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Engineering Strategies to Mimic the Glioblastoma Microenvironment

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Abstract

Glioblastoma multiforme (GBM) is the most common and deadly brain tumor, with a mean survival time of only 21 months. Despite the dramatic improvements in our understanding of GBM fueled by recent revolutions in molecular and systems biology, treatment advances for GBM have progressed inadequately slowly, which is due in part to the wide cellular and molecular heterogeneity both across tumors and within a single tumor. Thus, there is increasing clinical interest in targeting cell-extrinsic factors as way of slowing or halting the progression of GBM. These cell-extrinsic factors, collectively termed the microenvironment, include the extracellular matrix, blood vessels, stromal cells that surround tumor cells, and all associated soluble and scaffold-bound signals. In this review, we will first describe the regulation of GBM tumors by these microenvironmental factors. Next, we will discuss the various in vitro approaches that have been exploited to recapitulate and model the GBM tumor microenvironment in vitro. We conclude by identifying future challenges and opportunities in this field, including the development of microenvironmental platforms amenable to high-throughput discovery and screening. We anticipate that these ongoing efforts will prove to be valuable both as enabling tools for accelerating our understanding of microenvironmental regulation in GBM and as foundations for next-generation molecular screening platforms that may serve as a conceptual bridge between traditional reductionist systems and animal or clinical studies.

1. Introduction

Glioblastoma multiforme (GBM) is the most common and deadly form of primary brain cancer, accounting for approximately 54% of all brain tumors in the United States [1]. Despite its prevalence and lethality, there is currently no definitive treatment for patients afflicted with GBM. This lack of treatments is often attributed to the diffuse and unrelenting infiltration of tumor cells throughout the brain, [2] a phenomenon famously observed by neurosurgeon Dr. Walter Dandy in the 1920s, when he took the extreme step of surgically

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removing entire brain hemispheres of two comatose patients afflicted with GBM, only to see the tumor return post-resection [3].

While current treatment options are significantly more sophisticated than those exercised by Dr. Dandy, patient outcomes still remain poor. Standard therapy consists of the combination of tumor removal through surgical resection, radiotherapy, and chemotherapy. Following resection, image-guided radiotherapy is typically applied to the tumor margins, often including concomitant treatment with the alkylating agent temozolomide (TMZ) [4]. Despite this aggressive treatment regimen, tumor recurrence at the margin of the resection occurs in approximately 90% of patients and mean survival time is only around 21 months [4,5]. One of the main difficulties in effectively treating GBM with conventional therapies is that tumors that appear similarly in histopathological presentation are often in fact quite distinct at the cellular and molecular levels. For example, recent genomic analysis of many patient-derived GBM samples revealed at least three distinct subtypes of GBM, each of which contains specific genomic lesions relative to matched normal brain tissue (Figure 1) [6,7]. Furthermore, there is substantial cellular heterogeneity within a single tumor, with mounting evidence supporting the idea that tumor progression is driven by a subpopulation of glioma stem/initiating cells, which have high tumor-forming potential and express many neural stem cell markers [8]. Because cells in each tumor are distinct from other tumors classified as GBM, conventional treatments targeting intracellular signaling pathways, such as those regulating proliferation, will likely only be effective for a small subset of patients, and perhaps then only transiently as resistance evolves.

Motivated by these findings, recent clinical trials have begun to explore new directions in the treatment of GBM with the aim of targeting the few common features shared across GBM subtypes. Instead of targeting cell-intrinsic pathways, these trials seek to intervene by manipulating the extracellular environment and the interactions of tumor cells with this environment, which is beginning to be recognized as a critical regulator of tumor progression [9–11]. Important components of the microenvironment include: 1) the extracellular matrix (ECM), the biopolymeric scaffold surrounding tumor cells, 2) non-tumor cells near or within the tumor, such as astrocytes, macrophages, endothelial cells, and fibroblasts, and 3) soluble and scaffold-bound signals such as growth and differentiation factors. Particularly intriguing is treatment with anti-angiogenesis drugs such as bevacizumab, which targets vascular-endothelial growth factor (VEGF), thereby reducing tumor-induced vascular recruitment. Bevacizumab has been shown to increase progression-free survival in phase III clinical trials when added to a regimen of radio- and chemotherapy, but does not significantly improve overall survival [12–15]. In another novel modality of GBM treatment, directing cell migration towards an external chemotherapeutic sink with an implanted, migration-promoting hydrogel significantly reduced glioma tumor size in a mouse model [16]. The preliminary successes of these interventions hint at the promise of targeting the microenvironmental interactions of tumor cells as a viable treatment strategy in GBM. The identification of such targets for possible intervention therefore is a critical direction for GBM research, and this has motivated the development of advanced methodologies for studying interactions between GBM and the tumor microenvironment.

The extraordinary complexity of the tumor microenvironment has made it exceedingly difficult to determine how individual parameters in the microenvironment contribute to tumor progression in vivo. Because of this, there has been growing interest in developing simple, reductionist systems that model individual features of the microenvironment to isolate the effect of these features while offering a degree of reproducibility and interpretability not achievable with in vivo systems. This review will highlight the roles of the microenvironment on tumor development and progression, specifically focusing on the development of in vitro systems that mimic the tumor ECM. We will begin with a brief overview of how the microenvironment is thought to regulate GBM progression, then we will discuss in depth the experimental systems used to model this complexity in vitro. Finally, we will highlight existing technologies and model systems that may be adapted for the study of GBM.

2. Role of Microenvironment in GBM Progression

The microenvironment of GBM cells is extraordinarily complex and is composed of various types of ECM proteins and glycosaminoglycans (GAGs) as well as a diversity of cell types. This section provides a brief overview of the roles of the microenvironment in GBM, a subject that has been reviewed much more extensively elsewhere [17–19].

2.1 ECM Components of the Microenvironment

The ECM of the tumor microenvironment serves not only as a passive scaffold in which GBM tumor cells grow and migrate, but also plays a significant and active role in directing cellular behavior. The main component of brain ECM is the polysaccharide hyaluronic acid (HA) [20,21], which is a glycosaminoglycan made up of repeating disaccharides of D-glucuronic acid and N-acetyl-D-glucosamine. HA is constitutively produced within GBM tumors, [22,23] and its abundance is associated with cell proliferation and infiltration. Its cellular receptor CD44 is also overexpressed in GBM, and cells containing CD44 localize to the normal brain-tumor interface in vivo, suggesting that CD44-enriched cells are more efficient at invading the brain parenchyma [24,25]. Ligation of CD44 with HA activates key pro-tumorigenic signals including the Rho family of small GTPases [26,27], which are known to affect motility and proliferation, as well as PI3 kinase [28], which is known to affect cell growth, proliferation and differentiation. HA may also be endocytosed after CD44 binding, and its smaller degradation products have been shown to promote tumor progression through mechanisms that remain unclear [29].

The high vascularization of GBM also contributes ECM cues to GBM cells. For example, the basolateral membrane of GBM-associated vessels contains collagen IV, collagen V, fibronectin, and laminin, all of which are comparatively less abundant in avascular regions of the brain [30–33]. These molecules have been found repeatedly to enhance cell survival, proliferation, and migration in vitro and in vivo [34–38]. Furthermore, the proteoglycan tenascin-C is produced by tumor-associated endothelial cells and its presence correlates with angiogenesis and the progression from grade II to grade III glioma [39,40].

Attachment to many types of ECM, including fibronectin, laminin, and collagen, is controlled by a class of membrane-spanning proteins known as integrins. Interestingly,

adhesion of integrins to their extracellular ligands may significantly desensitize GBM cells to therapy [41]. For example, α_1 integrin signaling has been negatively correlated with drug induced apoptosis in GBM [42]. Additionally, α_6 integrin is necessary for GBM stem cell self-renewal, proliferation, and tumor-forming capacity [37,43].

In addition to responding to existing ECM-based cues, GBM tumor cells also have the ability to actively shape the ECM for optimal cell growth and infiltration. Specifically, tumor cells may remodel the ECM through cell-secreted proteases, which “prime” the microenvironment for tumor progression by removing steric barriers for cell migration and angiogenesis, leading to enhanced GBM growth and dissemination. Urokinase plasminogen activator (uPA), cathepsin B, and matrix metalloproteinases (MMP) have been shown to be upregulated in GBM and high levels of these proteases correlate with poor prognosis in patients [44,45].

2.2 The Mechanical Properties of the Extracellular Matrix and their Role in Tumor Progression

The mechanical properties of the ECM have been increasingly recognized as critical to the progression of advanced tumors in vivo. Cells interact with their mechanical environment, and these interactions have been shown to influence such tumor-critical processes as migration, differentiation, apoptosis, and proliferation.

Cells mechanically engage their environment and respond to microenvironmental forces using several distinct mechanisms whose actions are collectively termed mechanosensing or mechanotransduction [46]. In order to probe the local mechanical properties of its environment, a cell must deform it by actively applying a force. In one common paradigm, cells apply forces to the extracellular environment through the structural positioning [47] and contraction of actin-myosin complexes [48], with the resulting force being transmitted along actin filaments to membrane-spanning focal adhesion complexes that are physically connected to the extracellular environment through integrin-based complexes [49,50]. Focal adhesions then respond to the stress and/or strain across the adhesion, which initiates a signaling cascade with downstream targets such as changes in actin polymerization and crosslinking [51], changes in focal adhesion size [52], and regulation of gene transcription [53].

Numerous clinical observations support the hypothesis that tissue stiffness is critical for the progression of a variety of solid tumors, especially breast tumors [11]. GBM tumors are also thought to be stiffer than the normal brain tissue surrounding the tumor, as suggested by ultrasound imaging of strain magnitudes during neurosurgery [54]. Although the detailed spatial variation of stiffness in GBM tumor tissue and the contributing mechanisms are yet to be described, there is a strong possibility that increased tumor stiffness may contribute to malignancy as has been explored extensively in epithelial cancers [55,56]. Furthermore, observations by Hans Scherer in the 1940s identified key tracks for GBM invasion into the brain, such as the vasculature beds and white matter tracts [57,58]. Now known as Structures of Scherer, these components of the brain are known to be significantly stiffer than the surrounding parenchyma, suggesting GBM guidance may be influenced by the mechanical properties of the Structures of Scherer.

Equally important to these clinical observations is the fact that GBM cells often acquire abnormalities in the mechanosensory machinery, including aberrant expression of key molecular components. For example, the critical mechanosensory protein focal adhesion kinase (FAK) is overexpressed in many GBM tumors. [59]. Additionally, GBM often exhibits altered expression of integrins, which, as discussed above, are critical for the physical transmission of force to the extracellular matrix from the actin cytoskeleton, in addition to simply mediating attachment to the ECM [60–62]. Finally, the leaky vasculature of GBM tumors contributes to peritumoral edema and increased interstitial fluid pressure (IFP) [63,64], which routinely causes morbidity and hinders drug delivery to the tumor, but can also alter the mechanical microenvironment through modulation of cell-ECM tension in both tumor and stromal cells [65]. All of these observations imply that an intricate link exists between the mechanical environment of the brain and GBM progression, although the precise molecular details remain incompletely understood.

2.3 GBM Interactions with Cells

GBM tumors are often composed of not only tumor cells but also parenchymal cells, which contribute to tumor progression and may in fact contribute more than 30% of the mass of a tumor [17]. This section will provide an overview of the types of interactions observed between GBM cells and parenchymal cells.

2.3.1 Perivascular Niche Cells—There is increasing evidence that the vasculature of GBM serves not only to provide an exchange medium for nutrients and waste products, but also that anatomical structures formed by tumor-associated vessels provide a pro-survival environment for GBM cells. These structures, known as perivascular niches, are composed of endothelial cells, pericytes, and astrocytes [17]. GBM vasculature is different than non-diseased vasculature throughout the brain and is often characterized by endothelial cell proliferation and hyperplasia [66]. Recruitment of vascular smooth muscle cells and pericytes is critical for forming a perivascular niche and for the survival of tumor-associated endothelial cells [67] and have been implicated in the progression of malignant gliomas [68].

Perivascular niche-associated cells, particularly astrocytes, deploy autocrine signaling to promote tumor malignancy and survival. GBM cells residing in the perivascular niche are often associated with reactive astrocytes which may induce GBM growth through the secretion of stromal cell-derived factor 1 (SDF-1) [69] and astrocyte elevated gene-1 (AEG-1) [70], both of which are frequently overexpressed in human brain tumors [71,72]. Interestingly, suppression of AEG-1 activity in a GBM mouse model diminished tumor growth, highlighting the importance of the perivascular niche to GBM progression [71]. Additional chemokines may serve as malignancy-promoting signals transduced by receptors that have been found on glioma cells including, chemoattractant protein-1 (CCL2), interleukin-8 (CXCL8), and RANTES (CCL5) [73]. Conversely, gliomas are also known to influence the proliferation, motility, and secretion of MMPs from astrocytes through soluble signaling cascades, suggesting that gliomas may leverage astrocytes to enhance their invasion [74–76].

The perivascular niche may also be important for the maintenance of brain tumor stem cells [77]. Because neural progenitor cells also associate with perivascular niches [78,79], these environments have been hypothesized to incubate GBM tumor-initiating cells and/or induce de-differentiation of tumor cells to a tumor-initiator like state [80,81]. Tumor cells that express stem cell markers CD133 and nestin have been shown to preferentially associate with the vasculature [80,82] and, remarkably, preferentially proliferate at vascular branch points [83]. Paracrine nitric oxide signaling has been suggested as a potential mediator of tumor stem cell maintenance, working through the Notch signaling axis to enhance self-renewal [84]. Similarly, interleukin-8 secretion by endothelial cells has been shown to enhance the proliferation and migration of GBM tumor stem cells [85]. Intriguingly, this effect was enhanced when the endothelial cells were cultured as three-dimensional networks compared to conventional monolayer culture, underscoring the importance of microenvironment dimensionality and mechanics in the malignant behavior of tumor-associated stromal cells. The field's understanding of the cross-talk between tumor-associated endothelia and tumor stem cells is complicated by recent studies that suggest GBM stem cells may themselves transdifferentiate to become endothelial cells and incorporate into the tumor vasculature [86–88].

2.3.2 Microglia—The majority of non-tumor cells within a tumor are tumor-associated macrophages, which are most often differentiated macrophages known as microglia and have suppressed immune functions [89,90]. In fact, microglia may modulate the growth and migration of glioma cells [91,92]. Microglia have been found to produce MMPs, including MT-MMP1, in response to soluble factors secreted by glioma cells, likely through the activation of toll-like receptors on the microglia surface which further activates the MAPK pathway [92]. Additionally, in glioma mouse models that exhibit impaired microglia or lack microglia altogether, glioma tumor size was significantly less than those with normal microglia [93].

3. Engineering strategies to model the GBM microenvironment

As discussed above, the microenvironment of brain tumors plays a very significant role in GBM development, progression, and treatment. In this section, we will discuss strategies that have been adopted to recapitulate some of the key features of the GBM microenvironment in vitro. These strategies borrow technologies from surface science, polymeric materials, and microfabrication to manipulate the architectural and molecular features of biomaterial scaffolds and present cells with highly sophisticated and controlled microenvironmental cues.

3.1. Limitations of traditional cell culture systems

The role of ECM components in tumor pathophysiology has traditionally been studied using two-dimensional (2D) monolayer cell culture on glass or plastic substrates. This involves functionalizing the surface with the protein or proteoglycan of interest, typically by adsorbing it from solution, or by adding the molecule to the cell culture medium to observe effects of soluble signaling mediated by the ECM component. This simple method has enabled the study of tumor cell behavior in culture and has yielded a wealth of information

on the role of several ECM molecules in tumor initiation and progression, often laying the groundwork for further studies using more physiologically appropriate models. For example, Berens and co-workers adopted this technique in a series of pivotal studies delineating the role of ECM components such as fibronectin, vitronectin, and hyaluronic acid in promoting the adhesion and migration of astrocytoma cells [94,95]. Similarly, the role of the matrix metalloproteases (MMPs) in glioma invasion was first established using monolayer culture systems [96]. Despite their utility in basic functional assays, simple monolayer culture suffers from several significant drawbacks when employed to study complex cell-ECM interactions. First, it is now well understood that cells in three-dimensional (3D) culture behave very differently than on flat 2D substrates: essential differences in the presentation, organization, and polarity of ECM proteins in 3D matrix result in concomitant modifications in the architecture and composition of cell-ECM adhesions and downstream signaling events [97,98]. Second, the stiffness of typical plastic or glass substrates exceeds 3 GPa, which is many orders of magnitude stiffer than brain, a tissue whose stiffness typically ranges from 100–5000 Pa [55]. Third, essential features of the 3D tumor microenvironment including hypoxia [99], increased interstitial fluid pressure [100], and cytokine concentration gradients are poorly reproduced by 2D culture. Finally, flat monolayer culture by its very nature is not naturally suited to studying the effects of matrix remodeling or cell-cell interactions. To overcome these obstacles, the field has turned to engineered microenvironments that can offer exquisite control over material composition, stiffness, and architecture in both 2D and 3D culture to systematically study the effects of complex cell-ECM interactions on GBM tumor pathology.

3.2. Studying the role of ECM stiffness

As discussed earlier, a wealth of indirect evidence based on clinical and empirical observations led to a suspected role for cell-ECM biophysical interactions in the pathophysiology of glioma. However, systematic mechanistic studies in this area have only become possible due to the development of cell culture substrata with independently controllable elastic modulus and biochemical functionalization. This is typically achieved by controlling the elastic modulus via the degree of crosslinking of a polymeric hydrogel matrix, which is then chemically functionalized – either by covalent attachment or adsorption from solution – by the ECM-derived protein or peptide ligand of interest to facilitate glioma cell attachment. The most commonly used such system is based on crosslinked polyacrylamide (PAA) and was originally introduced by Wang and co-workers in seminal studies that established the role of ECM stiffness on fibroblast morphology and motility [52,101]. This platform has since become widely adopted and has motivated the creation of multiple step-by-step protocols for generating PAA gels of tunable elasticity and ligand density [102–105]. Using these tunable PAA gels functionalized with fibronectin, our group demonstrated for the first time that the morphology, cytoskeletal architecture, proliferation, and motility of human GBM cells were exquisitely sensitive to the stiffness of the underlying substrate [106]. Cells were rounded and largely immobile on soft substrates, whereas increasing ECM stiffness resulted in a dramatic increase in cell area, focal adhesion formation, proliferation, and motility. Further, we found that this mechanosensitivity was dependent on non-muscle myosin II-mediated cellular contractility, such that inhibition of contractility using the small-molecule drug blebbistatin rescued invasion on soft substrates.

In follow-up studies, we have explored the role of the focal adhesion proteins α -actinin [107] and talin [108] in transducing mechanical cues, as well as the central role of the Rho GTPase RhoA in mediating the resulting contractility-dependent cellular phenotype [109]. As a potential caveat, a recent study suggested that changing the stiffness of the PAA substrate is potentially communicated to cells through alterations in the anchoring density of the tethered ECM protein [110]. However, stiffness-dependent phenotypes observed on PAA can often be reproduced with other, unrelated materials, including hyaluronic acid [111] and PDMS-based micropost arrays [112], suggesting that these PAA-based behaviors may be correctly interpreted as mechanosensory events. In any case, these tunable-stiffness culture systems can be used to delineate the molecular mechanisms by which ECM-encoded cues are sensed and processed by glioma cells and therefore may help identify molecular targets against tumor progression. For instance, through combined studies of glioma invasion in brain slice cultures [113] and mouse models [114], Rosenfeld, Canoll, and colleagues have shown that pharmacologic inhibition of myosin can severely limit tumor invasion even in the presence of potent pro-motility cytokines.

3.3. Modeling the glioma microenvironment in 3D ECMs

There is an increasing impetus in the field of tumor biology to study cell behavior in fully three-dimensional matrices that are significantly better at recapitulating physiological features than 2D monolayer culture. In addition to the fact that cells in 3D ECMs adopt shapes and adhesions that are much closer to those *in vivo* [115], 3D culture also facilitates the investigation of processes such as matrix remodeling and proteolytic degradation, which are central to tumor invasion [116]. Consequently, there have been strong efforts to study glioma cell-ECM interactions in 3D matrices to elucidate the role of matrix density, stiffness, and architecture in glioma progression.

3.3.1 Self-assembled biopolymer gels—Much early effort to investigate glioma invasion in 3D matrices emphasized hydrogels composed of native biopolymers such as collagen I, or Matrigel, a laminin-rich ECM extract from mouse sarcoma tumors. For instance, initial studies of U87-MG glioma spheroid expansion in Matrigel indicated that the growing spheroid exerted compressive forces whereas invading cells exerted traction forces on the ECM, showcasing the varied nature of physical interactions involved [117]. To further analyze how glioma cells interact with the ECM, Kaufman and colleagues studied glioma spheroid invasion in collagen I gels of varying concentration [35]. Although increasing collagen I concentration facilitated spheroid invasion due to an increase in fibers available for cell-ECM adhesions and traction, spheroid growth was inhibited at higher collagen I concentrations, perhaps because of the inhibitory effect of growing against a dense matrix. This latter observation was broadly confirmed with a wider panel of glioma cell types, where the cellular levels of cadherin and matrix metalloprotease (MMP) expression were also found to impact the invasive pattern [118]. Indeed, degradation of the collagen matrix by MMP activation – for example, upon Epidermal Growth Factor (EGF) stimulation - is one mechanism by which glioma cells can overcome the inhibitory effect of a denser ECM on cell migration [119]. A recent study employed temperature-controlled nucleation of collagen I fibers to partly deconvolute collagen concentration and matrix pore size. Collagen matrices nucleated at 22 °C as opposed to 37 °C exhibited a more porous

network architecture, and permitted greater glioma spheroid invasion upon increasing collagen concentration, thus establishing the dominant role of matrix porosity in glioma invasion [120].

3.3.2 Synthetic ECMs—Although the studies described above using biopolymer gels have yielded a great deal of insight into the regulation of glioma by ECM, their interpretation and applicability to the physiological situation are complicated by two factors. One, the normal brain ECM, as discussed above, is almost entirely devoid of fibrillar collagens, being instead composed of a dense non-fibrillar matrix based on a HA-proteoglycan-tenascin network [121]. Further, as discussed above, changing the concentration of the gel-forming biopolymers simultaneously affects ECM ligand density, network architecture, and stiffness, making it difficult to assess the independent contributions of these parameters in regulating glioma invasion. These drawbacks of native biopolymer gels can be partly overcome by synthetic ECM platforms based on crosslinked natural or synthetic polymers, where ECM stiffness and biochemical ligand functionalization can be adjusted independently. Over the last few decades, a number of ECM platforms have been developed to address these needs (as previously reviewed [122] and [123]), some of which are now commercially available. Here we restrict our focus to the application of these biomaterials for studying glioma progression in 3D.

Our laboratory adopted an approach for decoupling ECM stiffness from protein concentration in collagen I hydrogels by mixing the protein with agarose, an inert biopolymer that forms a meshwork entangled with the collagen and serves to stiffen the hydrogels with modest alterations in the fiber architecture [124]. Using this platform, we found that increasing matrix stiffness in 3D inhibited and eventually abrogated glioma spheroid invasion – a result opposite to that observed on 2D substrates [106]. This apparent contradiction is explained by the fact that migration in 3D collagen matrices requires cellular traction-mediated remodeling of collagen fibers while navigating steric barriers within the matrix, which are both impeded by hydrogel stiffening due to added agarose [125]. Further, single-cell migration in denser, agarose-rich matrices showed a phenotype reminiscent of amoeboid motility, with cells exhibiting dynamic path-finding protrusions at the leading edge and constrictions in the cell body to pass through narrow spaces in the ECM. Amoeboid motility has previously been observed in cells lacking protease activity [126,127] and is thought to be a mechanism by which tumor cells can escape therapeutic interventions that inhibit MMPs [128]. To further elucidate the role of matrix density in governing the mode of 3D glioma cell motility, we sought to create synthetic ECMs that more closely mimicked the architecture of native brain tissue. To this end, we adopted an ECM platform consisting of methacrylated HA functionalized with ECM-derived peptide ligands, such as the cell-adhesive RGD sequence, and crosslinked by dithiols such as dithiothreitol (DTT), enabling independent control of cell adhesivity and stiffness over ~3 orders of magnitude [111,129]. Importantly, these HA ECMs were devoid of fibrillar structures and had a high density with submicron porosity, similar to brain matrix [121]. U373-MG glioma spheroid invasion in HA ECMs was again inhibited by increasing ECM stiffness, as observed before; further, cells invading in the dense HA ECMs displayed a phenotype unlike that observed in fibrillar collagen matrices but strikingly similar to that previously observed in brain slice

cultures [113] (Figure 2). This result raises the exciting possibility that HA-based synthetic ECMs can be used to dissect the signaling mechanisms that underlie the aggressive invasiveness of glioma cells, eventually leading to new molecular targets. These HA-based ECMs could also serve as a reductionist platform for studying the mechanisms by which the HA receptor CD44, which as described earlier activates intracellular signals that increase glioma cell proliferation and invasion [130,131].

3.4. Microfabricated platforms for studying cell-ECM interactions

An emerging trend in studies of tumor physiology *in vitro* is the use of microfabricated platforms that offer exquisite control over the topography, roughness, elasticity, and biochemical functionalization of the cell culture interface. Although these systems may not have the full complement of biochemical information encoded in native biopolymer gels or enable fully 3D studies of tumor invasion as in synthetic hydrogels, their specific advantage is in presenting a complex set of physico-chemical cues with control over spatial presentation at length scales relevant to cellular interactions, i.e. 10 nm – 10 μ m. (as previously reviewed [132], [133], [134]). Here we shall focus on the application of these microfabricated systems to elucidate key aspects of glioma pathophysiology.

Zhu *et al.* used direct laser irradiation to create periodic grooves spaced ~ 200 nm apart on a polystyrene film and reported robust alignment of C6 rat glioma cells along the groove orientation [135]. This phenomenon, known broadly as contact guidance, has been reported widely for many neural cell types in culture and verifies the sensitivity of cell shape and cytoskeletal structure to physical constraints imposed upon it by the topography of the environment. A recent study used soft lithography molding of poly(dimethylsiloxane) (PDMS) to create lines and grooves and showed that primary glioma tumor cells showed directionally persistent migration along the lines on the surface [136]. To directly investigate the interplay between topographical confinement as experienced by a cell migrating in a dense 3D environment, and the stiffness of the matrix – which are intricately coupled in 3D culture platforms – our laboratory studied glioma cell migration in microfabricated polyacrylamide channels with independently tunable channel width and wall stiffness [137]. We found that confinement in narrow channels (< 10 μ m width) increased glioma cell migration speed across all ECM stiffnesses, relieving the inhibitory effect of high ECM stiffness on 2D unconfined migration (Figure 3). Further, this confinement sensitivity was mediated by myosin II-mediated polarization of cytoskeletal traction forces, consistent with an established role for myosin II in enabling glioma cell migration in the dense brain environment [113]. We later used this platform to explore the interplay of these microenvironmental parameters with the expression of canonical oncogenes [138]. Another recent study from a different set of investigators used microcontact printing of adhesive fibronectin islands at different densities to simulate the physical compaction that is experienced by a growing tumor *in vivo* [139]. This study found that compaction increased expression of collagens IV and VI as well as the collagen crosslinking enzyme lysyl oxidase, which led to increased VEGF-mediated angiogenesis. Thus, microfabricated ECMs can be used to simulate several key features of the glioma microenvironment and uncover novel mechanisms that contribute to glioma progression.

4. Future directions in modeling GBM *in vitro*

One of the outstanding challenges in cancer drug discovery is to improve the success rate of potential anticancer agents that succeed in clinical trials, which is currently around 5% [140]. This low success rate is partly due to the poor predictive ability of preclinical models of human cancers, such as mouse xenograft models [141], which in turn is due to their inability to faithfully reproduce non-cell-autonomous contributions to tumor pathology. The field of ‘tumor engineering’ seeks to bridge the gap by building sophisticated models of human cancer *in vitro* by leveraging advances in tissue engineering and biomaterials to recapitulate the tumor microenvironment with greater fidelity while retaining the essential genetic and epigenetic background through the use of appropriately sourced human cells [142–144]. In the next section, we will discuss the ways in which this strategy may aid in modeling important features of GBM *in vitro*.

4.1. Modeling glioma motility in 3D

Because GBM aggressively invades brain tissue, effective inhibition of tumor cell migration is widely considered an important therapeutic objective [145,146]. Interestingly, glioma cells invading the dense brain parenchyma have been observed to exhibit a distinctive type of motility that features highly dynamic protrusions and saltatory forward movements and requires myosin II-mediated contractility to squeeze the cell-body through narrow spaces [113]. However, glioma cells undergoing perivascular migration along blood vessels exhibit conventional mesenchymal motility with an elongated cellular phenotype [147]. These observations reinforce the fact that many tumor cells display significant plasticity in their modes of motility, implying a redundancy in the underlying mechanisms that allows cells to ‘tune’ their motility apparatus to effectively migrate in a variety of environments [148,149]. It is apparent that the diversity in cellular motility modes is in part reflective of the varied microenvironmental barriers cells must overcome to migrate in 3D [150]; however, we still have a very limited understanding of how these extracellular inputs are processed and integrated to enable the proficient invasion that is the hallmark of glioma. Our work using collagen-agarose ECMs and HA-based hydrogels indicated that increasing density of the 3D matrix causes a transition from an elongated mesenchymal mode to a contractility-dominated amoeboid-like phenotype [111,124]; however, the molecular mechanisms underlying this switch are only beginning to be elucidated. Several studies have indicated a role for the Rho GTPases Rac1, RhoA, and proteins that regulate the balance of their activity in governing different modes of glioma migration [147,151–153]. It is also becoming evident that the size and deformability of the nucleus often represent the rate-limiting step in migration through dense environments such as brain tissue [113,154]. Future work in this area using the appropriate 3D ECM models is very much needed for obtaining a coherent overall picture of the regulation of glioma migration and how it may be effectively inhibited in prospective therapies.

4.2 Modeling Interactions between GBM Tumor Cells and Other Cells

4.2.1 Myelinated Axons—Conventional substrates for the study of GBM migration are problematic in that they do not adequately represent the migration along the Secondary Structures of Scherer, which are perhaps the most common routes of dissemination for GBM

in vivo. Myelinated axons, the main component of white matter tracts [155], have significant research value to many other research fields, such as the study of multiple sclerosis [156]. As such, significant effort has been made to create in vitro cultures that produce myelinated axons, primarily by the co-culture of immature oligodendrocytes with neurons [157]. Unfortunately, these myelinated axons have yet to be significantly incorporated into in vitro models of GBM, perhaps due to the significant technical burden associated with isolating, culturing, and patterning these cells. To achieve simpler and more reproducible systems, researchers have begun adapting approaches from materials science to mimic the aligned and discontinuous nature of the Structures of Scherer.

Electrospinning is a simple and effective method that applies high electrical potentials to polymer solution droplets to produce thin polymer fibers that can range from around 2 nm to hundreds of microns in diameter [158]. Electrospun fibers can be aligned as they are collected and functionalized to permit cell adhesion. Electrospun fibers have been widely exploited as tissue engineering scaffolds, specifically in the subfield of neural regeneration, and have been used effectively as substrates to investigate and promote neural stem cell differentiation [159], Schwann cell maturation [160], and as guides for neural migration following repair from injury [161,162]. Recently, these substrates have been adopted for the study of GBM[163,164]. For example, poly- ϵ -caprolactone (PCL) electrospun fibers have been shown to recapitulate important features of GBM migration observed in vitro. Using a core-spinning technique to independently vary the physical and chemical properties of PCL fibers, Rao et al. found that cell morphology, FAK expression, and myosin light chain-2 expression all strongly depend on fiber modulus [164]. Future effort will be needed to improve the degree to which these systems mimic tissue architecture, particularly with respect to incorporation of physiologically relevant ECM ligands and development of three-dimensional topologies.

4.2.2 Endothelial Cells—There has been substantial effort to understand the coordinated chemical signaling between blood vessels and glioma cells. Most in vitro efforts to model this have used simple co-culture models in which endothelial cells are cultured on the same surface as glioma cells [165–167]. These systems have revealed that endothelial and glioma cells can communicate through soluble paracrine signals and also physical signals when the cells are in direct contact. While these methods are useful for studying biochemical signaling between the vasculature and tumor, much work remains to be done to improve the degree to which these systems capture the cellular organization and architecture of the vascular-tumor cell interface.

GBM researchers may benefit from leveraging the methods developed by tissue engineers to produce realistic 3D vascular structures. Endothelial cells cultured on carrier beads and embedded in 3D hydrogels will spontaneously grow to form preliminary vessels [168]. Furthermore, spatially patterning PEG hydrogels with light to contain localized zones of bound integrin-adhesive RGD peptide or VEGF can guide the formation of blood vessels in vitro [169]. More sophisticated approaches have recently been developed that use a sacrificial 3D printed sugar-glass layer to form the structure of the endothelial cell network or microfluidics molds to produce realistic blood vessels [170–172]. Perhaps adopting these approaches to form model vasculature, in combination with glioma cells, will lead to further

insights into the mechanism of intravasation of GBM cells and the perivascular niche regulation of tumor initiating cells, as discussed previously [85].

4.2.3 Microglia, Astrocytes, and Tumor-Associated Fibroblasts—Microglia, astrocytes, and tumor-associated fibroblasts profoundly affect tumor formation *in vivo*, as discussed earlier [91–93]. While simple co-culture studies have revealed much about the nature of the soluble paracrine signaling between these cell types [173], it is likely that they may also physically communicate. As such, GBM researchers may benefit from the ability to control seeding density and cell-cell contact by using spatially controlled coculture systems developed for other cell types. There are many systems that have been developed to spatially localize multiple cell types *in vitro* [174], including simple selective patterning, most often using microcontact printing of multiple cell-adherent proteins, where one cells can bind one, but not both, of the patterned ligands [175]. Microfluidic platforms have also been designed to control spatial localization of cells. [176] Additionally, systems have been developed in which adhesion can be controlled both temporally and spatially, with heat or electrical charge, which allow seeding of one cell type followed by the exposure of more ligand that allows the second cell type to adhere in a spatially controlled manner [177,178].

Spatially controlled co-culture is also possible in three dimensions. When dispersed in mineral oil, small, cell-laden hydrogel building blocks will assemble into complex and controlled structures which may then be crosslinked into a continuous gel using UV light [179]. Alternatively, 3D tissues can be assembled by the sequential layering of detached cell monolayers [180]. Spatially controlled 3D cultures may also be formed through microfluidics-based approaches, where different solutions are slowly flowed through microchannels to spatially localize individual components [181]. The adoption by cancer biologists of these complex and controlled systems will likely aid in understanding the complex interactions between tumor and stromal cells.

4.3 High Throughput approaches to ECM Screening

As discussed previously, microenvironmental regulation of GBM is complex and multifactorial. Because of this, conventional low-throughput discovery platforms will likely not be able to fully uncover the specifics of the ECM-regulation of GBM cells. Furthermore, since chemoresistance is influenced by integrin adhesion to the ECM, multiplexed arrays with well-controlled cell-ECM interactions may facilitate both our fundamental understanding of how this adhesion drives biology and screening of chemotherapeutic drugs in a more physiologically-mimetic context than conventional screening assays.

Robotic spotting of ECM proteins has proven to be an extremely useful, versatile, and high-throughput means to test the effects of ECM on cells [182]. By combinatorially and sequentially varying the amounts of collagens I, III, IV, laminin and fibronectin, Alberti *et al.* found that specific combinations of these molecules controlled embryonic stem cell fate in ways that would have been nearly impossible to predict using traditional, lower-throughput paradigms [183]. Using the same approach, they were then able to devolve combinatorial effects of growth factor signaling and ECM signaling. Similarly, one can vary the mechanical properties of hydrogels using a robotic spotter and UV light to initiate

photopolymerization, with one study screening 17,000 distinct combinations of stiffness and ligand in one experiment (Figure 4) [184,185] These high-throughput platforms need to be leveraged for constructing more sophisticated and physiologically relevant in vitro models of GBM that can be used for drug discovery screens.

5. Conclusions

Despite recent revolutions in molecular and systems biology that have facilitated our fundamental understanding of cell-intrinsic regulation of GBM, the prognosis for this disease remains quite poor, suggesting that cell-extrinsic or microenvironmental interactions may also substantially contribute to disease progression. Extensive research has revealed a significant role for the microenvironment - including adjacent cells, the ECM, and the vasculature - in maintenance of GBM. The extreme complexity and potential importance of the microenvironment in vivo has motivated enormous effort to develop next-generation platforms for capturing key components of the GBM tumor microenvironment in vitro, which is in turn allowing unprecedented dissection of microenvironmental regulation of GBM.

While significant groundwork has now been laid in this direction, significant challenges remain. First, the extreme complexity of the in vivo environment remains to be modeled in a reproducible and highly controlled fashion. By integrating multiple reductionist approaches systematically and deploying them in a high-throughput fashion, researchers should be able to create more complete, reproducible, and controllable in vitro GBM microenvironments. In addition to the complexity of the microenvironment, significant knowledge gaps remain in our understanding of tumor complexity and heterogeneity, both within a single tumor (as exemplified in the increasingly recognized role of tumor stem cells) and across different GBM tumors (as embodied by the four distinct GBM subtypes). It is possible, perhaps even likely, that different tumor cell types will respond differently to the same constellation of microenvironmental cues. Finally, it will be important for investigators to leverage these increasingly complex in vitro systems for the high-throughput drug discovery and screening, which we envision will help bridge the gap often found between highly reductionist systems, animal studies, and clinical trials.

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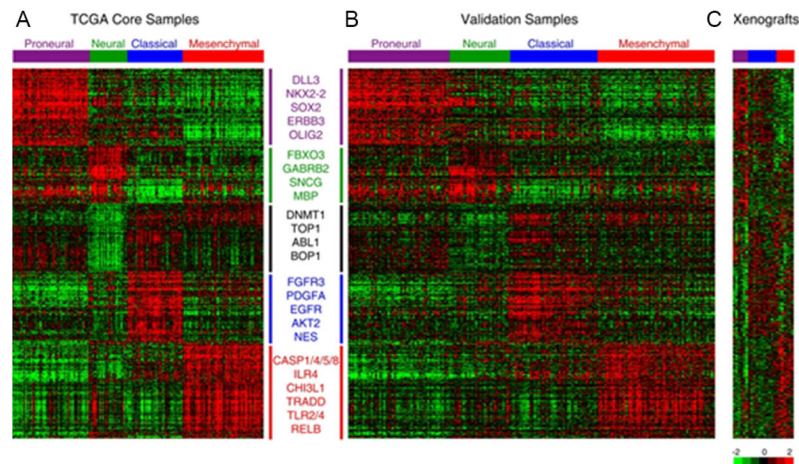


Figure 1. Heterogeneity in GBM tumors. Hierarchical clustering of 200 tumors and 1740 genes revealed four distinct, statistically significant subtypes in GBM samples, which can be minimally represented by a predictive 840 gene sample (A). Red depicts genes that are overexpressed relative to normal tissue, while green depicts genes that are underexpressed. The four subtypes are named according to the lineage the tumor type most resembles. Performing the same analysis on either previously published data (B) or xenografts taken from mice (C) confirm the presence of four distinct subtypes. Figure adapted from Verhaak et al (2010), with permission.

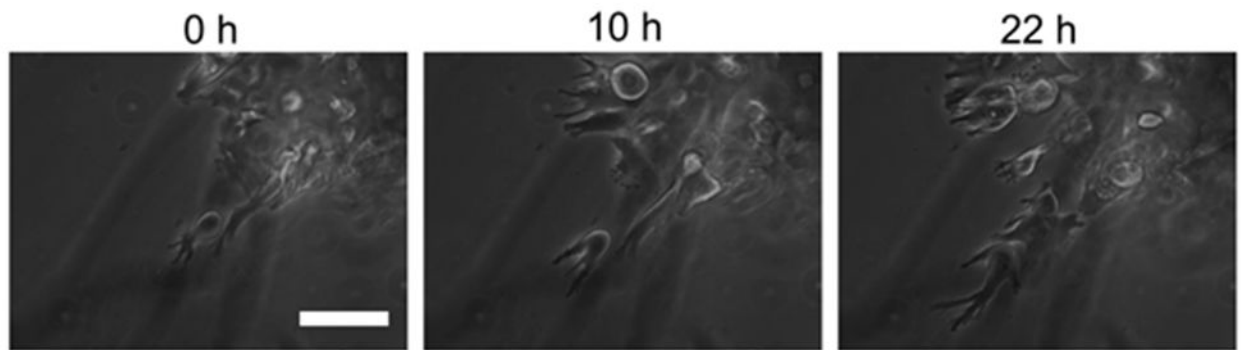


Figure 2.

GBM cell motility in dense ECMs. U373-MG cells migrating in dense 3D hyaluronic acid-based hydrogels adopted a phenotype displaying dynamic, branched leading protrusions with abrupt cell-body movements, distinct from mesenchymal motility in 3D Collagen gels but strikingly similar to the motility observed in brain tissue. Figure adapted from Ananthanarayanan et al (2011), with permission.

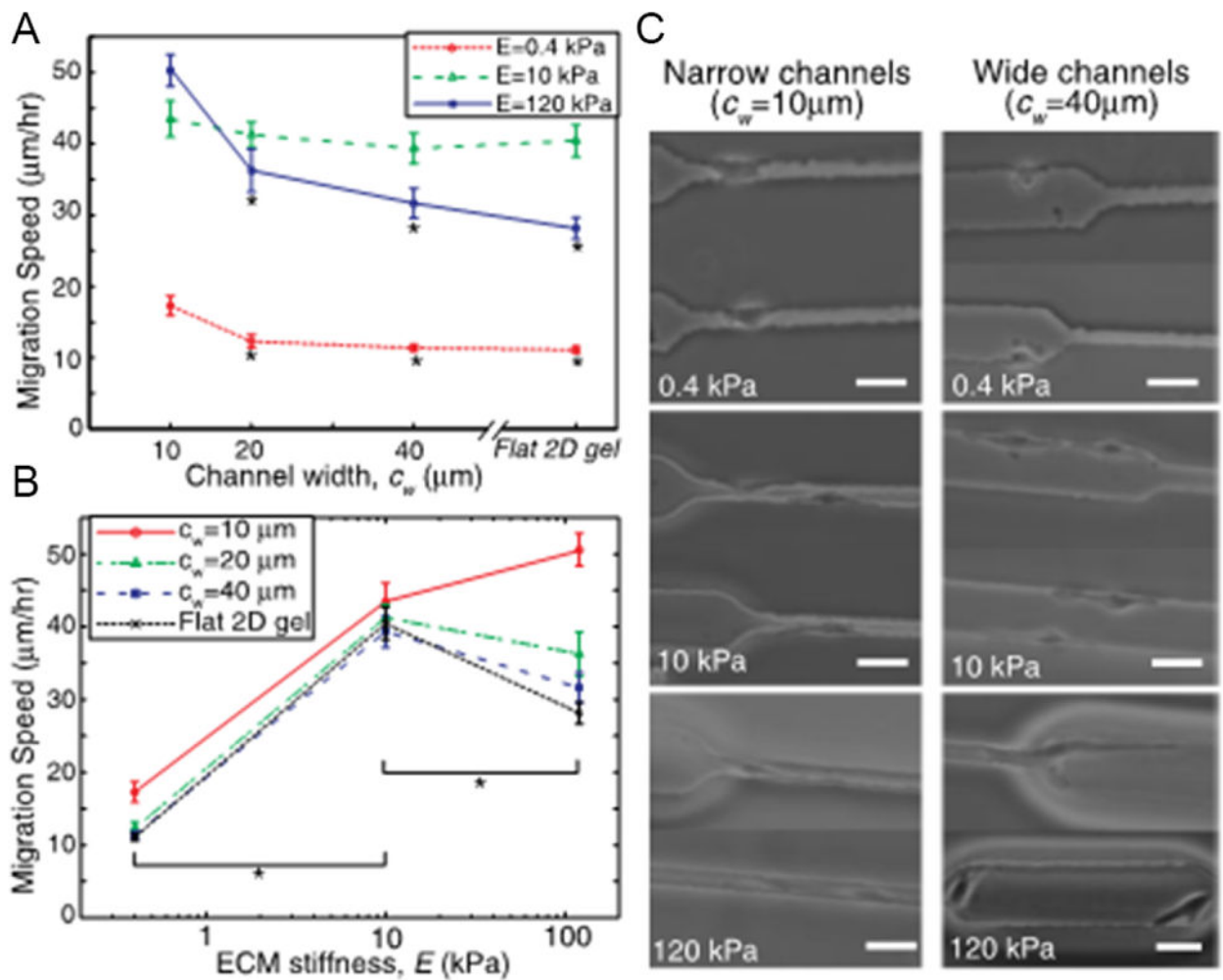


Figure 3.

Orthogonal regulation of GBM cell motility by ECM stiffness and topographical confinement. Migration of U373-MG cells was studied in microfabricated fibronectin-coated polyacrylamide substrates with independently varying channel widths c_w and wall stiffness E . Confinement in channels of narrow width increased migration speed for all values of wall stiffness (A) and relieved the inhibitory effect of high stiffness (B). Phase contrast images of the migrating cells shown in (C). Figure adapted from Pathak et al (2012), with permission

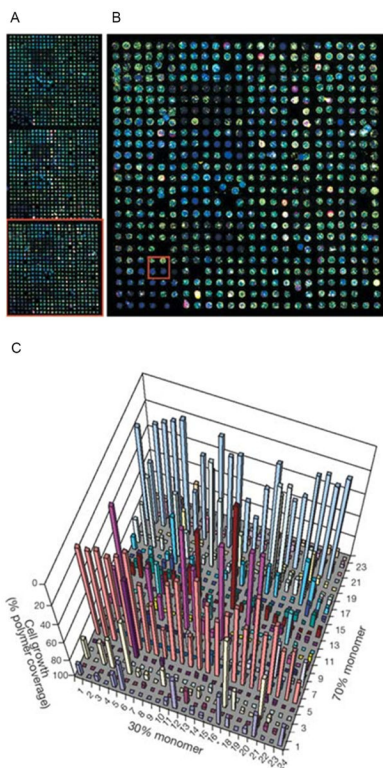


Figure 4. High throughput combinatorial screening of matrix configurations for human embryonic stem cell culture. Robotic spotting technologies were used to create highly precise arrays of 576 unique configurations of polymer gels (A, B). The arrays were then seeded with human embryonic stem cells and assayed for cell growth by quantifying the percent area of the island occupied (C). The high degree of multiplexing allowed for the identification of specific conditions that encourage embryonic stem cell growth in a manner that would have been largely impossible using conventional methods. This technology could be adapted to study the combinatorial effects of different ligand combinations on GBM behavior. Figure adapted from Anderson et al (2004), with permission.