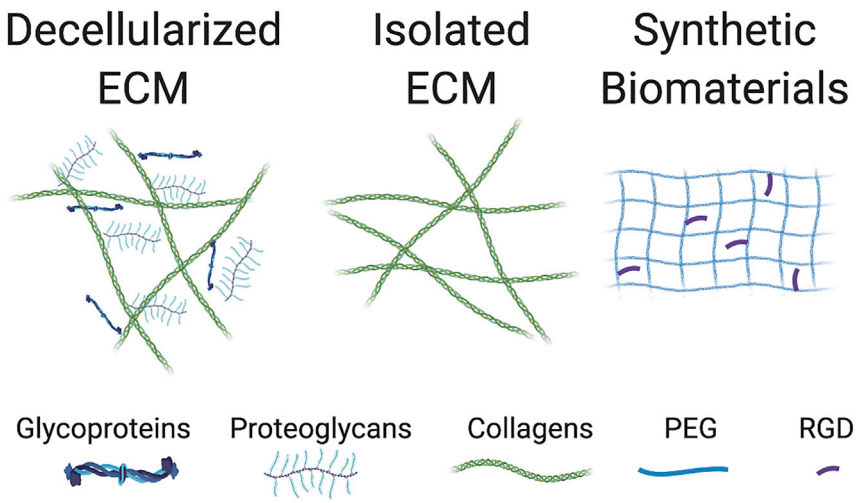

Figure 2. Example Methods Used to Generate ECM Scaffolds with Different Compositions
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therefore relevant to studying early tumor development or initial metastasis. However, many interesting biological questions still require investigators to generate their own decellularized matrix. In particular, there are disease states associated with elevated cancer risk that may result from ECM changes. For example, when decellularized ECM was obtained from cultured adipose stromal cells isolated from obese and lean mice, breast cancer cells had increased proliferation in the ECM from cells isolated from the obese background (Seo et al., 2015); this finding is consistent with increased risk/aggressiveness of breast tumors in obese patients (Hefetz-Sela and Scherer, 2013). Using decellularized ECM from healthy or cirrhotic human livers, it was found that cirrhotic liver ECM promoted EMT of hepatocellular carcinoma cells in a Smad-dependent manner (Mazza et al., 2019). These studies highlight the importance of using ECM specific to the question of interest and suggest the ECM as one mechanism linking pre-existing conditions to cancer risk.

Decellularized matrix from established tumors has also been investigated to determine its impact on tumor cell behaviors. Perhaps not surprisingly, such studies have generally supported that tumor-derived ECM imparts pro-tumorigenic activity. Glioblastoma cells underwent morphological changes and had increased migration in patient-derived decellularized tumor matrix compared with collagen gels (Koh et al., 2018). Colon cancer spheroids cultured on ECM derived from human liver metastases of colon cancer grew faster compared with spheroids grown on ECM from the colon of healthy patients (Romero-Lopez et al., 2017). Of course, one interesting question is how decellularized matrix isolated from the same tissue differs between the normal state and cancer or between tumors from different patients—to our knowledge, such studies have not yet been reported.

Isolated Extracellular Matrix Components

Although decellularized ECM retains the richness of the native ECM environment, it can be difficult to work with owing to limited tissue availability and the challenge of identifying the responsible components. As an alternative approach, scaffolds can be constructed from individual components of the ECM. Many foundational studies utilized collagen I gels to piece out mechanisms involved in breast cancer metastasis (Kai et al., 2019). More recently, scaffolds have been generated from combinations of purified ECM components using collagen I as the baseline material. For example, increased fibronectin is found in many tumors; the addition of fibronectin to collagen I gels enhanced migration of breast cancer cells (Oudin et al., 2016). A multi-cellular model of breast cancer cells and fibroblasts in collagen I or collagen I and fibronectin demonstrated a similar increase in tumor cell migration with the addition of fibronectin; however, the result here may have resulted from the production of additional MMPs by the fibroblasts in the collagen I plus fibronectin condition (Lugo-Cintron et al., 2020). The addition of collagen III into collagen I hydrogels led to increased invasion of fallopian tube epithelial cells relative to collagen I alone in the *in vitro* model of the cortical inclusion cyst described above (Fleszar et al., 2018).

Full-length ECM proteins possess multiple domains with distinctive functions that make it difficult to tease apart mechanisms regulating cancer cell behavior. In addition, they can be prohibitively expensive and difficult to incorporate into scaffolds owing to solubility issues. As an alternative, biomaterials can be engineered with fragments of the functional domains of ECM molecules, such as the minimal adhesion sequence RGD. For example, RGD incorporation into HA resulted in a cooperative effect to confer chemotherapy resistance to glioblastoma cells (Xiao et al., 2020). Photopolymerizable PEG-based hydrogel systems have been engineered with adhesion peptides including RGDS (fibronectin/vitronectin), GFOGER (collagen I), and IKVAV (laminin) to examine their effects on breast cancer cell cluster size, which acts as an indicator of proliferation (Sawicki et al., 2019). These materials demonstrated that cells were responsive to peptide identity but that these results differed between triple-negative breast cancer cells (MDA-MB-231) and luminal A cells (T47D). Although many PEG-based scaffolds are non-degradable, monitoring cancer cell invasion may require materials that respond to cell-mediated remodeling by MMPs. Therefore, MMP-degradable peptide linkers have been incorporated into PEG hydrogels. Glioblastoma cells cultured in MMP-degradable hydrogels had enhanced cell spreading with actin-rich cell protrusions compared with the rounded cell morphology found in non-MMP-degradable hydrogels (Wang et al., 2017).

Of course, many ECM changes in tumors involve altered concentrations, rather than the presence or absence of specific components. These questions can be asked using many of the same systems. For instance, fibronectin gradients generated in 3D collagen I gels demonstrated that Mena^{INV} increases directional movement of breast cancer cells toward higher fibronectin density regions (Oudin et al., 2016). Increasing the density of collagen I gels resulted in a loss of ductal-like morphology for breast cancer cells and increased glutamine entry into the TCA cycle, suggesting a role for the ECM in energy regulation (Morris et al., 2016). When collagen I density was matched to *in vivo* levels in the normal omentum, ovarian cancer cells were insensitive to the growth factor HB-EGF (Fogg et al., 2020). In contrast, ovarian cancer cells proliferated in response to HB-EGF when cultured on gels matching the density of metastatic tumors in the omentum (Fogg et al., 2020). Changes in ECM density may also be mimicked by altering the concentration of ECM-derived peptides in synthetic scaffolds. In the previously mentioned PEG-peptide work, decreased RGDS or IKVAV density led to increased growth of breast cancer cell clusters, suggesting enhanced proliferation; however, the effect of GFOGER density was cell-line dependent (Sawicki et al., 2019). As tumors metastasize, they encounter new tissues with different ECM compositions than the original microenvironment, in terms of both the type of ECM proteins present and the relative levels. Using *in vivo* data, *in vitro* mimics of these diverse environments have been generated to examine the tropism of different tumor cells for specific organs (Barney et al., 2015).

BIOENGINEERING APPROACHES TO STUDY THE MECHANICS OF THE TUMOR ECM

Increased deposition of fibrillar collagen is a hallmark of tumorigenesis and is associated with increased bulk stiffness; thus, many early *in vitro* systems aimed at modeling mechanical changes during tumorigenesis consisted of hydrogels formed using increasing concentrations of collagen I (Baker et al., 2009; Provenzano et al., 2009). Although increased collagen I density is reflective of the native tumor microenvironment, this approach does not permit tissue stiffness to be studied independently; collagen concentration affects not only stiffness but also fiber density, which is known to influence cell behavior (Berger et al., 2017; Lewis et al., 2018). Perhaps more importantly, the relatively weak non-covalent bonds that form collagen I gels provide only a limited range of elastic moduli (0.1–4 kPa), which is substantially less than the range of stiffnesses seen in most solid cancers (0.4–60 kPa) (Berger et al., 2017; Kawano et al., 2015). To overcome these limitations, various biomaterials approaches have been developed to examine the impact of bulk and local mechanics (Figure 3).

Synthetic Polymers to Model Changes in Bulk Tumor Stiffness

Synthetic polymers such as polyacrylamide (PAA) represent a facile approach to form elastic gels of precise, tunable stiffnesses that match the range seen in tumors (Tse and Engler, 2010). Using PAA gels modified with fibronectin, glioblastoma cells were observed to have increased proliferation as stiffness increased owing to stiffness-regulated EGFR clustering (Umesh et al., 2014). PAA gels are covalently modified with either intact ECM proteins or peptides to support cell adhesion; however, neither method provides full bioactivity as conjugation changes the protein conformation and minimal peptides do not support all functions of the intact molecule. Additionally, PAA gels can only be used with cells seeded on top of the gel, as its monomeric components are cytotoxic (Caliari and Burdick, 2016). PEG hydrogels can also be synthesized in a relevant range of stiffnesses and are compatible with 3D cell culture. Using a range of RGD

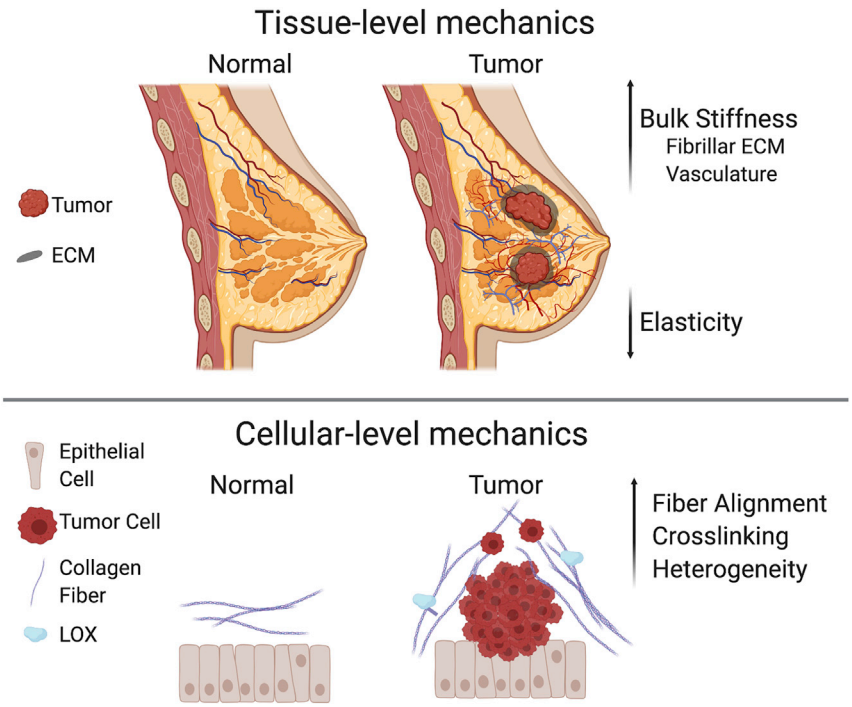

Figure 3. Comparison of Tissue-Level and Cellular-Level Mechanical Changes During Tumorigenesis

At the tissue level, mechanical changes during tumorigenesis are characterized by an increase in bulk stiffness due to increased vasculature, deposition of fibrillar ECM proteins, and a loss of tissue elasticity. At the cellular level, mechanical changes are facilitated through the remodeling of ECM architecture, including increased fiber alignment and cross-linking, which results in heterogeneity. Created with BioRender.com.

densities and degree of thiol-ene photo-cross-linking, fibrosarcoma cells were shown to respond to both ECM density and stiffness with respect to their migration speed (Singh et al., 2014). Interestingly, these cells tended to migrate from stiffer to softer areas more readily than softer to stiffer (Singh et al., 2014). Colon cancer cells embedded in 3D PEG gels modified with RGD peptides formed cyst-like structures, but only at higher stiffnesses (>8 kPa) (Enemchukwu et al., 2016). As native tissues and ECM molecules are viscoelastic and have a non-linear stress response to deformation, novel viscoelastic biomaterials have been developed to more accurately portray tissue mechanics in *in vitro* models. For example, PEG gels have been adapted to possess viscoelastic properties through employment of hydrazone bond cross-linking (Chaudhuri et al., 2020; McKinnon et al., 2014). In other work, viscous linear PAA was incorporated into elastic cross-linked PAA (Charrier et al., 2020), creating a matrix that allowed for independent control of viscous and elastic moduli. Using these viscoelastic PAA gels, it was observed that malignant prostate and brain cells had altered cell spreading, motility, and proliferation on viscoelastic gels compared with elastic gels of the same elastic modulus.

Modifications to Examine Changes in Bulk Tumor Stiffness Using Native ECM

As noted above, most gels made from native ECM have substantially lower stiffness compared with tumors. Low-density collagen I gels can be stiffened by non-enzymatic glycation (from 0.1 to 0.5 kPa), resulting in increased endothelial sprouting, branching in sprouts, and leakiness, which are all characteristic of tumor angiogenesis (Bordeleau et al., 2017). *In vivo*, elevated stiffness can result from cross-linking by enzymes such as lysyl oxidases. Therefore, one method to utilize native ECM molecules is to chemically modify the ECM in order to generate covalent cross-links that are more stable and therefore lead to stiffer materials. As an example of this approach, methacrylated hyaluronic acid (MeHA) gels have been developed that can be dynamically stiffened using a two-stage polymerization process (Ondeck et al., 2019). When the gel was stiffened from 0.15 to 3 kPa, encapsulated breast cancer cells gained a mesenchymal phenotype through activation of mechanosensitive transcription factors. Recently, dityrosine bonds were introduced into fibrin gels with a ruthenium-based photo-cross-linker to increase the modulus from 0.1 to 0.3 kPa (Hsieh et al., 2019). Although these gels were not tested with tumor cells, macrophages were

observed to have increased spreading and secretion of the inflammatory cytokine TNF-α (Hsieh et al., 2019), suggesting that ECM stiffening may support a pro-inflammatory tumor microenvironment.

An alternative approach is the use of interpenetrating networks (IPNs), gels that consist of blends of a material that can generate a range of stiffnesses and a material that provides cell adhesion and biochemical cues. For example, in an IPN consisting of alginate and recombinant basement membrane, the Young's modulus was increased from 0.09 to 0.95 kPa through calcium cross-linking of the guluronic acid chains in the alginate, while maintaining gel pore structure and ligand accessibility (Chaudhuri et al., 2014). Using this model, normal mammary epithelial cells in high stiffness networks underwent malignant transformation, including an increase in invasive clusters and Akt activation. In further work in this model, lung adenocarcinoma cells had increased proliferation and invasion in response to stiffening (Alonso-Nocelo et al., 2018). In an alginate-basement membrane IPN that was dynamically stiffened using calcium-loaded liposomes, breast cancer cells developed resistance to doxorubicin in response to stiffening (Joyce et al., 2018). However, in studies with MeHA or alginate IPNs, the range of stiffnesses attained is narrow and on the low end of the range of stiffnesses found in solid tumors (Kawano et al., 2015).

Methacrylated gelatin (GelMA) is a popular 3D *in vitro* model for modeling mechanical changes as it can be tuned to a large range of physiologically relevant stiffnesses (Nichol et al., 2010). Using a cross-linked network of GelMA and MeHA, increased stiffness was observed to inhibit glioblastoma cell invasion, although this effect was lessened with the incorporation of HA that is a key component of the native brain ECM (Chen et al., 2017). Owing to solubility issues, fibrillar ECM proteins are more challenging to integrate into IPNs. As gelatin and collagen I have the same biochemical sequence, they can be more easily blended than collagen I and other biomaterials. Utilizing these advantages, an IPN of GelMA and collagen I was created that allowed for the independent alteration of collagen I fiber density and stiffness (Berger et al., 2017). By modifying the extent of GelMA cross-linking and the ratio of GelMA to fibrillar collagen, stiffness and fiber density can be controlled independent of total protein concentration. When cells were embedded in these gels, it was found that fiber density and substrate stiffness independently impacted endothelial cell sprouting, but breast cancer cells required the presence of fibrillar collagen to invade the matrix (Berger et al., 2017). These findings highlight the importance of the fiber structure of the matrix when developing relevant *in vitro* models.

In Vitro Approaches to Model Tumor Associated Changes in Cellular-Level Mechanics

Although we often consider mechanical changes on a tissue-wide, bulk level, it is important to remember that cells actually sense their local environment. For example, within invasive ductal carcinoma samples, the invasive front of the tumor has been shown to be the stiffest by AFM (Acerbi et al., 2015). The local heterogeneity is largely a result of fibrillar matrix proteins, notably collagen I, that are actively remodeled into patterns that facilitate contact guidance and tumor cell invasion (Conklin et al., 2011; Tomko et al., 2018). To model the confinement presented to migrating tumor cells by aligned collagen I matrix, micropatterned PAA platforms with functionalized collagen I have been developed (Pathak and Kumar, 2013). When breast cancer cells were incorporated into the PAA microchannels of different stiffnesses and levels of confinement, it was observed that both increased confinement and increased substrate stiffness led to faster cell migration speed. Micropatterned ECM on 2D PAA and 3D PEG gels was used to confine melanoma or prostate cancer cells to distinct geometries (Lee et al., 2016). It was shown that, on the exterior perimeter of the geometry, cells expressed higher levels of markers of cancer stem cells (CSCs).

Of course, confinement and alignment can be observed at even lower length scales; to study this fiber-level scale stamp-casting has been used to create collagen I nanotextures on the surface of PAA gels (Tabdanov et al., 2018). To mimic this phenomenon in 3D, collagen I fibers can be aligned using magnetic beads (Guo and Kaufman, 2007); recent studies have clearly demonstrated that this method impacts local mechanics, whereas bulk mechanics remain constant (Taufalele et al., 2019). A 3D aligned collagen gel modeled after mouse mammary tumors was used to demonstrate that filopodia interacted with aligned collagen fibers, facilitating cellular anisotropy and migration (Carey et al., 2016). Another method to align fibers involves passing a collagen solution through an electrode as it gels to form aligned collagen bundles within the gel (Cheng et al., 2008). This method provides the unique ability to form larger groupings of collagen (up to 400 μm in diameter), which may be advantageous in modeling advanced, highly fibrotic cancers. Tunability of collagen I fibril bundling and gel porosity has been demonstrated through gelation of collagen I solutions at different temperatures (Doyle et al., 2015). Fibroblast cell adhesion stability and

migration was enhanced in a porous and bundled fibril architecture from 4°C polymerization compared with a highly reticular and heterogeneous collagen I matrix from 37°C polymerization. An alternative method to alter fibril organization in collagen gels without altering collagen density or stiffness involves incorporation of polyethylene glycol (PEG) into collagen gels to introduce molecular crowding. In these gels, fibrosarcoma cells had reduced adhesion and increased glycolytic activity, as cells could not degrade the more confined matrix architecture (Velez et al., 2019). Of course, collagen fibers can take many complex patterns in normal tissue and tumors (Wen et al., 2016); many of these patterns can be mimicked *in vitro* using multiphoton-excited polymerization in combination with GelMA and collagen I (Alkmin et al., 2019). Finally, a recent study relied on CAFs to align collagen I gels (Ray et al., 2018). This gel was then decellularized prior to seeding with triple negative breast cancer cells to study invasion, where it was found that the cells in the leading edge of invasion were more closely correlated with the aligned collagen fibers compared with cells in the bulk.

THE ENGINEERED MATRIX *IN VITRO* IS DYNAMIC, AS IS THE EXTRACELLULAR MATRIX *IN VIVO*

An important finding that has arisen from numerous studies is the confirmation that the initial engineered matrix that is presented to the tumor cells is quickly remodeled, as has been shown for other cell types (Loebel et al., 2019). However, the feedback that is activated depends on the initial scaffold composition. For example, glioblastoma cells were observed to produce higher levels of HA when cultured in gels generated from GelMA alone compared with gels composed of GelMA copolymerized with MeHA (Chen et al., 2017). Cells cultured on top of ECM mimics of different tissues have been used to screen for environments that support breast cancer cell dormancy (Barney et al., 2020). In this system, cells that generated a stable, fibrillar network of fibronectin maintained a dormant state, which could then be reversed through MMP-2 mediated degradation of this network (Barney et al., 2020). Additionally, stiffness can independently regulate matrix remodeling. Glioblastoma cells cultured in a PEG-based scaffold (modified with RGD peptides and MMP-degradable sequences) with interpenetrating HA upregulated HA synthase 1 and collagenase MMP-1 in stiff (26 kPa) versus soft (1 kPa) gels (Wang et al., 2014). Recently, our group demonstrated that Mena, an actin regulatory protein, was upregulated when breast cancer cells were embedded in stiff (12 kPa) but not soft (2 kPa) gels and that the increase in Mena facilitated organization of cell-secreted EDA-fibronectin to overcome the inhibition to invasion observed in the stiff matrix (Berger et al., 2020).

FUTURE DIRECTIONS

Owing to mounting evidence that ECM fibers are critically influential in tumor cell behavior and observations that omission of fibers may result in incorrect predictions of cellular outcomes (Berger et al., 2017; Davidson et al., 2020), an emphasis on fiber-tunable scaffolds has been emerging in the evolution of tumor tissue engineering. Many techniques allow alteration of fiber size independently of overall collagen concentration or bulk Young's modulus (Carey et al., 2012; Oh et al., 2020; Seo et al., 2020), although these approaches have been limited by issues such as poor control over fiber size modulation, non-physiological fiber sizes, or use of specialized equipment. There has been recent progress on this front, wherein simple modifications to the common collagen gelation protocol yielded physiological fiber sizes (Gong et al., 2020), although there remains a need for accessible technologies capable of modulating fiber features in a controlled manner. Another limitation that remains across these fiber-focused studies is that they are typically conducted with collagen-only materials and have not included other ECM cues prevalent in the tumor environment (e.g., fibronectin, laminin, HA). Moving forward, there is an immediate opportunity to blend fiber-tuning techniques with copolymerization of ECM cues, thereby creating multi-component scaffolds with independently tunable fiber density, fiber thickness, ECM composition, and bulk modulus. Interpreting these complex, multivariate relationships will be aided by computational approaches (Kreeger et al., 2018).

Studies that examine the influence of fiber features on cell behavior have also raised the intriguing question of whether the biological identity of the fiber even matters, or if it is the physical features of the structure that dominates the delivery of ECM information to the cells (Baker et al., 2015). Synthetic materials have been used to produce controlled, fibrillar environments to study fibrosis (Baker et al., 2015; Davidson et al., 2020), and these platforms may represent another path forward in advancing tumor-mimicking research. For example, a modified, non-native sugar (dextran vinylsulfone) can be electrospun to create scaffolds with mechanical tunability at both the single fiber and bulk scaffold level, while also allowing modulation of fiber density and alignment (Davidson et al., 2020). These fibers can be functionalized

with ECM-derived peptides or proteins to increase their biomimicry. This type of system has the potential to address many of the challenges of teasing apart the relative contributions of changes in architecture, composition, and mechanics on tumor progression.

There is also room for growth in developing dynamic scaffold environments that can undergo user-controlled temporal changes in ECM stiffness or composition. Dynamic stiffening of scaffolds can be achieved by multiple means (Madl and Heilshorn, 2018) and has been used to examine the role of matrix stiffness in tumor progression (Ondeck et al., 2019). Additionally, recent years have seen the development of numerous bio-orthogonal scaffold manipulations (Madl and Heilshorn, 2018) that enable dynamic modification of scaffold composition, which have not yet been applied in the context of cancer. A wide range of cytocompatible modification chemistries has been employed to temporally control the *in situ* addition, removal, and reversible patterning of ECM cues (DeForest and Anseth, 2011; Madl and Heilshorn, 2018; Shadish et al., 2019). As we learn more about the ECM changes that accompany tumorigenesis, these highly flexible techniques may be implemented to create defined, temporally tunable microenvironments that allow researchers to probe complex questions regarding the signals and mechanisms that drive tumor progression. Of course, one remaining complication is the difference in time scales between tumor ECM changes (e.g., days) and chemical modification of biomaterials (e.g., minutes).

Finally, although the aforementioned platforms are able to mimic many elements of the native ECM environment, the only scaffold that can truly recapitulate the complexity of the ECM is the native tissue itself. Although *ex vivo* organ cultures are typically seen as lacking tunability, opportunities exist to modulate the ECM in these intact tissues. Approaches to selectively deplete ECM components have been explored for other applications (Rodriguez et al., 2011). Meanwhile, although ECM enrichment of *ex vivo* tissues has not previously been described, the successful application of gene delivery to *in vivo* structures (Mueller et al., 2018) suggests that "ECM editing" of organ cultures is an achievable goal. The ECM complexity of these "top-down" scaffolds would complement the defined environments provided by "bottom-up" approaches. Together, the emerging technologies discussed in this section have the potential to provide robust tumor-mimicking platforms that will significantly advance our understanding of tumor biology and progression.

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AUTHOR CONTRIBUTIONS

Conceptual development was done by K.S.M. and P.K.K., literature review of ECM composition was conducted by M.R.V., literature review of mechanics was conducted by H.M.M. All authors contributed to writing and final editing of the manuscript. Funding was obtained by K.S.M. and P.K.K.

DECLARATION OF INTERESTS

P.K.K. has a sponsored research agreement with Novartis International AG. H.M.M., M.R.V., and K.S.M. declare no competing interests.

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