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Application of Engineered Cell Culture Models to Study Glioma Stem Cell Motility

by

Kwasi Yeboa Amofa

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Joint Doctor of Philosophy

with the University of California, San Francisco

in

Bioengineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Sanjay Kumar, Chair

Professor Lydia Sohn

Professor Zev Gartner

Summer 2024

Abstract

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Professor Sanjay Kumar, Chair

In the past two decades, glioma stem cells (GSCs) have been increasingly implicated in driving tumor initiation, resistance, recurrence, growth and invasion in glioblastoma (GBM). Despite *in vivo* evidence identifying GSCs at invasive regions of GBM tissue, GSC invasion and by extension motility has been less well appreciated. Furthermore, GBM tumor invasion is informed by a multitude of biochemical factors, such as cytokines and growth factors, and biophysical factors, such as matrix and stroma geometry and mechanics. GBM tumors invade slowly through the hyaluronic acid (HA)-rich parenchyma and then rapidly along microvascular tracks of varying geometries. How biophysical and biochemical factors together contribute to driving invasion of GBM tumor cells such as GSCs within these regions is not well understood. Progress in understanding this combinatorial effect is limited by a lack of physiologically representative cell culture models that can enable systematic investigations to gain better understanding of regulators of invasion.

In this dissertation, we first applied an HA-based hydrogel model of the brain parenchyma to study transforming growth factor beta (TGF- β) induced invasion of GSCs. We demonstrate that in response to TGF- β , GSCs differ in their ability to invade HA in a way that can be predicted from TGF- β receptor 2 expression and SMAD2 phosphorylation. Additionally, we found an association between TGF- β responsiveness and GSC subtype. Interestingly, TGF- β stimulated GSC invasion exhibited a strong dependence on the presence of RGD peptides. Next, we deployed protein micropattern lines with vessel-like geometries to understand the emergent cell migration behavior of GSCs along a confined environment. We tested multiple GSC lines and found that vessel-like geometries enhanced both migration speed and persistence in GSCs. However, no individual GSC line demonstrated both enhanced migration speed and persistence, suggesting that vessel-like geometric confinement differentially influence migration dynamics of GSCs.

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Chapter 1: Regulation of Tumor Invasion by the Physical Microenvironment: Lessons from Breast and Brain Cancer

Parts of this chapter are excerpted with permission from Annual Reviews, from the article “Regulation of Tumor Invasion by the Physical Microenvironment: Lessons from Breast and Brain Cancer,” by Garrett F. Beeghly, Kwasi Y. Amofa, Claudia Fischbach, and Sanjay Kumar in Annual Review of Biomedical Engineering, 24, 29-59 (2022).

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1.1 Abstract

The success of anticancer therapies is often limited by heterogeneity within and between tumors. While much attention has been devoted to understanding the intrinsic molecular diversity of tumor cells, the surrounding tissue microenvironment is also highly complex and coevolves with tumor cells to drive clinical outcomes. Here, we propose that diverse types of solid tumors share common physical motifs that change in time and space, serving as universal regulators of malignancy. We use breast cancer and glioblastoma as instructive examples and highlight how invasion in both diseases is driven by the appropriation of structural guidance cues, contact-dependent heterotypic interactions with stromal cells, and elevated interstitial fluid pressure and flow. We discuss how engineering strategies show increasing value for measuring and modeling these physical properties for mechanistic studies. Moreover, engineered systems offer great promise for developing and testing novel therapies that improve patient prognosis by normalizing the physical tumor microenvironment.

1.2 Introduction

Cancer was once viewed as a tumor cell–autonomous disease in which the accumulation of successive genetic mutations drives uncontrolled cell proliferation and resistance to cell death.¹ In time, scientists and clinicians gradually began to appreciate the contribution of surrounding noncancerous tissue, known as the stroma, to disease progression.² Stephen Paget first proposed this seed and soil hypothesis in 1889, positing that a given tissue environment acts as soil to either promote or restrict the growth of so-called tumor seeds. However, this theory was not widely embraced for another century, as cancer became increasingly framed as a systems-level disease. Initially, the microenvironment was viewed solely in terms of biochemical interactions between tumor and stromal cells, including secreted cytokines and growth factors that drive disease progression.^{3,4} It has now become clear that physical remodeling of the stroma also powerfully contributes to tumor progression.^{5,6} However, uncovering the exact mechanisms by which physical properties of the tumor microenvironment mediate disease outcomes requires further investigation.

Developing tumors induce numerous physical changes in the stroma, including (a) aberrant tissue microarchitecture, (b) altered material properties such as stiffness, (c) increased solid stress, and (d) elevated interstitial fluid pressure (IFP).⁵ Increased tissue stiffness is the most clinically accessible of these changes, with lesions that are firm,

irregular, and palpable classically drawing the most suspicion. Tissue stiffening results from increased total cell density and extracellular matrix (ECM) deposition by both tumor and stromal cells. As a developing tumor expands, it also deforms and displaces existing structures within the peritumoral space, exerting tensile and compressive forces that generate solid stress within the surrounding tissue.⁷ Notably, increased solid stress disrupts normal tissue microarchitecture and leads to the collapse of blood and lymphatic vessels within the tumor and nearby stroma.⁸ As a result, blood flow and lymphatic drainage are compromised, leading to fluid accumulation and increased IFP. This elevated IFP in turn increases interstitial fluid flow (IFF) from the tumor into surrounding noncancerous tissues in which pressure is lower, facilitating the transport of tumor-secreted factors in the process.⁹ To identify how these changes impact the development, progression, and prognosis of cancer, it will be critical to integrate experimental approaches across the biological and physical sciences.

In this review, we discuss physical changes to the tissue microenvironment that drive cancer progression and highlight how bioengineering strategies can be used to fundamentally improve our understanding of these phenomena. Although many of the concepts we cover are relevant to all solid tumors, we compare breast and brain cancer as instructive examples. In particular, we highlight how physical aspects of the breast and brain stroma modulate invasion with a focus on how tumor cells in both diseases exploit structural guidance cues, contact-dependent heterotypic cell–cell contact, and elevated IFP. We then outline how specific bioengineering strategies have contributed to our ability to measure and model these mechanical properties for mechanistic studies.

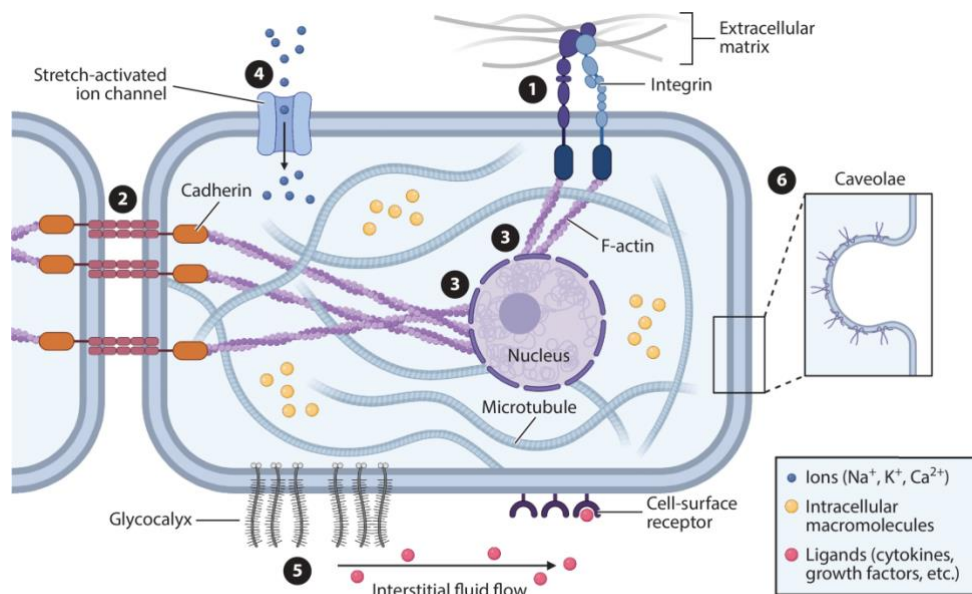
1.3 Breast cancer and Glioblastoma as instructive comparison

Mechanistic discovery in cancer research has almost exclusively focused on dissecting the molecular events that drive the development of specific cancer types and subtypes.¹ These efforts have deeply enriched our understanding of tumorigenesis and facilitated the development of targeted therapies. However, efforts to generalize findings have been frustrated by the diverse molecular backgrounds of each tumor and patient, yielding variable and unpredictable responses to treatment.¹⁰ For example, breast cancer originates from epithelial tissue and has favorable patient survival for early-stage disease but poor prognosis when tumors metastasize.¹¹ Glioblastoma (GBM), in contrast, originates from neuroglial tissue, universally exhibits poor patient survival, and almost never metastasizes.^{12,13} In contrast to molecular events, physical changes are often shared between solid tumors regardless of the tissue of origin. Both breast cancer and GBM exploit structural guidance cues, contact-dependent heterotypic cell–cell interactions, and dysregulated IFP and IFF to mediate tumor invasion. In breast cancer, invasion precedes entry into the vasculature and the formation of distant metastases. In GBM, invasion produces diffuse tumor margins that make surgical resection difficult and recurrence more probable. Breast cancer cells align collagen fibers in the stroma to regulate their invasion, while GBM cells co-opt existing vascular structures to infiltrate the brain parenchyma.^{14–16} Invasion is further aided by direct cell–cell contact of breast cancer cells with myofibroblasts and GBM cells with neurons via heterotypic cadherin-based or microtubule-dependent junctions, respectively.^{17,18} Similarly, breast cancer and

GBM cells must both overcome physical barriers provided by the myoepithelium and astrocytic processes that impede invasion.^{19,20} The collapse and dysfunction of tumor-associated vessels elevate IFP and IFF for both tumor types, leading to the formation of chemotactic gradients that drive cancer cell migration out of the tumor bulk.^{21,22} Moreover, increased IFP and IFF also impede delivery of chemotherapies to the tumor core.²³ Thus, understanding and targeting these shared physical phenomena that collectively modulate invasion could offer a novel approach to restrict disease progression and improve patient prognosis across otherwise distinct tumor types.

1.4 Review of mechanotransduction

Both tumor and stromal cells respond to physical cues via mechanotransduction, the process by which cells convert mechanical inputs into biochemical signals (**Figure 1**).²⁴ One of the most-studied mechanisms by which cells sense physical cues is through the formation of focal adhesions (FAs) and consequential remodeling of the cellular cytoskeleton.²⁵ FAs are micron-scale, multiprotein complexes that include clusters of transmembrane integrin heterodimers.²⁶ Outside the cell, integrins bind to ECM proteins such as collagen, fibronectin, and laminin. Inside the cell, scaffolding proteins such as talin, tensin, and vinculin connect integrins to the cytoskeleton. While the actin cytoskeleton is often emphasized in discussions of FA biology, FAs also coordinate the transmission of mechanical force to microtubules and intermediate filaments.^{27–29} External mechanical forces transmitted to FAs change the conformation of specific intracellular proteins (e.g., talin), thus altering binding kinetics and recruiting downstream signaling molecules such as FA kinase (FAK), paxillin, and Src that elicit biochemical responses. Similarly, adherens junctions are cell–cell adhesions formed between transmembrane cadherins that bind to the actin cytoskeleton via catenin proteins.³⁰ In addition, forces may also be transmitted between cells through intermediate filament–based complexes, such as desmosomes.^{31,32} Applied forces at cell–matrix or cell–cell adhesions can propagate through the cytoskeleton and act directly on the nucleus via linker of nucleoskeleton and cytoskeleton (LINC) complexes.³³ The resulting deformation of the nucleus is thought to alter chromatin packing and transport of biomolecules via nuclear pores to either promote or inhibit the transcription of mechanoresponsive genes.^{34,35}



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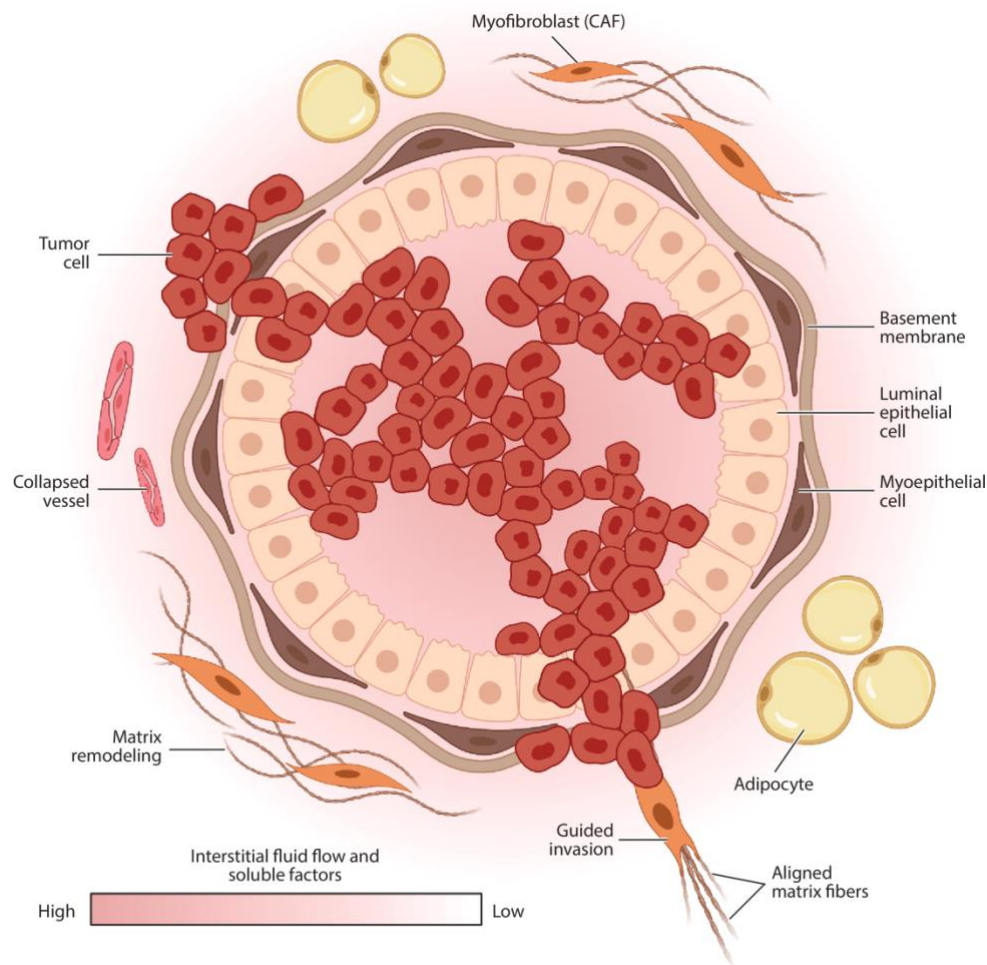
Figure 1. Mechanisms of cell mechanotransduction. Both tumor and stromal cells use a variety of mechanisms to sense mechanical signals within their microenvironments. These include force transmission at cell–matrix and cell–cell adhesions, propagation of force through the cytoskeleton to the nucleus, activation of ion channels in response to membrane tension, transduction of fluid shear stress by surface-bound glycocalyx macromolecules, and conformational changes of caveolae in the plasma membrane. Figure created with BioRender.com.

In addition to cytoskeleton-dependent mechanisms, stretch-activated ion channels help mediate the cellular response to mechanical stimuli.³⁶ These transmembrane channels, such as Piezo1, undergo a conformational change and open upon the application of membrane tension, transporting ions from the extracellular space into the cytosol.³⁷ In turn, altered intracellular ion concentrations and membrane potentials elicit biochemical responses. Moreover, a layer of membrane-bound proteoglycans, known as the glycocalyx, deforms in response to fluid shear stress and is known to transduce these forces in endothelial and tumor cells.^{38,39} Interestingly, extension or compression of the plasma membrane in and of itself can modulate cell signaling by unfolding or refolding small membrane invaginations known as caveolae.⁴⁰ These conformational changes release otherwise sequestered biomolecules or enable the intracellular docking of curvature-sensing proteins that initiate downstream signaling cascades.^{40–42} Moreover, compression is associated with cell volume reduction that, in turn, increases the effective concentration of intracellular molecules.⁴³ This phenomenon, termed macromolecular crowding, slows diffusion within the cytosol by reducing unoccupied void space, resulting in altered biochemical reaction kinetics.⁴⁴

1.5 The Physical Microenvironment of Breast Cancer

While breast cancer arises from the genetic transformation of mammary epithelial cells, the properties of the surrounding stroma influence whether benign disease becomes malignant and invasive.¹¹ Indeed, irradiated fibroblasts promote tumor formation from

otherwise nonmalignant epithelial cells, while implantation of malignant cells into normal embryos prevents tumor development.^{45,46} The mammary duct, where over 80% of breast cancers originate, consists of an inner layer of polarized luminal epithelial cells and an outer layer of contractile myoepithelial cells. These epithelial layers are encased by a dense, collagen IV- and laminin-rich basement membrane.⁴⁷ In early-stage disease, premalignant epithelial cells become highly proliferative and fill the central lumen of the mammary duct but stay confined within the basement membrane. As tumors progress, the basement membrane degrades, enabling transformed cells to migrate into the surrounding stroma (Figure 2). Subsequently, the biochemical and physical properties of the stroma further promote or restrict tumor invasion and entry into nearby blood and lymphatic vessels. These processes are key rate-limiting steps of metastasis, which accounts for up to 90% of breast cancer-related deaths.⁴⁸



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Figure 2. Schematic of invasive breast cancer. Breast cancer cells invade through the mammary gland basement membrane and along aligned collagen fibers. Both tumor cells and activated myofibroblasts contribute to fiber alignment and remodeling. Elevated interstitial fluid pressure due to dysfunctional vasculature drives interstitial fluid flow from the tumor into the nearby stroma, resulting in chemotactic

gradients of soluble factors. Figure adapted from Adaptive Immunity During Breast Cancer Progression by BioRender.com, retrieved from <https://app.biorender.com/biorender-templates>.

1.5.1 Structural guidance cues in breast cancer.

The breast stroma refers to connective tissue that surrounds mammary ducts and becomes altered in the context of cancer. It typically consists of adipocytes, fibroblasts, and immune cells as well as blood and lymphatic vessels embedded in a network of ECM molecules.⁴⁷ In the 1980s, clinicians identified a population of α -smooth muscle actin (α SMA)-positive myofibroblasts that was contained in malignant but not healthy breast tissue.⁴⁹ These cells, also termed cancer-associated fibroblasts (CAFs) (the term we use here), develop from resident or recruited fibroblasts or mesenchymal progenitor cells when exposed to tumor-secreted transforming growth factor- β 1 (TGF β 1).^{50–52} CAFs deposit and remodel ECM components, including collagen I, fibronectin, and hyaluronic acid (HA), that drive changes in tissue architecture and stiffness.⁵³ Epidemiologically, dense breast tissue caused by increased deposition and structural remodeling of fibrotic ECM is one of the most significant risk factors for breast cancer and is used to detect tumors clinically.^{54,55} One hallmark of breast cancer-associated ECM remodeling is that collagen fibers adopt distinct patterns of orientation (**Figure 2**).⁵⁶ Densely aligned collagen fibers oriented perpendicular to the tumor border correlate not only with increased invasion but also with poor patient prognosis regardless of tumor grade, size, receptor status, or subtype.⁵⁷ Thus, the physical organization of peritumoral collagen fibers can serve as a prognostic biomarker to help predict clinical outcomes for breast cancer patients.

Several mechanisms contribute to the correlation between collagen fiber alignment, tumor cell invasion, and patient prognosis. For example, local fiber alignment and cross-linking increase tissue stiffness, which drives tumor progression and malignancy via enhanced cytoskeletal tension and integrin signaling.^{58,59} Similar changes increase the activation of resident fibroblasts into CAFs in response to soluble TGF β 1, further promoting tumor progression.^{60,61} Independent of stiffness, aligned collagen fibers can promote tumor cell invasion by directing cellular protrusions to increase migration persistence in the direction of alignment.¹⁴ Importantly, ECM stiffness and fiber orientation are reciprocally linked with cell phenotype via a positive feedback loop.^{62,63} As cells pull on and align nearby collagen fibers, collagen strain-stiffens, which induces a reciprocal tensile strain on the tumor cells, activating calcium ion channels and Rho signaling pathways to further enhance cell contractility and migration. This interplay between the material properties of collagen and cellular mechanosignaling increases not only tumor cell invasion but also the activation of fibroblasts into CAFs.^{62,64} In addition, fiber alignment impacts cellular confinement, which can independently drive tumor cell invasion.^{65–68} For example, cells confined in 3D collagen microtracks form larger, vinculin-rich adhesions that correspond with higher cell contractility than those produced by cells in unconfined conditions. Thus, as tumor and stromal cells become more contractile while aligning dense peritumoral collagen, fiber reorganization may simultaneously confine cell polarity and increase tumor cell migration.

In addition to modulating migration speed, ECM architecture influences the mechanisms by which tumor cells invade. Invasion depends on a combination of mesenchymal migration, in which cells degrade the surrounding ECM to create spaces to invade, and amoeboid migration, in which cells squeeze through preexisting gaps in their microenvironments.⁶⁹ Studies (with viscoelastic hydrogels indicate that breast cancer cells can also irreversibly widen ECM pores.⁷⁰ Given that these studies also found tumor-associated tissue to be more plastic than normal tissue, this mechanism may allow breast cancer cells to create permanent microtracks that facilitate invasion by subsequent cells. Moreover, altered cell–cell adhesions and matrix-dependent confinement jointly regulate whether breast cancer cells invade individually or as collective strands.⁶⁸ For tumor cells without cell–cell adhesions, dense ECM patterns confine migration paths to collective strands of moving particles, much like the molecules of an active fluid. In less confined regions of matrix, however, cells decondense or individualize into a pattern of gas-like, single-cell dissemination. Collectively, this suggests that tumor-associated ECM can act as a deformable scaffold for leader cells to mediate the migration of follower cells and confine invasion to collective strands even for tumor cells that have lost the strong cell–cell adhesions characteristic of epithelia.

1.5.2 Direct heterotypic cell–cell contact in breast cancer.

In addition to the structural guidance cues provided by the ECM, direct interactions between breast cancer cells and noncancerous stromal cells influence tumor invasion. For example, normal fibroblasts have been shown to restrict tumor progression, while CAFs mediate the opposite effect.⁷¹ In collagen-based coculture studies, CAFs promote the collective invasion of carcinoma cells in a manner that requires direct cell–cell contact.⁷² Indeed, CAFs form heterotypic cadherin-based adhesions with carcinoma cells, generate intercellular forces, and physically pull tumor cells to guide and facilitate collective invasion (**Figure 2**).^{17,73} Forces generated by CAFs can further promote tumor cell invasion by mechanically deforming the basement membrane to enable cancer cell migration independent of proteolysis.⁷³ While these studies underscore the role of physical interactions between CAFs and tumor cells, similar mechanisms can promote migration of otherwise noninvasive cells. Indeed, fibroblast-like adipose stromal cells isolated from obese breast tissue express CAF markers and are more contractile than their lean counterparts, enabling more effective invasion of premalignant mammary epithelial cells.^{74,75} These mechanisms likely contribute to the clinical observation that obesity is associated with a worse clinical prognosis for breast cancer patients.

Similar to CAFs, heterotypic interactions with myoepithelial cells regulate invasion, but often in the opposite direction. For example, direct contact between breast cancer cells and normal myoepithelial cells promotes the cancer cells' self-assembly into noninvasive, growth-arrested acini.⁷⁶ This antitumorigenic effect depends on the formation of heterotypic cell–cell adhesions between both cell types that direct cell sorting and morphogenesis. Additionally, normal myoepithelial cells can act as a dynamic barrier to the protrusion of tumor cells.¹⁹ Myoepithelial cells localize to the surface of mammary duct organoids, restrain protrusions of migrating cancer cells, and even recapture cells that escape from the organoid bulk. However, given sufficient exposure to tumor-secreted

signals, myoepithelial cells lose their anti-invasive properties and ability to direct assembly of transformed epithelial cells into growth-arrested acini.⁷⁷ Indeed, the disruption of normal myoepithelial adhesion or contractility results in a patchier surface layer on mammary duct organoids with frequent gaps that are unable to restrain invasive cancer cells.¹⁹ These findings are clinically relevant, as the upregulation of $\alpha v \beta 6$ integrins in myoepithelial cells—used as a surrogate marker for a cancer-associated phenotype—strongly correlates with tumor progression and reduced median time to recurrence in a cohort of breast cancer patients.⁷⁸

1.5.3 Elevated interstitial fluid pressure and flow in breast cancer.

Elevated IFP in human breast tumors compared to adjacent noncancerous tissue was first documented in 1994 for women undergoing biopsy.⁷⁹ Subsequent studies in mice indicated that rapidly expanding tumors generate sufficient solid stress to collapse nearby blood and lymphatic vessels.⁸⁰ Coupled with vessel dysfunction and the increased deposition of hygroscopic HA, fluid readily accumulates around developing tumors. This creates a net pressure gradient that drives interstitial fluid into the surrounding stroma.^{23,81} Historically, the resulting IFF was thought to promote disease progression primarily by impeding delivery of cytotoxic therapies to the tumor core. However, more recent studies found that IFF can independently induce tumor malignancy by generating gradients of autocrine factors, including CCL19 and CCL21, that stimulate tumor cell migration in the direction of flow via activation of CCR7 (**Figure 2**).²¹ Somewhat surprisingly, these effects are most pronounced at low tumor cell densities, with increasing cell densities hypothesized to flatten local chemotactic gradients due to simultaneous secretion of the same cues by neighboring cells.⁸²

Interestingly, subsequent experiments demonstrated that IFF can also induce upstream tumor cell migration (i.e., against the direction of fluid flow).⁸² This phenomenon, termed rheotaxis, depended on asymmetric tension generated on cellular adhesions by fluid drag forces. The application of higher tension on the upstream edges of cells resulted in the local activation of FAK, the polarized recruitment of FA complexes, the formation of actin-rich membrane protrusions, and finally directed migration against fluid flow.⁸³ The rheotactic response of breast cancer cells may also be controlled by upregulating the mesenchymal markers vimentin and Snail without losing expression of the epithelial marker E-cadherin, as might be expected for complete epithelial-mesenchymal transition.⁸⁴ These studies indicate that IFF can direct the downstream chemotactic migration of cancer cells away from the tumor during early-stage disease when cell densities are low. Subsequently, the upstream rheotactic migration of cancer cells against transmural fluid flow radiating from intact capillaries could mediate homing to the nearby stromal vasculature.

In addition to influencing tumor cell migration directly, elevated IFF can modulate stromal cells in the peritumoral stroma to create a proinvasive environment. Fluid flow levels similar to those generated by tumor-associated IFP, for example, promote fibroblast-to-myofibroblast conversion and collagen alignment in a manner that depends on autocrine TGF β 1 secretion and integrin $\alpha 1 \beta 1$ signaling.⁸⁵ These changes are functionally relevant,

as protease- and contractility-dependent remodeling and alignment of collagen by fibroblasts in response to fluid flow enhanced tumor cell migration via similar mechanisms.⁸⁶ Hence, fluid flux out of the tumor bulk and into the adjacent stroma synergistically promotes invasion both by influencing tumor cells directly and by reinforcing the structural guidance cues described in section 1.5.3. Moreover, elevated IFF also induces the formation of new lymphatic vessels and increases the secretion of chemotactic ligands by existing lymphatic vessels—providing additional stimuli to drive cancer cell migration toward the stromal vasculature and thus metastatic spread via the circulation.^{21,87}

1.6 The Physical Microenvironment of Glioblastoma

GBM develops from the genetic transformation of neural stem cells and glial progenitor cells including astrocytes and oligodendrocytes.^{88,89} While GBM tends to be quite heterogeneous within and between patients, tumors are often classified in terms of mutation [e.g., isocitrate dehydrogenase (*IDH*)], amplification [e.g., epidermal growth factor receptor (*EGFR*) and platelet-derived growth factor receptor alpha (*PDGFRA*)], or promoter methylation (e.g., *MGMT*) of specific genes.^{90,91} Next-generation sequencing has also produced a broad classification scheme enabling stratification of GBM into transcriptional subtypes (classical, mesenchymal, or proneural), although single-cell sequencing has painted a more nuanced picture in which bulk tumors are composed of distinct cell populations that represent different subtypes.^{90,92} Functionally, GBM exhibits highly malignant growth and rapid invasion, in which bulk tumor expansion precedes rapid cellular infiltration into neighboring healthy brain tissue. In early-stage disease, tumor expansion caused by highly proliferative tumor cells leads to the compression of intratumoral blood vessels, which contributes to the development of a hypoxic, necrotic tumor core (**Figure 3**). Hypoxic signaling then induces pseudopallisading infiltration, in which tumor cells slowly invade from the tumor bulk into the brain parenchyma and subsequently shift to a faster invasion scheme along white matter tracts. Finally, the tumor cells eventually reach the brain perivascular space and rapidly invade along blood vessels via vascular co-option.⁹³

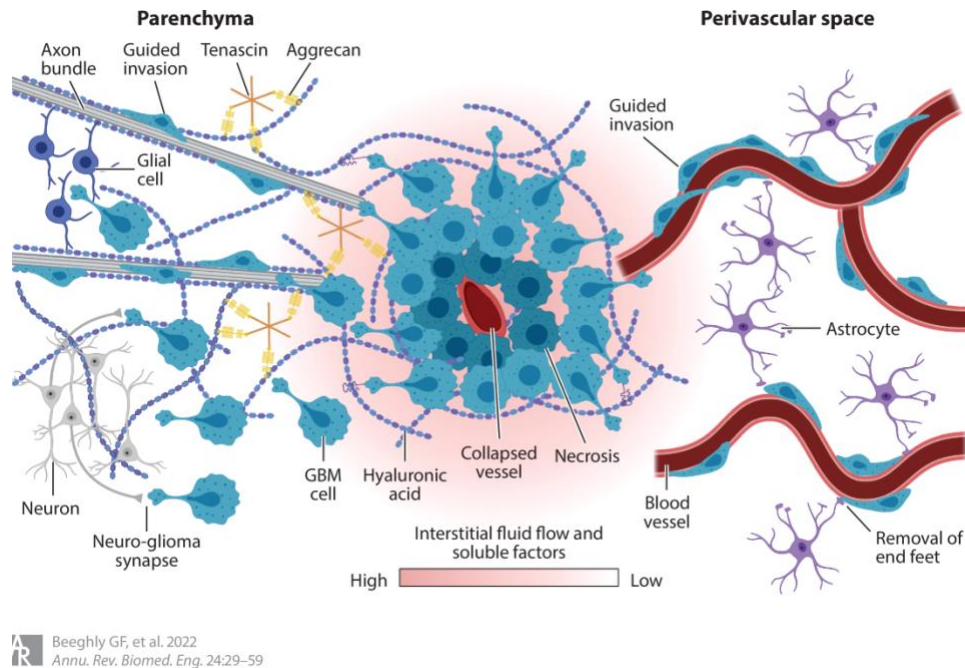


Figure 3 Schematic of invasive glioblastoma (GBM). GBM cells invade the parenchyma and perivascular space of the brain after escaping the tumor core. The brain parenchyma contains a nanoporous matrix including hyaluronic acid as well as numerous cellular protrusions that physically hinder and confine invasion. Alternatively, the linear structures of white matter tracts in the parenchyma and blood vessels in the perivascular space encourage the rapid invasion of GBM cells along these guidance cues. Figure created with BioRender.com.

1.6.1 Structural guidance cues in glioblastoma.

While breast cancer invasion is largely driven by structural guidance cues provided by the ECM, GBM invasion is guided by the unique architecture and structural features of the brain parenchyma and vasculature. Many *in vivo* studies using GBM mouse models report that tumor cells associated with blood vessels and white matter tracts achieve higher net displacements than cells not associated with these structures, contributing to the idea that these cues promote infiltration.^{20,94–96} How vascular structures and white matter tracts facilitate invasion may be understood in terms of the contrast between these structures and the surrounding parenchyma. The gray matter of the brain parenchyma consists of an isotropic polysaccharide- and glycoprotein-rich ECM (e.g., HA, tenascin-c, aggrecan, and osteopontin) and densely packed cells (e.g., neuronal cell bodies and glia) that collectively pose significant steric barriers to invasion (**Figure 3**).^{15,16} In contrast, white matter tracts (i.e., axons) are surrounded by a linearized ECM and space that more easily accommodates tumor cell infiltration.⁹⁷ Similarly, blood vessels are embedded within the subarachnoid space, which lowers the physical resistance to GBM invasion and provides a linear geometry that preferentially directs cell migration.¹⁵

As GBM cells invade brain tissue, they alternate between a variety of motility strategies, or modes, depending on the specific constraints of the local microenvironment. While these modes differ in their speed and molecular mechanisms, all involve adhesion and

transmission of force between the ECM and cytoskeleton through specialized molecular machinery. The HA adhesion receptor CD44 is particularly important for GBM cells to navigate through the brain parenchyma and can serve as a predictor for disease outcomes.^{98,99} GBM cells use CD44 to engage and transduce mechanical signals from HA, which in turn facilitate migration through ECM pores.¹⁰⁰ For example, CD44-dependent microtentacles, analogous to those sometimes seen in circulating tumor cells, mechanically couple the actin and microtubule cytoskeleton to HA through a molecular motor-clutch system that includes the actin-binding protein IQGAP1 and the microtubule-binding protein CLIP170.¹⁰¹ Elsewhere, GBM cells have been reported to extend pseudopodia-like protrusions to navigate through the brain parenchyma. These protrusions depend on the Rho GTPases Rac1 and Cdc42, which direct actin polymerization, and Lck-I, which supports FA generation through the regulation of paxillin phosphorylation.^{94,102}

Within the perivascular space, GBM cells adopt a unipolar morphology with prominent anterior processes to linearly invade along blood vessels in a saltatory fashion.⁹⁵ As a result, vascular architecture influences GBM invasion dynamics. For example, during invasion, cell division often takes place near vascular branch points, and GBM cells that interact with multiple vessels are more invasive.^{95,97} Interestingly, GBM cell migration is also driven by engagement with less common ECM components, including collagen IV and laminin on the abluminal surface of vessels.¹⁰³ Interactions with vascular ECM often involve integrins, which are typically upregulated by invasive GBM cells. However, integrin suppression only partially inhibited vascular migration in preclinical studies and yielded disappointing results in clinical trials.^{103,104} The residual invasion following integrin suppression could be due to physical guidance cues provided by vessels or cell adaptation to other adhesive systems such as formins, which have been implicated in GBM migration on laminin-coated tracks.^{103,105} Additionally, vascular engagement by tumor cells leads to local vascular deformation through an actomyosin-driven, motor-clutch invasion mechanism to propagate tumor growth.¹⁰⁶ However, it is not clear whether these invasion-driven deformations also contribute to vascular dysfunction. In summary, GBM cells adopt unique morphologies driven by ECM adhesion proteins and cytoskeletal components to invade along vessels and within the brain parenchyma.

1.6.2 Direct heterotypic cell–cell contact in glioblastoma.

Direct cell–cell contact between GBM cells and brain stromal cells including astrocytes, endothelial cells, and neurons also supports GBM growth and invasion. While the vasculature is a permissible route for invasion overall, astrocytic processes that physically maintain the integrity of the blood–brain barrier hinder tumor cell migration. To overcome this obstacle, perivascular GBM cells physically displace astrocytes from the vasculature (**Figure 3**), which concurrently contributes to vessel dysfunction and fluid accumulation.²⁰ Remarkably, a single perivascular GBM cell is sufficient to disrupt the vasculature by displacing astrocytic end feet.²⁰ Moreover, removal of astrocytic end feet interferes with vascular tone regulation (i.e., vasodilation or vasoconstriction), resulting in vessels that are unable to respond to vasoactive molecules released by astrocytes. GBM cells can thus usurp this vasomodulatory role by mobilizing calcium-activated potassium ion

channels to constrict vessels, increase perivascular space, and enable greater perivascular invasion.²⁰ However, some instances of GBM-induced vascular modulation have also been reported to induce vessel dilation.⁹⁶

Furthermore, vessel architecture and barrier function are disrupted due to direct contact between GBM and endothelial cells. Notably, GBM vascular co-option is associated with the loss of endothelial cell tight junction proteins, including zonula occludens-1 and claudins, which otherwise maintain vascular integrity.^{20,107} Additionally, direct contact with endothelial cells supports the growth and self-renewal of GBM stem-like cells. For example, endothelial cells that express Notch ligands are often found adjacent to Nestin- and Notch-receptor-positive cancer stem-like cells.¹⁰⁸ Knocking down the Notch ligands JAG or DLL in endothelial cells decreases the presence of CD133-positive GBM stem-like cells adjacent to blood vessels in vivo. This corroborates previous findings that the microvasculature supports the maintenance of a stem-like state for GBM.¹⁰⁹

Physical and biochemical interactions with neurons have also been recently reported to promote GBM progression. Indeed, direct GBM contact with the subventricular zone of the neural stem cell niche is associated with decreased patient survival.¹¹⁰ Two recently published studies reported that neuron-to-glioma synaptogenesis, mediated by the transmembrane glutamate receptor AMPAR, regulates tumor growth and invasion.¹¹¹ Here, electrochemical cell communication was mediated by AMPAR-dependent neuro-glioma synapses, where GBM cells were often found on the postsynaptic side of these junctions. Elsewhere, neuro-glioma synapses have been observed to connect with microtubules on GBM cells. Thus, targeting neuro-glioma synapses by the genetic and pharmacological perturbation of AMPAR-mediated calcium activation attenuated disease progression in GBM mouse models.¹⁸

1.6.3 Elevated interstitial fluid pressure and flow in glioblastoma.

Similar to most solid tumors, elevated IFP contributes to invasion in GBM.¹¹² Vascular abnormalities, including loss of barrier function in GBM, constitute one of the primary causes of elevated IFP, along with an impaired fluid drainage system. The compromised GBM-associated vasculature permits fluid accumulation and immune cell infiltration in the interstitial space surrounding the tumor. Subsequent immune cell secretion of proinvasive growth factors such as TGF β 1, epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF), together with elevated IFP and IFF, amplify invasion.^{113–115} Similar to the observations in breast cancer described previously, the combination of IFF and cytokine secretion can create chemotactic gradients used by GBM cells to invade healthy brain tissue.²²

Despite its role in regulating invasion, the biological mechanisms through which IFF drives GBM invasion remain underexplored. CXCR4-, CXCL12-, and CD44-based mechanisms are known means by which GBM cells migrate in response to flow. Specifically, the presence of IFF enhances GBM cell motility by activating CXCR4, which responds to autocrine CXCL12 gradients.²² However, this response is not universal, as specific subpopulations of GBM cells instead respond to flow by CD44-dependent

mechanisms.¹¹³ These results were reproduced in a mouse model of GBM in which IFF induced by convection-enhanced delivery increased tumor cell invasion and the population of CXCR4-positive cells, whereas administration of a CXCR4 antagonist abrogated these effects.¹¹⁶ Additionally, irradiation led to an increase in CXCR4-positive cells, which could then respond to IFF-induced gradients of CXCL12 at the tumor border to potentiate invasion.¹¹⁶ As a result, how candidate therapies influence IFP and IFF must be considered moving forward, given their implications for tumor invasion.

Elevated IFP in GBM also leads to cerebral edema and frustrates the successful delivery of therapeutic agents to the tumor core. Efforts to improve drug delivery by decreasing IFP include normalizing the tumor vasculature with anti-angiogenic agents that inhibit VEGF signaling.¹¹⁷ For example, administration of anti-VEGF monoclonal antibodies reduced IFP and inhibited tumor growth in a xenograft model of GBM.¹¹⁸ Similarly, in recurrent GBM, anti-VEGF therapies reduced edema and resulted in improved survival when delivered with other chemotherapies in a phase II clinical trial.^{119,120} Thus, normalizing the peritumoral vasculature increases uptake of cytotoxic drugs while reducing cerebral edema. However, anti-angiogenic therapies can also produce undesirable compensatory effects, such as the elevated secretion of other proangiogenic factors, including basic fibroblast growth factor (bFGF) and stromal cell–derived factor 1 alpha (SDF1 α), the expansion of treatment-resistant GBM progenitor cells, and even increased tumor cell invasion.^{119,121} Thus, more research is needed to understand how treatment-induced vascular modulation translates into clinical outcomes.

1.7 Engineering strategies to measure physical aspects of the tumor microenvironment

Established methods from the physical sciences have been adapted to quantify and improve our understanding of the physical tumor microenvironment. For example, atomic force microscopy (AFM), first developed to characterize nanoscale materials in the 1980s, has been applied to measure the viscoelastic properties of cancer cells, stromal cells, and ECM components.¹²² Likewise, the bulk mechanical properties of tumors have been assessed via extensional and shear rheology.^{70,123} Beyond preexisting technologies, new tools to probe cell and tissue mechanics have been engineered with specific biological questions in mind. For an overview of these strategies, see **Table 1**.

Table 1: Example systems used to measure physical traits of the tumor microenvironment

Category	Measurement systems	Physical traits	References
Cell mechanics	Micropipette aspiration, real-time deformability cytometry, nanoindentation, optical tweezers	Generalized cell material properties	124–126, 128–134
Subcellular mechanics	Atomic force microscopy, directed micropipette aspiration	Organelle cell material properties	123, 127–129
Substrate mechanics	Atomic force microscopy, (micro)rheology, nanoindentation, optical tweezers	Extracellular matrix properties	14, 51, 63, 64, 70, 73, 74, 124
Cell adhesion forces	Spinning disc assays, single-cell force spectroscopy, dual micropipette aspiration, magnetic tweezers	Cell-substrate and cell–cell adhesion forces	17, 144–151
Cell traction forces	Traction force microscopy, microfabricated post array detectors, optical tweezers	Cell-generated traction forces and strain-stiffening	17, 51, 63, 136–141, 143
Flows, pressures, and stresses	Pressure transducer systems, contrast-enhanced imaging, deformable microdroplets, molecular tension sensors	Fluid flow, fluid pressure, and solid stress	8, 9, 152–157, 159

1.7.1 Measuring the material properties of cells.

The material properties of cells can be measured by assessing cell deformation in response to forces applied with an external object (e.g., a cantilever or piston) or by exposing cells to fluid shear stress and pressure. Micropipette aspiration, in which cells in suspension are partially aspirated into narrow glass pipettes, is perhaps the most established of these methods.¹²⁴ After aspiration, the extent of cell distension into the pipette is measured with a microscope and is used to calculate the viscoelastic properties given the applied suction pressure. In practice, micropipette aspiration is sensitive enough to distinguish low-grade from high-grade tumor cells due to their differential mechanics acquired over malignant transformation.¹²⁵ These methods are complementary, as micropipette aspiration integrates whole cell mechanics while AFM provides higher-resolution measurements that can detect differences in subcellular mechanics.¹²⁶ Recent advances in micropipette aspiration have focused on improving the speed at which cells can be sampled and spatially directing aspiration to measure the mechanical properties of specific organelles such as the nucleus.^{127,128} Similar in principle, real-time deformability cytometry (RT-DC) assesses cell mechanics by monitoring the shapes of

individual cells flowing through pressurized channels in response to shear stress.^{129,130} RT-DC is higher throughput (hundreds to thousands of cells per minute) than micropipette aspiration and simultaneously measures relative cell size and granularity like conventional flow cytometry. In practice, RT-DC has been applied to distinguish malignant from normal blood cells in samples collected from patients with leukemia based on relative differences in cell deformability.¹³¹

The development of advanced imaging systems has enabled optics-based measurements of cell mechanical properties. For example, optical tweezers and related technologies mechanically manipulate particles and cells by taking advantage of small forces generated by light refracting and changing momentum as it passes through an optical interface, such as between extracellular fluid and the cytoplasm.¹³² By measuring the deformation of a given cell along the applied axis of light, the relative elasticity can be inferred. Studies using these approaches found that tumor cell deformability correlated with invasive potential, and that malignant cells altered their cellular mechanics in response to substrate mechanics while nonmalignant cells did not.^{133,134} Similar principles have been applied to measure ECM mechanical properties via optics-based microrheology.¹²³ By optically pulling on spherical beads embedded in a substrate of interest, microscale viscoelastic properties can be calculated. For collagen substrates composed of fine, uniform fiber networks, this approach yielded comparable results to conventional shear rheology. However, for substrates with large pores and thick fibers, microscale elasticities were often much larger than bulk elasticities, given that individual fiber mechanics dominate at the microscale. Independent studies using a similar setup measured the degree to which collagen fibers stiffen when elongated by contractile tumor cells.⁶³ When both beads and tumor cells were embedded in collagen gels, bead-conjugated fibers located near cells resisted optical deformation more than those far away from cells or those located near noncontractile control cells. These results indicate that tumor cell-generated traction forces led to local ECM stiffening in the immediate vicinity of a given cell.

1.7.2 Measuring cell–matrix traction forces.

Traction force microscopy (TFM) is widely used to estimate the forces that adherent cells generate against their substrates. In this method, cells are cultured on top of substrates that contain fluorescent tracer particles.¹³⁵ Cell-generated forces produce net displacement of these tracers relative to their relaxed reference positions. If the mechanical properties of the substrate material are known, the stress distribution can then be inferred from the net bead displacements by solving an inverse problem. TFM was first established to examine the traction stresses generated by migrating fibroblasts on 2D collagen-coated polyacrylamide gels and has since been applied to interrogate diverse cell-substrate interactions in applications ranging from tissue morphogenesis to tumor invasion.^{135,136} In a similar manner, culturing cells on elastomeric micropost arrays enables the direct measurement of 2D cellular traction stresses by measuring the deformation of the underlying posts.^{137,138} Enabled by the improved axial resolution of modern confocal microscopes, the more recent developments of 2.5D and 3D TFM permit traction forces to be measured in all three spatial dimensions by cells seeded on or within

3D substrates, respectively.^{139,140} Newer methods such as optical coherence microscopy (OCM)-based TFM enable label- and tracer-free measurements of cell-generated traction forces by directly imaging collagen fiber deformation.¹⁴¹ Given that OCM uses near-infrared light, this method is also well suited for time-lapse analysis of biological samples (e.g., those with large volumes embedded in scattering media). In addition, the use of astigmatic imaging, which infers 3D spatial information within a few micrometers of a given focal plane, has enabled 2.5D and 3D TFM to be performed without collecting multi-image stacks.¹⁴² Thus, adopting advanced imaging modalities has greatly improved both the spatial and temporal resolutions of traditional TFM methods.

1.7.3 Measuring cell adhesion forces

Early methods for measuring cell adhesion forces involved culturing cells on spinning discs and tracking cell detachment due to the applied rotational forces.¹⁴³ While this approach proved extraordinarily valuable for semiquantitative population-level measurements, it does not yield absolute single-cell measurements of adhesion. To this end, AFM-based, single-cell force spectroscopy has emerged to measure adhesion forces in the subnanonewton range.^{144–146} Here, a modified AFM cantilever is functionalized and coupled to a cell of interest. The conjugated cell is then lowered onto an opposing cell or substrate until adhesion occurs, the cantilever is retracted, and the force required for separation is recorded.¹⁴⁷ Similarly, in dual pipette assays, cells are held in place by micropipette-generated suction and brought into contact to facilitate adhesion.^{148,149} The cell doublet is then pulled in opposing directions and the suction pressure is increased in a stepwise fashion until the cells detach from each other instead of from the pipettes. In addition, magnetic tweezers have been used to detach micron-scale magnetic beads conjugated with E-cadherin from epithelial cells to examine the role of cell–cell adhesion strength in tissue dynamics.¹⁵⁰ In the context of tumor invasion, researchers extended this platform to demonstrate that CAFs bind and pull cancer cells to enhance their migration via the formation of heterotypic cadherin-based adhesions.¹⁷

1.7.4 Measuring fluid pressure and solid stress.

While many strategies have been developed to measure microscale mechanical properties and cell-generated traction and adhesion forces, relatively few methods exist to measure tissue-scale changes in IFP and solid stress associated with tumor development.⁵ Elevated IFP within tumors relative to that in normal tissue has been quantified by direct insertion of wick-in-needle, fiberoptic, or piezoelectric pressure transducer systems.^{151–153} However, these techniques are invasive, and there is some debate about the extent to which their readings reflect pressure contributed by free interstitial fluid, bound interstitial fluid, or artifacts of solid stress. More recently, fluid pressure has been noninvasively inferred by imaging IFF (driven by relative differences in pressure) via contrast-enhanced imaging modalities.¹⁵⁴ Monitoring solid stress within tumors has proven even more challenging. Injection of deformable fluorescent microdroplets into tissues and ectopic expression of molecular tension sensors have enabled local stresses to be calculated from microdroplet deformation and changes in sensor fluorescence, respectively.^{155,156} However, these methods measure forces at

subcellular or cellular scales and are subject to the optical limitations of confocal microscopy. To measure tissue-level solid stress, researchers have developed protocols to embed resected tumors in agarose and monitor tissue displacement following tumor slicing as a metric of stress dissipation.^{9,157} As this technique is destructive, the evolution of tumor solid stress over the course of disease progression cannot be monitored longitudinally. Moving forward, improved strategies to noninvasively measure tissue-level mechanics in real time will help to reveal new mechanisms of tumor pathophysiology and to identify physical targets for clinical intervention.

1.7.5 Engineering Strategies to Model Physical Aspects of the Tumor Microenvironment

While cell and tissue engineering strategies were initially developed with an eye toward regenerative medicine, these approaches are similarly valuable for cancer research.^{158,159} Indeed, tumor engineering publications increased 24-fold from 2000 to 2020 and have isolated specific physical characteristics of the tumor microenvironment for mechanistic studies.^{160,161} Key design considerations for these systems include the ability to independently tune mechanical properties, to define ECM composition and architecture, to integrate multiple cell types, and to visualize outcomes in real time with live cell imaging techniques. For an overview of these strategies, see **Table 2**.

Table 2: Example systems used to model physical traits of the tumor microenvironment

Model system	Physical traits	References
Advanced biomaterials	Material properties, cleavable cross-links, ligand presentation, and microarchitecture	64, 70, 160, 165–168, 172–186, 203
Protein micropatterns	Geometric constraints and heterotypic cell–cell contacts	191–196
Microfabricated devices	Structural guidance cues, material properties, and confinement	14, 67, 84, 198, 199, 202, 204, 206, 207
Tumor-on-chips, microfluidics	Heterotypic cell–cell contacts, interstitial fluid flow, and pharmacodynamics	85, 128, 198, 205, 208–210, 212
Pressure-driven flow systems	Interstitial fluid flow and heterotypic cell–cell contacts	21, 22, 39, 82–84, 86, 113, 212

1.7.6 Advanced biomaterials to mimic extracellular matrix mechanics and microarchitecture.

Natural biomaterials, such as solubilized collagen and reconstituted basement membrane, were among the first substrates to enable the 3D culture of cells. Studies with these hydrogels enabled key insights into how ECM-derived cues regulate cell behaviors ranging from morphogenesis to malignant transformation.^{162,163} However, the physical and biochemical properties of natural biomaterials cannot be easily decoupled, making it difficult to interrogate how physical motifs in the microenvironment regulate cell phenotype.¹⁶⁴ For example, altering the stiffness of collagen matrices by modulating collagen concentration simultaneously changes the density of adhesion ligands and the pore size between collagen fibers, as well as fiber length and thickness, all of which can independently affect cell behavior.^{64,165} Moreover, tissue-derived matrices, including those derived from epithelial basement membranes, often include growth factors and cytokines and suffer from batch-to-batch variability.^{166,167} Synthetic or semisynthetic biomaterials can address these limitations by independently tuning parameters such as ligand density, microarchitecture, and stiffness.

Synthetic biomaterials are often functionalized with bioactive components to recapitulate specific properties of the ECM.^{168,169} For example, biocompatible, photopolymerizable polyethylene glycol (PEG)-based hydrogels have been modified with integrin-binding adhesion peptides and enzymatically cleavable cross-links to enable cell-ECM interactions and proteolytic remodeling of hydrogels following cell encapsulation.^{170,171} The stiffness of such PEG-based systems depends on the degree of photoinducible cross-linking and can be patterned to explore how substrate mechanical gradients impact tumor cell migration. For example, tumor cells that started in regions of lower stiffness migrated rapidly and reversed directions upon encountering regions of higher stiffness, while cells that started in regions of higher stiffness migrated more slowly but readily invaded into regions of lower stiffness.¹⁷² These results indicate that, above a certain threshold, the degree of ECM stiffness restricts cell migration. However, note that the response to stiffness varies as a function of ECM remodeling, as cell migration depended on cleaving cross-links in stiff but not soft PEG-based substrates.¹⁷³ PEG-based systems have also increased our understanding of how adhesion ligand density affects encapsulated cell behavior.¹⁷⁴ While increasing the density of RGD adhesion ligands at a constant stiffness prompted metastatic carcinoma cells to self-assemble into structures that resembled healthy acini, increasing adhesion ligand density in the presence of TGF β 1 stimulated epithelial-mesenchymal transition.¹⁷⁵ This discrepancy reveals the complex and often unexpected interplay between physical and biochemical cues on cell behavior. Importantly, the material properties of synthetic PEG-based platforms can also be adjusted in vivo, enabling direct comparison with in vitro findings. For example, the application of transdermal light to subcutaneous PEG-maleimide scaffolds freed previously unavailable RGD adhesion sites from photolabile cages, prompting the inflammation and vascularization of the implanted material.^{176,177}

Given that tumors and tumor-associated stroma often stiffen over the course of disease progression, dynamic biomaterials that can alter their physical properties on demand

represent promising platforms to research the consequences of evolving tumor mechanics.⁵⁸ Methacrylated HA hydrogels coated with collagen can be dynamically stiffened from 100 to 3000 Pa in response to stepwise doses of ultraviolet irradiation. Premalignant epithelial cells cultured on soft substrates that were later stiffened to pathological levels resisted adopting malignant phenotypes when compared to cells directly seeded onto prestiffened substrates, indicating that cells respond to both the timing and degree of matrix stiffening.¹⁷⁸ Using methacrylated HA to adjust substrate stiffness is particularly attractive for studies of GBM invasion, as HA is an essential component of the native brain ECM.^{179,180} For example, GBM cells plated onto stiff 5-kPa HA spread, adhered, and migrated more than those on soft 150-Pa HA. Interestingly, CD44-HA adhesions alone could facilitate the migration of GBM cells.¹⁰⁰ Introducing RGD ligands to enable integrin-based adhesions only increased the rate of invasion when CD44 was inhibited. These findings indicate that CD44-based mechanisms result in greater GBM invasion speeds, while integrin-based mechanisms facilitate a slower, alternative mode of migration. Finally, HA-based platforms can also be leveraged to examine other aspects of the tumor microenvironment such as the induction of angiogenesis. Studies with RGD-functionalized tunable HA hydrogels, for example, suggested that substrate stiffness and ECM degradation are both essential for robust endothelial cell sprouting and vascular tube formation.^{181,182}

In addition to photopolymerization, dynamic substrate stiffening has been accomplished with interpenetrating networks (IPNs) of alginate and Matrigel that contain liposomes filled with soluble calcium.¹⁸³ Near-infrared irradiation released calcium from these liposomes, creating ionic cross-links within the alginate and increasing the elastic moduli of the hydrogels from 150 to 1200 Pa. This on-demand stiffening promoted encapsulated breast cancer cells to downregulate epithelial markers, to adopt more protrusive morphologies, and to form into large, disorganized clusters. IPNs of alginate and Matrigel also expanded our understanding of how viscoelastic substrate properties influence tumor cell invasion.⁷⁰ IPNs with high plasticity (i.e., nonreversible deformation in response to cell-generated forces) induced tumor cells to extend invadopodia-like protrusions and create permanent pores in the surrounding alginate-Matrigel network that were large enough for cells to squeeze through in a proteolysis-independent manner. Consequently, these materials revealed a new mechanism of tumor cell migration under confined conditions, an insight that would not have been possible with conventional approaches.

While the described synthetic or semisynthetic biomaterials offer many advantages over natural biomaterials, most of them polymerize into nanoporous structures that lack the fibrous microarchitecture characteristic of native ECM.¹⁸⁴ These ECM microarchitectural cues (i.e., interfibrillar pore size, fiber alignment, length, and thickness) influence malignancy independently of bulk material properties.^{67,165} Thus, there is a need to develop platforms that combine the tractable nature of synthetic biomaterials while also recapitulating the fibrillar architecture of native ECM. One approach is to form fibers by electrospinning synthetic biomaterials into fine, nanoscale filaments.¹⁸⁵ For example, reducing the stiffness of spun methacrylated dextran (DexMA) fibers encouraged spreading, proliferation, and FA formation by encapsulated cells. The authors determined that cells exerted traction forces to recruit fibers and increase the local concentration of

RGD peptides to elicit these effects, a phenomenon that was hindered when fibers became too stiff to easily remodel. Increasing the bulk stiffness of similar scaffolds inhibited myofibroblast differentiation, while increasing the concentration of spun fibers promoted fibroblast-to-myofibroblast conversion upon exposure to TGF β 1.¹⁸⁶ Notably, both studies contradict previous findings on traditional substrates, in which increased stiffness promotes cell spreading, proliferation, and FA assembly as well as myofibroblast differentiation.^{58,61} These discrepancies indicate that changes to bulk substrate mechanics do not necessarily influence cell behavior in the same manner as do similar changes to microscale fiber mechanics. Moreover, matrices composed of spun DexMA fibers have also been leveraged to discover new mechanisms of tumor cell migration via live imaging studies.¹⁸⁷ Breast cancer cells intermittently stopped migrating in these matrices to pull fibers in front of them toward their cell bodies, storing elastic energy in the process. Eventually, adhesions at the trailing edge of a given cell failed and the tumor cell ricocheted forward as the recruited fibers returned to their starting positions. Despite the stationary period, this mode of tumor cell migration was ultimately faster and resulted in greater net displacement than did continuous mesenchymal migration.

1.7.7 Microfabricated models of the tumor microenvironment.

How tumor cells respond to the geometric and mechanical constraints of their microenvironments has been extensively studied with protein micropatterns.^{188,189} Typically, protein micropatterns are printed onto glass or polyacrylamide substrates using photolithography approaches that employ either an elastomeric polydimethylsiloxane (PDMS)-based or deep ultraviolet (UV) irradiation-based technique.^{188,190} The former uses a PDMS template with defined geometric features to print proteins onto a given substrate, which determines the features, location, and resolution of the resulting micropattern.¹⁸⁹ The latter approach relies on deep UV exposure through a photomask that permits targeted irradiation of a given substrate coated with a photosensitive protein repellent, thus enabling protein adsorption to irradiated areas.¹⁸⁸ Both techniques achieve a patterning resolution on the order of 1–10 μm .^{186,187} In one example, fibronectin micropatterns were used to demonstrate that imposed interfacial geometry can guide tumor cells toward a stemlike state via altered integrin $\alpha 5 \beta 1$ signaling.¹⁹¹ Recent advances in micropatterning technologies have also enabled real-time patterning to examine how cells adapt to the dynamic presentation of adhesion sites within their microenvironments. In this study, real-time patterning was achieved using pulsed lasers that oxidized a PEG-based substrate to create new adhesion sites via subsequent protein adsorption onto oxidized regions.¹⁹²

While micropatterning has been used to present proteins in controlled 2D spatial configurations, other microfabrication methods have been implemented to model 3D aspects of the ECM microarchitecture. For example, injecting collagen into narrow microfabricated PDMS channels aligns the resulting fibers in the direction of the channel.^{14,193} As the channel width increases, the fibers lose their aligned orientation and default to a randomly organized architecture. Studies with this platform demonstrated that collagen fiber orientation enhances tumor cell migration efficiency, directional persistence, and distance traveled. One alternative method includes casting collagen into

PDMS molds that are stretched during polymerization to orient the resulting fibers, an approach that affected the migratory and morphogenic behavior of preseeded tumor and stromal cells.¹⁹⁴ To more accurately mimic the 3D environment of developing tumors, a coaxial rotating cylinder platform was used to encase breast cancer spheroids with both perpendicularly and tangentially aligned collagen fibers.¹⁹⁵ This method leveraged the nucleation and elongation phases of collagen polymerization to produce a system in which the spheroids contacted two common fiber orientations found in breast cancer. In particular, fiber alignment in this system was driven by horizontal laminar Couette flow in the nucleation stage of polymerization, while gravitational forces guided fiber growth during elongation. In agreement with previous observations, breast cancer spheroids preferentially invaded along perpendicularly rather than tangentially aligned collagen fibers.

Numerous engineered model systems have leveraged microfabricated channels with defined dimensions to examine confined tumor cell migration in vitro.¹⁹⁶ Notably, PDMS can be functionalized with cell adhesion proteins and is optically transparent, permitting real-time imaging that enables mechanistic studies of confined migration dynamics. Studies using these platforms found that tumor cells polarize their cytoskeleton and physically soften to achieve faster migration speeds when confined.^{197,198} Furthermore, these microchannels can be modified to introduce additional physical constraints that tumor cells may encounter during invasion. These include physical gradients, in which cells migrate from a wider into a narrower channel, or bifurcations, in which cells are presented with a channel that splits into two channels of different widths.^{196,198} In addition, microfabricated platforms have been used to examine nuclear deformation during confined tumor cell migration.¹⁹⁹ Here, nuclear deformation compromised nuclear envelope integrity, leading to envelope rupture and subsequent DNA damage.²⁰⁰ Interestingly, follow-up studies found that mechanical deformation of the nucleus also damaged DNA without nuclear envelope rupture.²⁰¹ These findings suggest that mechanically induced DNA damage contributes to the genomic instability commonly associated with metastatic tumor cells.

Many engineered systems take a reductionist approach to modeling cancer, restricted to studying tumor cells in isolation. While this approach enables the fundamental behavior of tumor cells to be studied without confounding factors, its application to studying tumor–stroma interactions is limited. Microfluidic-based, tumor-on-chip systems address this challenge by introducing other stromal compartments such as blood vessels, immune cells, and fibroblasts. These platforms enable direct heterotypic cell–cell interactions to be studied by integrating multicellular compartments on a single platform. In addition to stromal cells, other aspects of the tumor microenvironment, such as pressure gradients, specific ECM components, and defined mechanical properties, can also be incorporated.²⁰² Using these methods, an intravasation model consisting of tumor cells, endothelial cells, and macrophages demonstrated that impaired endothelial barrier function facilitates rapid tumor cell intravasation via direct heterotypic cell–cell interactions.²⁰³ Here, macrophage secretion of tumor necrosis factor alpha (TNF- α) disrupted endothelial cell tight junctions, which subsequently enhanced tumor cell transmigration by increasing tumor–endothelial cell interactions. Moreover, such tumor-on-chip systems are a

promising method to systematically evaluate the efficacy and toxicity of candidate therapies.²⁰² Interestingly, by integrating tumor cells and cardiac muscle cells on a single culture platform, researchers were able to more accurately predict both the direct and off-target effects of tyrosine kinase inhibitors.²⁰⁴

PDMS-based systems have also been used to model IFP and IFF in vitro. For example, IFP was simulated in a PDMS-based system by varying the volume of media between two reservoirs connected by a channel seeded with tumor cells. This setup established a pressure differential of 1.2 mmHg and generated fluid flow with a velocity of $1 \mu\text{m}\cdot\text{s}^{-1}$. The authors found that IFF alters the expression of mesenchymal genes, which influences the invasion phenotype of tumor cells.⁸⁴ Alternatively, transwell-based assays, in which tumor cells are seeded on top of a porous membrane, have been used to study IFF-mediated invasion.²² In this setup, tumor cells were encapsulated in a hydrogel on top of the transwell membrane and a pressure head was introduced to establish fluid flow through the gel. More recently, a modified version of this system was established to stimulate a monolayer of lymphatic endothelial cells (LECs) with both transmural (perpendicular) and luminal (shear) flow.²⁰⁵ In this system, LECs simultaneously experienced transmural flow via an applied pressure head and luminal flow induced by a peristaltic pump. Moreover, this system enabled real-time monitoring of tumor cell transmigration across the LEC monolayer as a model of intravasation. Ultimately, the authors found that transmural and luminal flow synergistically upregulated LEC expression of CCL21 to promote breast cancer cell transmigration.²⁰⁵

1.8 Clinical translation and future perspectives

While the impact of the physical microenvironment on tumor progression is now well established, targeting these phenomena in the clinic remains challenging. For example, cilengitide, a peptide inhibitor of integrins $\alpha\beta3$ and $\alpha\beta5$, initially demonstrated anti-invasive effects in preclinical tumor models and phase I/II clinical trials but failed to improve patient survival in a phase III clinical trial.^{206–208} Perhaps the most successful example of targeting physical tissue properties thus far is the use of anti-angiogenic agents, which were shown to normalize tumor vasculature, reduce IFP, and improve drug penetration in preclinical studies.^{23,209} Bevacizumab, a humanized anti-VEGF monoclonal IgG1 antibody, was the first anti-angiogenic agent approved for clinical use by the US Food and Drug Administration (FDA) in 2004.²¹⁰ While still recommended for numerous cancer types, including GBM, the FDA revoked bevacizumab's approval for advanced breast cancer in 2011.²¹¹ This decision resulted from a failure to improve survival while exposing recipients to adverse hemodynamic effect.^{212–214} Indeed, the efficacy of bevacizumab is highly variable, and it is unclear whether patients who respond to treatment benefit from vascular normalization or secondary, angiogenesis-independent effects such as immunomodulation.^{215,216} Ultimately, while bevacizumab is often considered the most successful therapy that targets the tumor microenvironment to date, it has not become a universal treatment for solid tumors as researchers once hoped.

Antifibrotic agents that target the ECM comprise another class of emerging therapies directed toward the physical tumor microenvironment.²¹⁷ These therapies tend to (a)

enzymatically degrade specific ECM components, (b) normalize or deplete ECM remodeling cells such as CAFs, or (c) interfere with the ability of cells to sense aberrant ECM by inhibiting mechanosignaling pathways.²¹⁸ Despite success in preclinical studies, therapies that directly modulate the ECM such as PEGPH20, a modified hyaluronidase, or simtuzumab, an inhibitor of the collagen cross-linker LOXL2, have largely failed clinical trials due to a lack of therapeutic benefit and a high incidence of adverse events.^{219–221} Strikingly, therapies that deplete CAFs worsened outcomes in preclinical studies.²²² These negative outcomes underscore the complexity of tumor–stroma interactions, which can either promote or restrict disease progression. Therapies that interfere with profibrotic or mechanosignaling pathways offer an alternative to target tumor-associated ECM. Indeed, multiple inhibitors for FAK, Rho-associated kinase, and TGF β 1 are currently under investigation.^{223,224} For example, the use of galunisertib, a small molecule inhibitor of profibrotic TGF β 1, improved overall survival for patients with unresectable pancreatic cancer and advanced liver cancer in phase I/II clinical trials.^{225,226} Collectively, these findings reveal the clinical potential of targeting the physical tumor microenvironment but also emphasize our incomplete understanding of these phenomena, which impedes the successful translation of candidate therapies from bench to bedside.

Bioengineers are well positioned to address outstanding questions in cancer mechanobiology given their multidisciplinary training and ability to translate between basic scientists, technicians, oncologists, and other healthcare professionals. Moving forward, interrogating physical parameters with more representative models of cancer, such as patient-derived organoids, will be critical to further our understanding of how the tumor microenvironment interfaces with the molecular heterogeneity of human disease.^{227,228} In particular, combining patient-derived tissues with humanized mouse models or organ-on-chip systems will enable better screening of candidate therapies in the preclinical setting and facilitate clinical translation to improve patient prognosis.^{229–231} Moreover, integrating spatial transcriptomics with high-resolution mapping of tissue mechanics in these studies will reveal how molecular and physical heterogeneity synergize to coordinate disease behavior. As we move toward adaptive strategies that aim to control tumor growth and prevent genetic selection of therapy resistance, we must also consider how the physical microenvironment evolves in response to treatment to collectively regulate clinical outcomes.^{232,233}

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Chapter 2: Dissecting TGF- β -induced glioblastoma invasion with engineered hyaluronic acid hydrogels

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2.1 Abstract

Glioma stem cells (GSCs) contribute to rapid cellular invasion in glioblastoma (GBM). Transforming Growth Factor- β (TGF- β) has been strongly implicated in supporting key GSC functions, including stemness, immunosuppression, and resistance. Although TGF- β is well-known as a driver of cancer invasion, how TGF- β supports the invasion of GSCs is not well understood. Progress in understanding mechanisms of TGF- β -driven invasion in GSC-derived tumors has been limited by an absence of three-dimensional (3D) culture systems that support TGF- β -stimulated invasion. Here, we show that 3D hyaluronic acid (HA) matrices can address this need. We perform bioinformatic analysis of human glioma datasets, which reveals progressive enrichment of TGF- β -related gene expression with increasingly aggressive glioma grade and GBM subtype. We then experimentally screen invasion of a panel of human GSC spheroids through a set of 3D matrix systems including collagen I, Matrigel, and HA and find that only HA recapitulates TGF- β -induced invasion. We then show that GSCs differ in their ability to invade HA in a way that can be predicted from TGF- β receptor 2 expression and SMAD2 phosphorylation. GSC spheroid invasion depends strongly on the presence of RGD peptides on the HA backbone but is surprisingly independent of matrix metalloprotease degradability. Finally, we demonstrate that TGF- β stimulates invasion through SMAD-dependent signaling, consistent with recent observations that TGF- β /SMAD signals drive tumor microtubule formation and invasion. Our work supports further development of HA as a matrix platform for dissecting contributions of TGF- β and other cytokines to GBM invasion and screening of cytokine-dependent invasion in human tumors.

2.2 Introduction

Glioblastoma (GBM) is an incurable primary brain tumor marked by rapid cellular invasion into surrounding brain tissue, which is in turn promoted by a combination of biochemical and biophysical factors within the tumor microenvironment (TME).^{180,234} Biochemical factors found in the TME include secretory cytokines, such as Transforming growth factor – β (TGF- β), Tumor necrosis factor - α (TNF- α) and interleukins, many of which can prime tumor cells for invasion through receptor-mediated signaling.^{114,235,236} Successful invasion also typically involves engagement with the extracellular matrix (ECM) through integrins, CD44, and other adhesion receptors, as well as remodeling of the ECM through protease degradation and nascent ECM deposition.^{100,101,103,237}

Over the past two decades, glioma stem cells (GSCs) have been increasingly implicated in driving GBM initiation, growth, recurrence, and resistance.^{238,239} Although the importance of GSCs in invasion is less well appreciated, GSCs are found at the invasive peritumoral region of GBMs and can directly contribute to evasion of therapeutic agents and seeding of secondary tumors.²⁴⁰ The cytokine TGF- β appears to play a particularly potent role in the maintenance of GSC stem-like characteristics and tumor-initiating capacity, with TGF- β -dependent signaling contributing to local immunosuppression and resistance to ionizing radiation and chemotherapies.^{241–245} While TGF- β signaling regulates GSC properties, how TGF- β stimulates GSC invasion has been minimally explored.^{246–248} This is in part due to a lack of *in vitro* culture platforms that can enable systematic dissection of intracellular and extracellular regulators of TGF- β -mediated invasion. The development and application of such platforms could offer new mechanistic insight into how TGF- β (and, by extension, other cytokines) drive GBM pathology, uncover new prognostic markers and therapeutic targets, and establish a foundation for personalized screening.

Traditional *in vitro* culture platforms to study invasion include two-dimensional (2D) tissue culture dishes and Boyden chamber assays. While these simplified paradigms have enabled novel insight into GBM pathology, they lack the full three-dimensional (3D) ECM microenvironment characteristic of the tissue microenvironment. To address this limitation, there has been a growing effort to model invasion using 3D *in vitro* culture models, including embedding tumor spheroids within 3D hydrogels, which are typically composed of tissue ECM preparations or purified ECM proteins such as collagen I.^{249,250} While native ECM-based hydrogels incorporate important structural and bioactive features of tissue ECMs, these materials are often heterogeneous, incompletely defined, and not amenable to precise modulation of biophysical properties. These needs have paved the way for engineered ECMs that enable tight control of viscoelastic properties and presentation of bioactive peptides for adhesion, capture of growth factors, and protease digestion. 3D invasion platforms may be built from ECM-derived biopolymers, such as alginate or hyaluronic acid (HA) or fully synthetic polymers such as polyethylene glycol (PEG).^{251–253}

HA is an especially attractive platform for modeling GBM invasion. HA is the most abundant matrix component in normal brain and plays a critical role in defining brain structure and mechanics as well as directly engaging cellular adhesion receptors such as CD44. Moreover, HA is highly enriched in GBM relative to healthy brain tissue, with the most aggressive forms of gliomas exhibiting greater levels of HA and altered HA molecular weight.^{180,254} HA-based adhesive signaling through CD44, and other receptors can drive GBM progression and infiltration into brain tissue.^{101,180,255} HA also retains the chemical versatility of synthetic polymers as it contains multiple orthogonal functional groups (e.g. hydroxyl, carboxyl) that can be exploited for conjugation of crosslinking agents and bioactive peptides.²⁵⁵ To this end, we and others have productively used HA-based scaffolds to model, deconstruct, and conduct molecular screens in GBM.^{16,179,256–258} Additionally, HA-CD44 signaling can also directly and indirectly modulate TGF- β signaling. For example, CD44 has been shown to directly bind and stimulate signaling through TGF- β receptor I, with TGF- β receptor I reciprocally phosphorylating and

activating CD44 to promote HA-based adhesion and migration.^{259–262} Thus, HA is not only an ideal material for modeling GBM invasion but particularly suitable for investigating contributions of TGF- β to the process.

Here, we combine engineered HA hydrogels, bioinformatics analysis, GSC culture, and mechanistic perturbations to investigate the interplay between TGF- β signaling and HA scaffold properties in driving invasion. We successfully classify GSCs according to their ability to invade HA in spheroid culture and correlate that invasive potential to expression of elements within the TGF- β signaling pathway. We also identify matrix conditions necessary for invasion, with invasion being strongly reliant on the presence of integrin-ligating peptides but surprisingly independent of proteolytic degradability. Finally, we show that TGF- β -induced GSC invasion in HA is mediated by SMAD2 phosphorylation, which is amplified when RGD peptides are conjugated to the HA backbone.

2.3 Results

2.3.1 Expression of TGF- β -related transcripts correlates with disease aggression in human gliomas.

Although much direct and indirect evidence supports a role for TGF- β in GBM invasion, many of these studies are preclinical in nature. Thus, we began by querying the Cancer Genome Atlas (TCGA) and Repository for Molecular Brain Neoplasia Data (Rembrandt) to confirm if tumor aggressiveness is correlated with enhanced expression of TGF- β -associated transcripts in a broad cohort of human GBMs. Using the Gliovis (<http://gliovis.bioinfo.cnio.es/>) to access the Rembrandt patient dataset, we first assessed relative expression of TGF- β ligands (TGFB1, TGFB2) and associated receptors (TGFR1, TGFR2) in different glioma grades (Oligodendroglioma, Astrocytoma, GBM) relative to normal brain. The highest-grade glioma, GBM, exhibited the highest expression of both TGF- β ligands and receptors (**Fig. 1A**).

We then focused our analysis on GBM. GBM tumors have been transcriptionally classified into subtypes (proneural, classical, mesenchymal), with mesenchymal-subtype tumors typically associated with more aggressive phenotypes and poorer patient survival.²⁶³ We first compared the expression of TGF- β ligands (TGFB1, TGFB2) and associated receptors (TGFR1, TGFR2) in mesenchymal-subtype tumors to the less aggressive proneural subtype, which is associated with a more favorable patient survival time but often recurs as the mesenchymal subtype.²⁶³ Mesenchymal tumors expressed higher levels of both TGF- β ligands and receptors relative to proneural tumors (**Fig. 1B**). Next, we assembled a panel of TGF- β responsive genes in glioma tissue from the literature and compared the expression of these genes in mesenchymal and proneural GBMs.^{248,264} Mesenchymal GBMs expressed most of these TGF- β targets at higher levels than proneural GBMs (**Fig. 1C**). We also broaden our search to TGF- β related genes reported in the KEGG Pathway Database and Molecular Signatures Database (MsigDB) and found a few genes were upregulated in the mesenchymal GBM tissue compared to the proneural GBM tissue (**Fig. S6**). Thus, expression of TGF- β pathway components and

transcriptional targets correlates with increasing glioma grade and is further enhanced in GBM subtypes that is considered most aggressive.

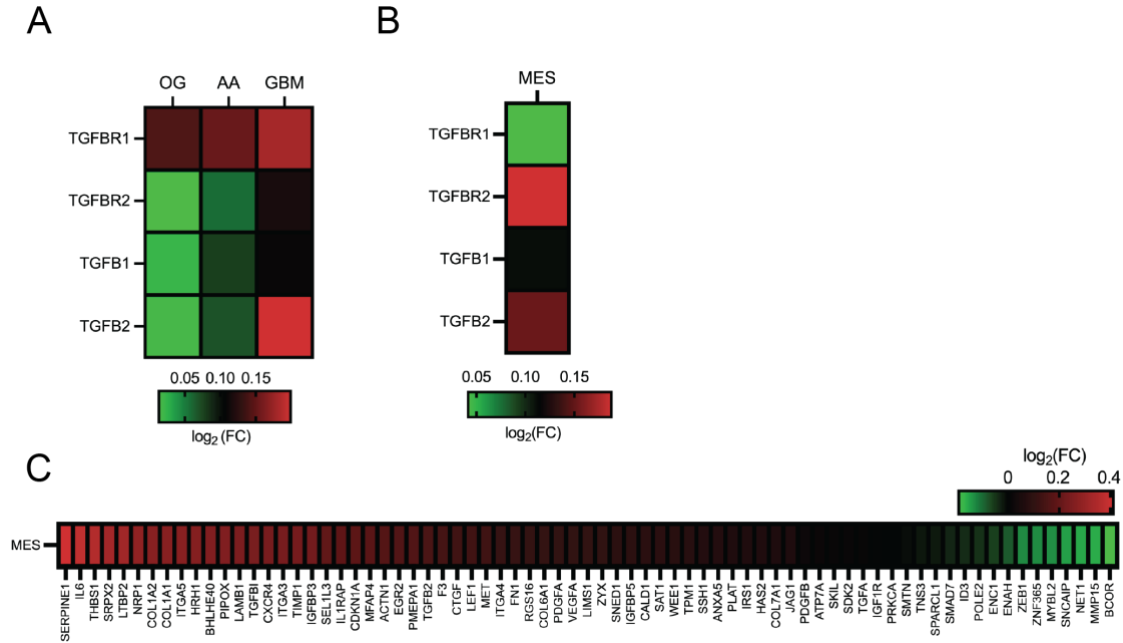


Figure 1. Expression of TGF- β -related transcripts correlates with disease aggression in human gliomas. (A) Correlation of TGF- β receptors and ligand expression with glioma grade. Oligodendroglioma (OG), Astrocytoma (AA), and Glioblastoma (GBM) expression of TGF- β ligands and receptors normalized to non-tumor tissue. (B) Expression of TGF- β ligands and receptors in GBM mesenchymal tumors (Mes). (C) Expression of TGF- β target genes (curated from literature) in mesenchymal GBM tumors. In (B) and (C), the expression of each gene was normalized to values for proneural tumors. In (A)-(C) expression data was acquired from TCGA using Gliovis.

2.3.2 HA matrix supports TGF- β responsive invasion in a GSC subtype-specific fashion.

Given that HA is highly expressed in glioma tissue and can influence TGF- β signaling, we next investigated the use of HA as a platform for studying TGF- β -induced GSC invasion. We first selected an HA hydrogel in which RGD-based peptides were conjugated to the HA backbone and hydrogel assembly was mediated by a protease degradable crosslinker to achieve a stiffness of ~ 450 Pa. Our parameter selections were guided by our past HA formulations for studying GBM invasion.^{101,179,258} We then selected a panel of patient GSCs derived from mesenchymal (GSC20, GSC28) and proneural (GSC262, GSC295) tumors and subjected spheroids of each GSC type to 3D invasion assays in engineered HA matrices. In the absence of TGF- β stimulation, all GSC spheroids remained circumscribed and did not invade as qualitatively defined by the absence of protrusions, multicellular fingers, or escaped cells into the ECM (**Fig. 2B-C**). This non-invasive phenotype persisted over 14 days of culture in HA (**Fig. S1**). Despite the absence of invasion, the spheroids continued to grow in volume indicating ongoing proliferation (**Fig. S1**). Interestingly, we observed that the non-invasive phenotype of the GSC20 and GSC28 in HA was independent of stiffness over a range of 100 Pa – 1.2 kPa, which

nominally brackets the stiffness range of brain and glioma tissue (**Fig. S8**)^{265–267}. Addition of TGF- β 1 ligand stimulated GSC invasion through extended and branched protrusion into the matrix (**Fig. 2B**), although only for a subset of GSCs tested. Notably, the responsive GSCs (GSC20 and GSC28) were both derived from mesenchymal tumors (Fig. 2B), while the non-responsive GSCs (GSC295 and GSC262) were both derived from proneural tumors (Fig. 2C). Thus, in this limited subset of GSCs, HA recapitulates the expected hierarchy of subtype-specific invasive behaviors.

To determine whether other commonly used 3D ECM platforms can similarly support subtype-specific TGF- β invasion, we repeated GSC spheroid invasion studies in two naturally derived matrices: collagen I and Matrigel. We chose these matrices because of their common use in tumor invasion studies rather than their mimicry of brain tissue; the brain is typically understood to be collagen I-poor relative to other tissues and Matrigel is derived from mouse sarcoma basement membrane.^{268,269} In contrast to HA matrix, all the GSCs rapidly invaded collagen I and Matrigel matrices within 24 hours of seeding, with or without TGF- β 1 stimulation (**Fig. 2E-F, H-I**). In collagen, GSC migratory protrusions were linear and directional, whereas in Matrigel, protrusions displayed a diversity of invasion morphologies, including directional, branched, and single cell modalities that varied across GSC lines. Again, these morphologies were present with and without TGF- β 1 stimulation. Nonetheless, collagen did preserve some differences across GSC lines previously identified as TGF- β responsive/non-responsive in HA (GSC20, GSC28), including longer protrusions for these GSCs under TGF- β stimulation, contributing to a modest TGF- β -dependent increase in the spheroid invasion index.

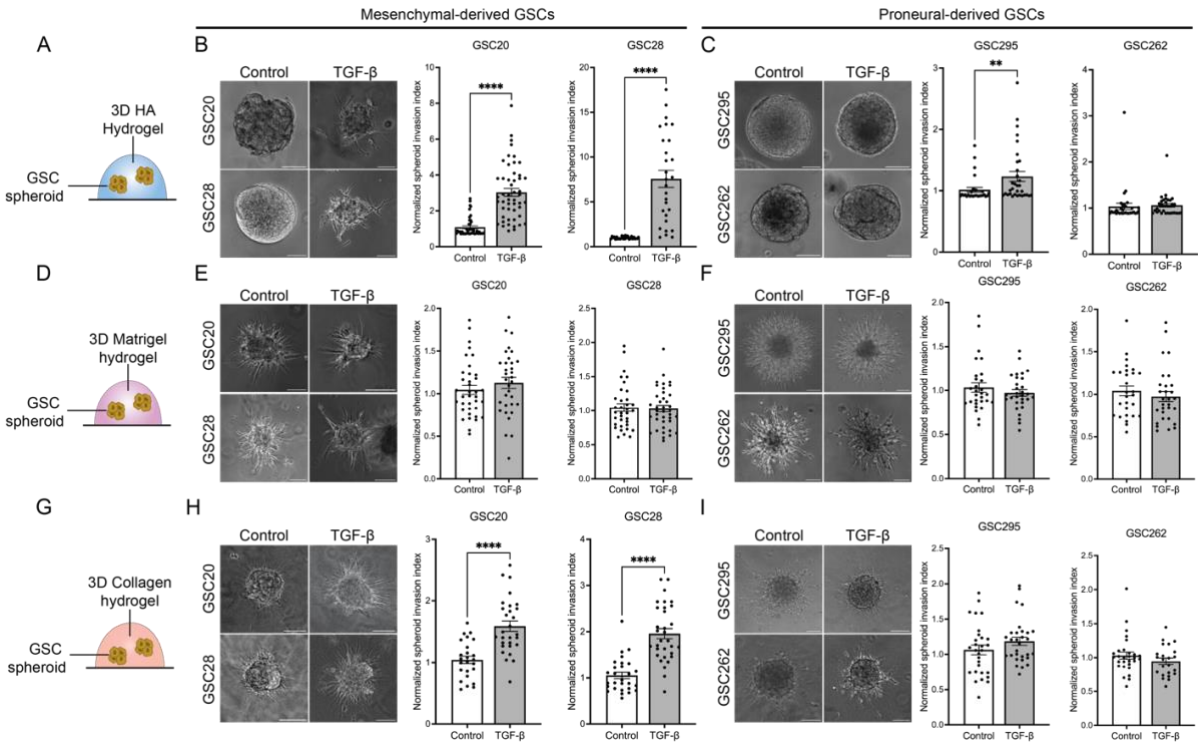


Figure 2. HA matrix supports TGF- β responsive invasion in a GSC subtype-specific fashion. (A) Schematic of HA GSC spheroid invasion assay. (B-C) GSCs spheroid invasion in HA. GSCs were cultured in HA with or without TGF- β stimulation. Data represent $n = 44$ and 50 spheroids (GSC20), 31 and 28 spheroids (GSC28), 27 and 32 spheroids (GSC295), and 32 and 33 spheroids (GSC262) for control and TGF- β stimulation conditions, respectively. Values display mean $\pm$ standard error of the mean (SEM) (D) Schematic of GSC spheroid invasion assay in Matrigel. (E-F) GSC spheroid invasion in Matrigel. GSCs were in Matrigel cultured with or without TGF- β stimulation. Data represent $n = 37$ and 33 spheroids (GSC20), 35 and 39 spheroids (GSC28), 29 and 30 spheroids (GSC295), and 28 and 30 spheroids (GSC262) for control and TGF- β stimulation conditions, respectively. Values display mean $\pm$ SEM (G) Schematic of GSC spheroid invasion assay in collagen. (H-I) GSC spheroid invasion in Collagen. GSCs were in collagen cultured with or without TGF- β stimulation. Data represent $n = 25$ and 29 spheroids (GSC20), 28 and 32 spheroids (GSC28), 29 and 30 spheroids (GSC295), 29 and 23 spheroids (GSC262) for control and TGF- β stimulation conditions, respectively. Values display mean $\pm$ SEM * $p < 0.033$, ** $p < 0.002$, *** $p < 0.0002$, **** $p < 0.00001$ [Mann-Whitney Test]. Scale bar: $100\mu\text{m}$.

2.3.3 TGF- β -induced invasion of GSCs in HA is mediated by SMAD phosphorylation.

To gain additional insight into the mechanistic origins of the differential HA invasion response across GSCs, we measured expression of the TGF- β receptors TGFBR1 and TGFBR2 by qPCR. The expression of TGFBR2 but not TGFBR1 was higher in responsive GSCs (GSC20, GSC28) relative to nonresponsive GSCs (GSC295, GSC262) (**Fig. 3A, 3B**). Interestingly, when assessing the levels of TGFBR1 and TGFBR2 expression in each of the GSC lines, we found that the relative expression of TGFBR2 expression was 2-3 fold higher than TGFBR1 in the responsive GSCs (GSC20, GSC8). This contrasted with the non-responsive GSCs (GSC295, GSC262) where TGFBR2 expression was 2-9x lower than TGFBR1 (Fig. 3C).

Having correlated TGF- β -responsiveness with TGFBR2 expression, we next investigated whether this correlation extended to TGF- β pathway effectors by measuring SMAD2 phosphorylation via western blot. Responsive GSCs displayed SMAD2 phosphorylation (Fig. 3D) when treated with TGF- β 1 ligand whereas the nonresponsive GSCs lacked SMAD2 phosphorylation (Fig. 3E). To test the functional contributions of SMAD phosphorylation to invasion, we repeated our 3D HA tumor spheroid invasion studies in the presence of a SMAD2 inhibitor (Galunisertib) that has been explored clinically for GBM.²⁷⁰ Galunisertib treatment suppressed TGF- β -stimulated invasion by six-fold for GSC20 and three-fold for GSC28, confirming that TGF- β induced SMAD2 phosphorylation drives invasion (Fig. 3F-G). This finding corroborates recent evidence pointing to SMAD activation as a mediator of TGF- β induced formation of tumor microtubes, which support GBM invasion *in vivo*.²⁴⁸

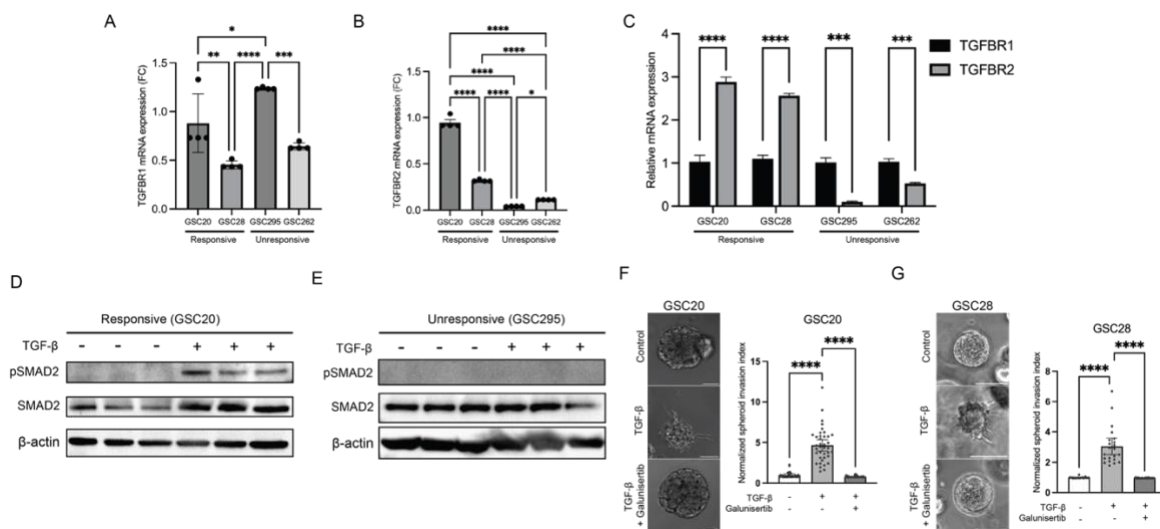


Figure 3. TGF- β -induced invasion of GSCs in HA is mediated by SMAD phosphorylation. (A) Relative TGFBR1 mRNA expression in GSC20, GSC28, GSC295, and GSC262. Expression from all GSCs was normalized to GSC20. N=4 biological replicates. (B) Relative TGFBR2 mRNA expression in GSC20, GSC28, GSC295, and GSC262. N = 4 biological replicates. Expression from all GSCs was normalized to GSC20. (C) TGFBR2 mRNA expression relative to TGFBR1 expression. The expression of TGFBR1 was used to normalize TGFBR2 expression within each of the GSCs used (GSC20, GSC28, GSC295, and GSC262). N = 4 biological replicates. (D-E) Comparison of SMAD2 activity from GSCs with and without TGF- β treatment. Western blot probing pSMAD, SMAD2, and β -actin expression in representative TGF- β responsive (D) and non-responsive GSCs. Western blots are representative example from n = 3 biological replicates for both responsive and non-responsive GSCs. (F-G) SMAD2 inhibition of TGF- β stimulated invasion. Spheroid invasion of GSC20(F) and GSC28 (G) in HA under TGF- β stimulation, with or without SMAD2 inhibitor (galunisertib). Data represent n = 28 (control), 38 (TGF- β), and 34 (TGF- β + galunisertib) spheroids for GSC20 and 24 (control), 22 (TGF- β), and 27 (TGF- β + galunisertib) spheroids for GSC28. *p<0.033, **p<0.002, *** p<0.0002, ****p<0.00001 by (A-B) One way ANOVA with Tukey Multiple Comparison Test; (C) Unpaired t-test; and (F-G) Kruskal-Wallis test with Dunn's multiple comparison test. Values display mean $\pm$ SEM. Scale bar: 100 μ m.

2.3.4 TGF- β induced GSC invasion in HA matrices requires conjugation of RGD peptides.

Finally, we sought to deconstruct matrix-based contributions to TGF- β -stimulated 3D cell motility in HA. 3D invasion is supported by a combination of cell-matrix adhesion and ECM remodeling, including matrix digestion. In our engineered HA platform, we have previously established that at least two adhesive systems contribute to adhesion: binding of CD44 to the HA backbone and binding of integrins to HA-conjugated RGD peptides.^{100,101} Additionally, our HA matrix is crosslinked with a peptide crosslinker that may be cleaved by matrix metalloproteinases (MMP) (**Fig. 4A**). To understand how these three modalities contribute to TGF- β -stimulated invasion, we first measured transcriptional changes of responsive GSCs (GSC20 and GSC28) cultured in our HA platform with and without TGF- β stimulation via qPCR. We selected a set of genes related to ECM adhesion and remodeling including hyaluronidases, HA synthase, MMPs, and integrin subunits expected to be relevant in our HA matrix. We observed an overall increase in expression of all the genes with the highest fold change captured in MMP2 and ITGB3 for both GSC20 and GSC28 (**Fig. S3C, Fig. S4C**). Interestingly, we noticed that genes relating to matrix degradation (MMP2, HYAL1) were upregulated in both GSCs, while some genes relating to matrix synthesis (HAS2) and adhesion (ITGAV, ITGB1) were only upregulated in GSC28 but not GSC20. CD44 expression was not altered with TGF- β treatment in either cell line (**Fig. S3C, Fig. S4C**). We further compared these findings to patient transcriptomic data based on GBM subtype and found an upregulation of these genes in mesenchymal tissues compared to proneural tissue (**Fig. S7**). Only HYAL1 was upregulated in proneural tissue (**Fig. S7**). Furthermore, we selected another gene set that is commonly reported to be associated with the GBM mesenchymal signature, is targetable by TGF- β , and includes multiple regulators of ECM remodeling^{271–276}. We found that all genes in this set were upregulated in both GSCs when treated with TGF- β in HA. Only CHI3L (YLK-40) exhibited reduced expression in GSC20 (**Fig. S3A, Fig. S4A**).

Next, we subjected GSC20 and GSC28 to HA tumor spheroid invasion assays in which we either included or excluded RGD peptides from the HA backbone and used protease-degradable vs. protease-non-degradable peptide crosslinkers. Surprisingly, TGF- β -induced invasion was nearly completely suppressed when RGD was removed from the HA backbone but was unaffected when MMP degradability was removed. We saw that TGF- β stimulated invasion was reduced two-fold when MMP degradability was removed and reduced three-fold when RGD was removed for GSC20 (**Fig. 4B**). For GSC28 cells, TGF- β stimulated invasion was reduced three-fold when MMP degradability was removed and reduced six-fold when RGD was removed (**Fig. 4C**). Because integrin signaling can strongly affect TGF- β signaling, we asked whether the lack of invasion in RGD-free matrices was directly due to loss of integrin-adhesive function or some broader, RGD-dependent dysfunction in TGF- β signaling or matrix invasion capacity. First, we did not detect a difference in TGFBR2 expression between responsive GSCs cultured in HA-RGD (+) and HA-RGD (-) matrices (**Fig. S2**), implying that loss of RGD did not compromise the ability to bind TGF- β . We then probed expression of genes from our panel of ECM remodeling and GBM mesenchymal genes in GSCs cultured in HA-RGD (-

) with and without TGF- β stimulation. We found that transcript expression within the panel mirrored the HA-RGD (+) conditions, in that genes upregulated by TGF- β treatment in HA-RGD (+) matrix were also upregulated in HA-RGD (-) matrix (**Fig. S3B, Fig. S3D, Fig. S4B, Fig. S4D**). Additionally, we performed qPCR measurements of the same gene set to directly compare TGF- β treated GSCs cultured in HA-RGD (+) and HA-RGD (-) matrices, which revealed no significant differences (**Fig. S5**). The results of our qPCR measurements were similar in both TGF- β -responsive GSCs (GSC20 and GSC28). These findings suggest that RGD presentation supports the conversion of transcriptional changes induced by TGF- β treatment into functional invasion of GSCs in HA matrix.

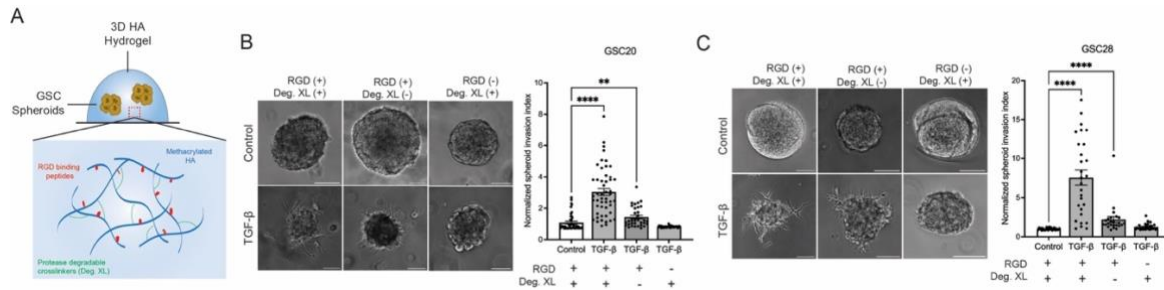


Figure 4. TGF- β induced GSC invasion in HA matrices requires conjugation of RGD peptides. (A) Schematic of engineered HA matrix showing RGD peptides and protease-degradable crosslinkers. (B-C) TGF- β stimulated HA spheroid invasion in the absence of MMP degradability and RGD for (B) GSC20 and (C) GSC28. Data represent n = 44 (Control, RGD (+) and Degradable XL (+)), n = 50 (TGF- β , RGD (+) and Degradable XL (+)), n = 33 (TGF- β , RGD (+) and Degradable XL (-)), n = 32 (TGF- β , RGD (-) and Degradable XL (+)) spheroids for GSC20 and n = 31 (Control, RGD (+) and Degradable XL (+)), n = 28 (TGF- β , RGD (+) and Degradable XL (+)), n = 24 (TGF- β , RGD (+) and Degradable XL (-)), n = 30 (TGF- β , RGD (-) and Degradable XL (+)) spheroids for GSC28. Values display mean $\pm$ SEM. * $p < 0.033$, ** $p < 0.002$, *** $p < 0.0002$, **** $p < 0.00001$ by Kruskal-Wallis test with Dunn's multiple comparison test. Scale bar: 100 μ m.

2.4 Discussion

While TGF- β -mediated signaling has been demonstrated to support resistance, stemness-like qualities, and tumor initiating capacity of GSCs, the field's understanding of how TGF- β promotes 3D invasion of GSC-derived tumors remains somewhat limited. This knowledge gap is partially due to a lack of modular 3D culture platforms that enable systematic dissection of cell- and matrix-based regulation of TGF- β during invasion. We show that engineered HA-based matrices can address this unmet need. Using HA-based materials, we successfully classify GSCs into TGF- β -responsive and non-responsive categories and show that TGF- β induces GSC spheroid invasion through SMAD2 phosphorylation. We also show that invasion requires the presentation of integrin ligands (RGD peptides) but surprisingly not MMP-degradable crosslinks.

One important advantage of HA as a platform for studying TGF- β -mediated invasion is that invasion is limited in the absence of TGF- β stimulation, providing a clean "baseline" to assess stimulated invasion in GSCs. This contrasts with collagen I and Matrigel, which support robust invasion even the absence of exogenous cytokine treatment (**Fig. 2**). The

strong matrix-dependence of invasion may be due to the very different architectures of the three matrix systems. Collagen I and Matrigel hydrogels have been reported to have pore sizes on the order of micrometers whereas we have reported the pore size of our HA system to be on the nanometer scale.^{179,277} Additionally, collagen I hydrogels containing fibers that can be used as contact-guidance cues for rapid invasion. Moreover, Matrigel frequently contains – and collagen I contains binding sites for – pro-migratory cytokines, which could potentially blunt effects of exogenous TGF- β .^{179,277}

An important advantage of our biomaterial platform is the ability to systematically tune matrix factors to test their influence on invasion. In doing so, we found that conjugation of RGD peptides to the HA backbone was needed for TGF- β -stimulated invasion (Fig. 4). While the strong integrin-dependence of invasion was not perhaps in itself surprising, it was notable that the presence or absence of RGD did strongly influence the expression of nominally pro-invasive genes (**Fig. S3, Fig. S4**). This raises the possibility that invasion-competent GSCs are transcriptionally “primed” to invade but cannot execute an invasion program without the appropriate ECM environment. This priming hypothesis is supported by two observations in RGD-free matrices: first, integrin expression increases upon stimulation in TGF- β -responsive GSCs; and second, that tumor spheres exhibit jagged edges suggesting increased membrane ruffling (Fig. 4). The strong RGD-dependence of invasion raises the question of the role played by HA-CD44 interactions in this system. CD44 expression did not change following TGF- β stimulation (**Fig. S3C, Fig. S4C**); however, the interpretation of this finding is complicated by the fact that GSC20 and GSC28 have been reported to have high basal expression of CD44, leaving little dynamic range for a stimulated increase.^{101,263} We have also previously reported that CD44-HA is an early and short lived (0.5 h) matrix adhesion system, whereas integrin-based adhesions take longer to mature.¹⁰⁰ Therefore, it is plausible that CD44-HA adhesion alone in RGD-free matrices can initiate but not sustainably support adhesion and adhesion-dependent processes necessary for TGF- β -stimulated invasion over the time scale of invasion (10-14 days).

Finally, our findings add to a growing body of work illustrating the heterogeneity of GSCs with respect to TGF- β -sensitivity in other contexts.^{246,278,279} GSCs tumors that are responsive to TGF- β in vivo, as identified by TGFBR2 expression and lack of 33phosphor-SMAD2, exhibit increased infiltration of immune cells.^{278,279} Implications of differential TGF- β -sensitivity for invasion have been difficult to dissect in vivo due to the complexity of the microenvironment.²⁴⁶ Our study offers a roadmap to tease apart this heterogeneity and further suggests that TGF- β -sensitivity may be cross-correlated with the traditional hierarchy of GBM subtypes (proneural, classical, mesenchymal). Our study also suggests that changes in SMAD2 phosphorylation status strongly predict TGF- β -sensitivity, with SMAD2 phosphorylation not increasing with TGF- β stimulation in non-responsive GSCs. This heterogeneity is consistent both with past studies on the role of TGF- β in inducing mesenchymal transitions and the emerging finding that cells between and within GBM tumors may show characteristics of multiple distinct subtypes, potentially supported by state-switching of GSCs.^{92,246,263} It will be valuable to explore these sources of heterogeneity more fully in the future and ask how TGF- β -sensitivity maps to other GBM classification categories such as IDH mutation and methylation status. We expect that our

HA hydrogel platform and other synthetic invasion platforms can play an important role in reaching these goals.

2.5 Supplementary Material

A

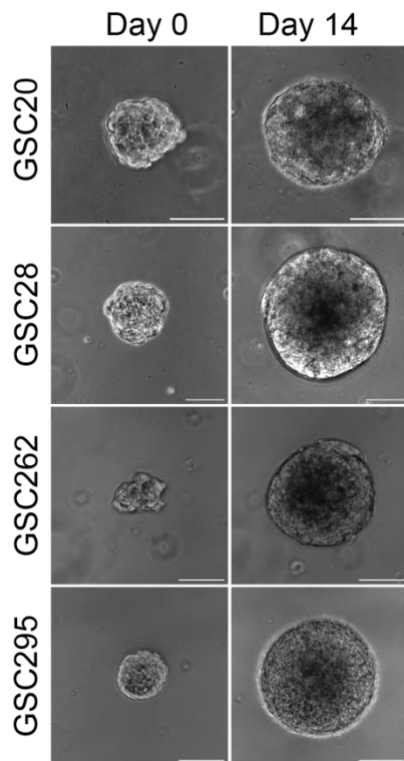
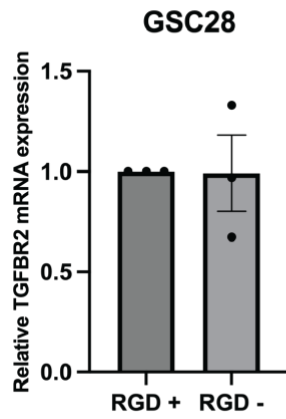


Figure S1. GSCs do not invade at baseline in HA over the course of a 14-day culture. GSC20, GSC28, GSC262, and GSC295 was cultured in HA for 14 days and all the GSCs remained circumscribed, showing no signs of protrusion formation over the course of the culture period in ~500 Pa HA-RGD (+) hydrogels. Scale bar: 100 μ m.

A



B

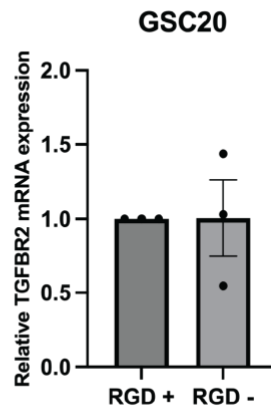


Figure S2. TGFBR2 expression is independent of RGD conjugation. TGFBR2 expression of GSCs in HA-RGD (+) was used to normalized TGFBR2 expression of GSCs in HA-RGD (-) for both GSC28 (A) and GSC20 (B) under TGF- β stimulation. Values display mean + SEM. N= 3; $p > 0.05$ by paired t-test.

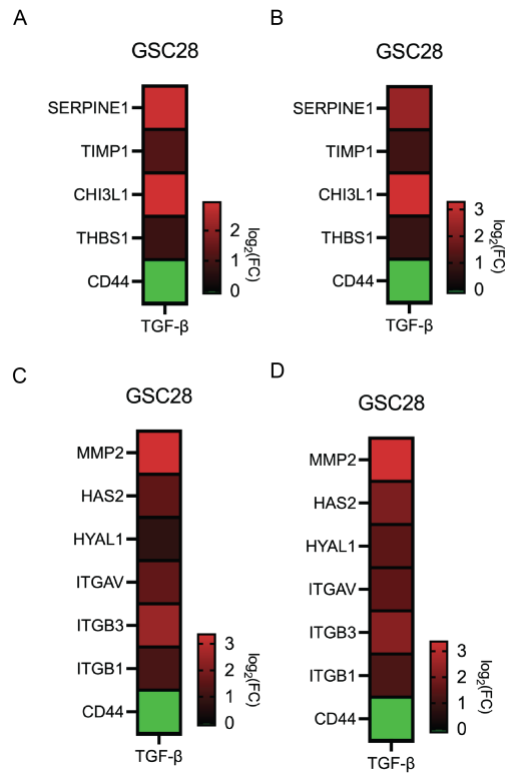


Figure S3. GSC28 GSCs cultured in the presence and absence of conjugated RGD share common transcriptional responses to TGF- β . Relative expression of mesenchymal subtype (A-B) and matrix remodeling genes (C-D) of GSC28 with and without TGF- β stimulation cultured in HA-RGD (+) (A,C) and HA-RGD (-) (B,D) matrices. Non-TGF- β -treated cells were used as a control condition to normalize data and more clearly identify gene expression changes associated with TGF- treatment. Values mean $\pm$ SEM (n = 3 biological replicates) and represented as $\log_2(\text{fold change})$.

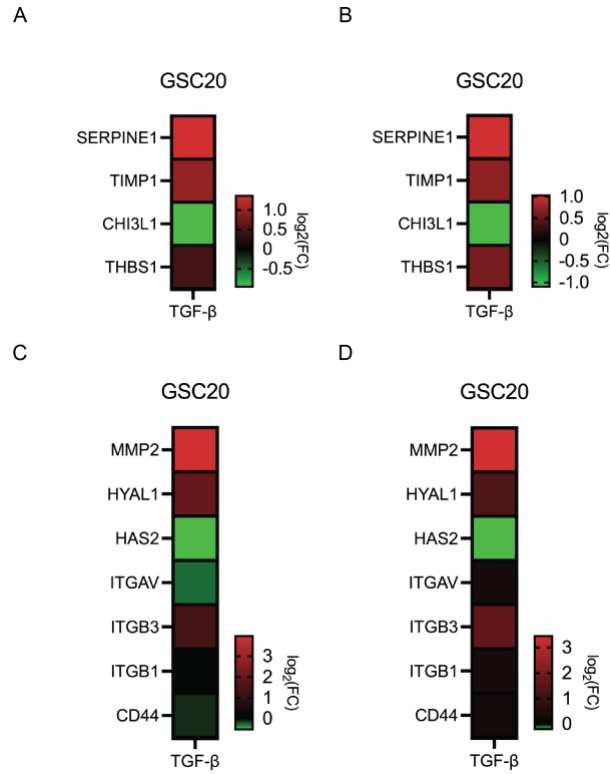


Figure S4. GSC20 GSCs cultured in the presence and absence of RGD share common transcriptional responses to TGF- β . Relative expression of mesenchymal subtype (A-B) and matrix remodeling genes (C-D) of GSC20 with and without TGF- β stimulation cultured in HA-RGD (+) (A,C) and HA-RGD (-) (B,D) matrices. Non-TGF- β -treated cells were used as a control condition to normalize data and more clearly identify gene expression changes associated with TGF- treatment. Values mean $\pm$ SEM (n = 3 biological replicates) and represented as log₂(fold change).

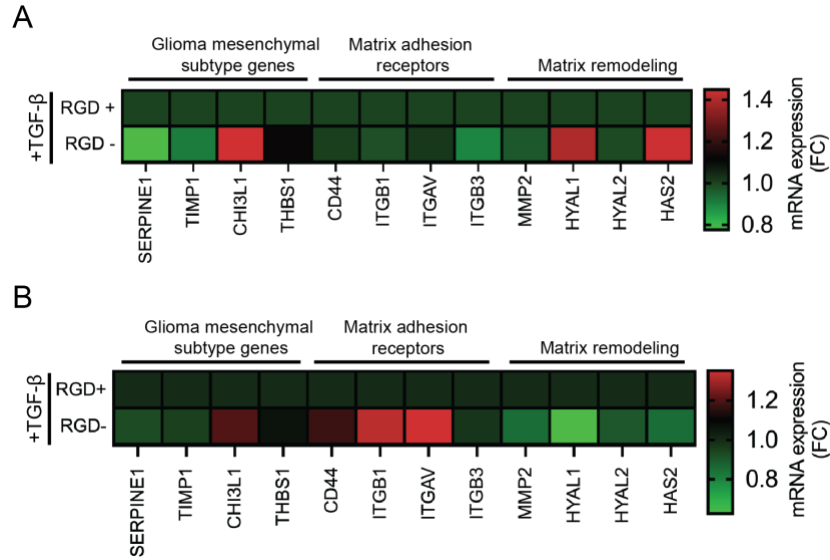


Figure S5. GSCs cultured in HA with or without RGD share common transcriptional responses to TGF- β . Relative expression of mesenchymal subtype, matrix adhesion, and matrix remodeling genes of GSC28 (A) and GSC20 (B) under TGF- β stimulation cultured in HA-RGD (+) and HA-RGD (-) matrices. Cells cultured in HA-RGD (+) condition were used as a control condition to normalize data and more clearly identify gene expression changes associated with removal of RGD from the HA backbone. Values displayed are represented as $\log_2(\text{fold change})$. Mean $\pm$ s.e.m. N=3. Paired T-test.

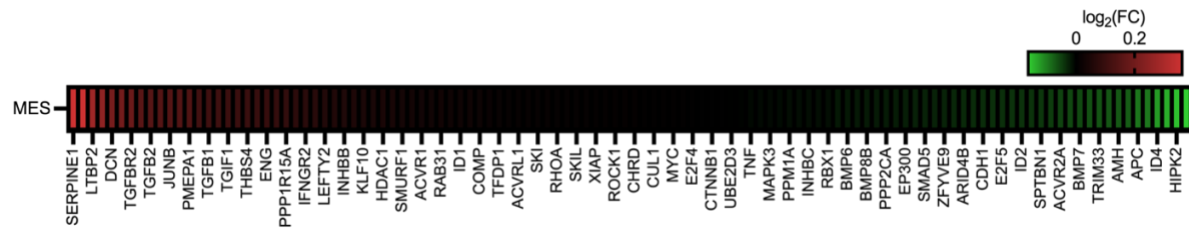


Figure S6. TGF- β related genes are upregulated in mesenchymal GBM tissue compared to proneural GBM tissue. Expression of TGF- β target genes (curated from Molecular Signatures Database and KEGG Pathway database) in mesenchymal GBM tumors. The expression of each gene was normalized to values for proneural tumors. Expression data was acquired from TCGA using Gliovis.

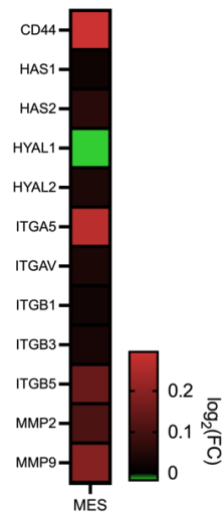


Figure S7. Matrix adhesion and remodeling transcripts are upregulated in mesenchymal tumor subtype relative to proneural subtype. Expression of matrix adhesion and remodeling related genes in mesenchymal GBM tumors. The expression of each gene was normalized to values for proneural tumors. Expression data was acquired from TCGA using Gliovis.

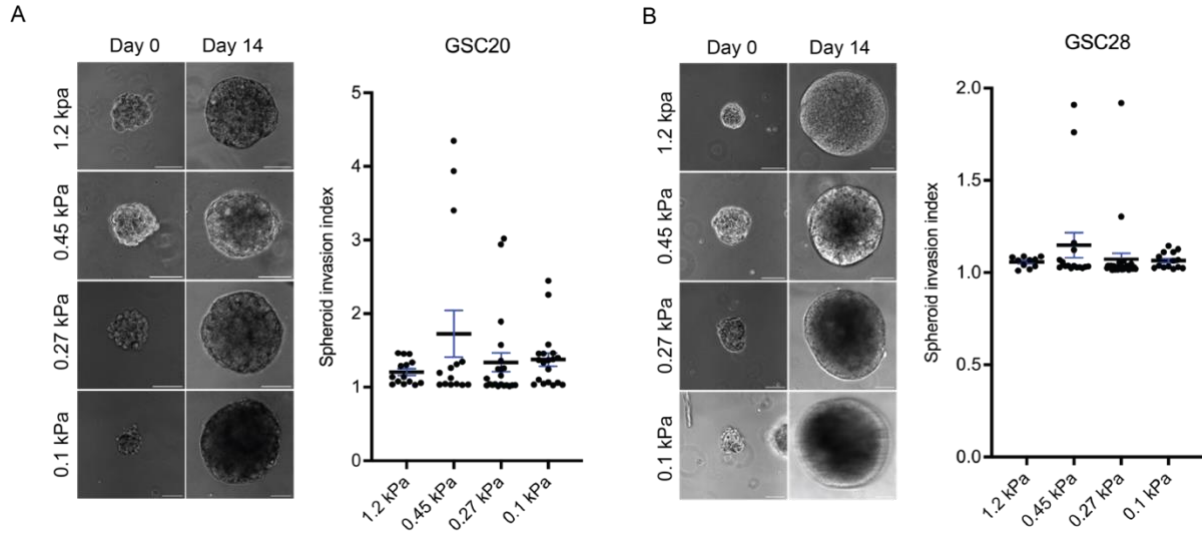


Figure S8. Mesenchymal GSCs do not exhibit 3D stiffness-dependent invasion at baseline. GSC20 (A) and GSC28 (B) cultured in HA over the course of 14 days at different stiffness values (100 Pa – 1.2 kPa). Scale bar: 100 μ m. Values display mean $\pm$ SEM

Supplementary Table 1. qPCR primer sequences.

Gene	Forward Primer	Reverse Primer
RPS9	GACTCCGGAACAAACGTGAGGT	CTTCATCTTGCCCTCGTCCA
CD44	CGAACAAGGTGTGGGCAGAA	CCGTTGAGTCCACTTGGCTT
ITGB1	CAAGAGAGCTGAAGACTATCCCA	TGAAGTCCGAAGTAATCCTCCT
CHI3L1 ((YLK40)	AAGCAACGATCACATCGACAC	TCAGGTTGGGGTTCCTGTTCT
SERPINE1	TTTCAGAGGTGGAGAGAGCC	CTGATTTGTGGAAGAGGCGG
TIMP1	ACCACCTTATACCAGCGTTATGA	GGTGTAGACGAACCGGATGTC
TGFBR1	ACGGCGTTACAGTGTTTCTG	GCACATACAAACGGCCTATCTC
TGFBR2	GTAGCTCTGATGAGTGCAATGAC	CAGATATGGCAACTCCCAGTG
OLIG2	TGGCTTCAAGTCATCCTCGTC	ATGGCGATGTTGAGGTCGTG
ITGB3	CAGATGCCTGCACCTTTAAGAAA	TCACGGCAGTAACGGTTGC
ITGAV	ATCTGTGAGGTCGAAACAGGA	TGGAGCATACTCAACAGTCTTTG
hTSP1	TGCCTGATGACAAGTTCCAAG	CCAGAGTGGTCTTTCCGCTC
HAS2	TCGCAACACGTAACGCAAT	ACTTCTCTTTTCCACCCCATT
HYAL1	ACCACCGTCTGGAATGCAAA	ACCACATCGAAGACACTGACAT
MMP2	AGCGAGTGGATGCCGCCTTTAA	CATTCCAGGCATCTGCGATGAG

2.6 Materials and Methods

Cell culture

GSC20, GSC28, GSC262, and GSC295 cells were obtained from MD Anderson Cancer Center and propagated as neurospheres in DMEM/F12 basal medium (Corning, catalog no. 10-090-CV) supplemented with 2% (vol/vol) B-27 supplement (Gibco, 17504-044), 20 ng/mL EGF (R&D Systems, 236-EG-01M), and 20 ng/mL FGF (R&D Systems, 233-FB-025/CF). For 3D HA gel experiments, the medium was supplemented with 0.1% penicillin/streptomycin (Thermo Fisher Scientific, catalog no.15140-122). Cells were screened for mycoplasma and validated by Short Tandem Repeat (STR) analysis every six months.

HA synthesis and RGD conjugation

HA hydrogels were synthesized as previously described.¹⁷⁹ Briefly, methacrylic anhydride (Sigma-Aldrich, 94%, catalog no. 276685) was used to functionalize sodium hyaluronate (Lifecore Biomedical, Research Grade, 66 kDa – 99 kDa, HA60K) with methacrylate groups. The extent of methacrylation per disaccharide was quantified by ¹H NMR as detailed previously and found to be ~85% for materials used in this study. For studies involving RGD peptides, methacrylated HA was conjugated via Michael Addition with the cysteine-containing RGD peptide Ac-GCGYGRGDSPG-NH₂ (Anaspec, catalog no. AS-62349) at a concentration of 0.5 mmol/L. Peptide crosslinker details are described in the Spheroid Encapsulation section below.

Generation of spheroids

Glioma stem cell (GSC) spheroids were generated using AggreWell Microwell Plates (the STEMCELL Technologies, catalog no. 34415). Briefly, the wells of the microwells were treated with non-adherent rinsing solution (STEMCELL Technologies, catalog no. 07010). GSCs were then seeded as single cells at ~200k per well for 2-3 days to allow the cells to aggregate and form uniform tumor spheroids. The spheroids were then encapsulated in hydrogels to perform the 3D spheroid invasion assay. For Matrigel and collagen experiments, cells were seeded ~300k per well and cultured for 2-3 days.

Spheroid encapsulation in HA

Spheroids were harvested from AggreWell plates, resuspended in non-phenol red DMEM (Thermo Fisher Scientific, Catalog no.31053028), and mixed with HA hydrogel precursor solution consisting of HA-RGD (+) or HA-RGD (-) polymer, 10wt% stock of protease degradable (KKCGGPQGIWGQGCKK, Genscript) or 10wt% stock of non-degradable (KKCGGDQGIAGFGCKK, Genscript) crosslinker containing a bifunctional cysteine peptide. Methacrylated HA was dissolved to a concentration of 1.5% (wt/vol), and the ratio of thiols: HA monomer was varied to achieve a shear modulus of ~450 Pa unless mentioned otherwise. Approximately 0.3 thiol:HA monomer ratio was enough to reach the desired shear modulus for both protease degradable and non-degradable crosslinker. The mixture was then plated at 10μL droplets in a non-adherent well plate and incubated for 1 hr at 37 °C, 5% CO₂ before introducing cell culture medium. TGF-β1 ligand (10ng/ml, R&D Systems, catalog no. 7754-BH) and Galunisertib 10μM, Selleckchem, catalog no.

S2230) was added to the cell culture medium as needed for each experiment. Media was changed every 2-3 days.

Spheroid encapsulation in Matrigel

Spheroids were harvested from AggreWell plate resuspended in Matrigel (Fisher Scientific, catalog no. CB40230) at ~9 mg/ml. The mixture was then plated as droplets in a non-adherent cell culture plate and incubated for 50 min. at 37 °C, 5% CO₂ before introducing cell culture medium. TGF- β 1 ligand (10 ng/ml, R&D Systems, catalog no. 7754-BH) was added to the cell culture media as needed for each experiment.

Spheroid encapsulation in Collagen

Spheroids were harvested from AggreWell plate resuspended in non-phenol red DMEM (Thermo Fisher Scientific) and mixed with Bovine collagen type I (Advanced Biomatrix , catalog no. 5010-50ML), 1N NaOH (Fisher Scientific, LC245004), 1N HEPES (Life Technologies, catalog no. 15630-080), to yield a 2mg/ml final collagen concentration. The mixture was then plated as droplets in a non-adherent cell culture plate and incubated for 50 min at 37 °C, 5% CO₂ before introducing cell culture medium. TGF- β 1 ligand (10ng/ml, R&D Systems, catalog no. 7754-BH) was added to the cell culture media as needed for each experiment.

Imaging and spheroid index invasion quantification

Spheroids were tracked for up to 14 days in HA, 24-48 hr in Matrigel, and 24 hr in collagen using Eclipse TE2000 Nikon Microscope with a Plan Fluor Ph1 $\times$ 10 objective. Images were acquired using NIS-Elements Software. Invasion was quantified using ImageJ by outlining the invasion front of the tumor spheroids to acquire a circularity value using the ImageJ function “shape descriptors.” Then the inverse of the circularity was taken and reported as spheroid invasion index. Condition where TGF- β was not added was used to normalize the plotted data.

Quantitative real time polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) experiments were setup using paired sample experimental setup and complimentary analysis.²⁸⁰ Cells were dissociated and encapsulated in hydrogel at a concentration of 10k cells/10uL of hydrogel for 7 days of culture at 37 °C, 5% CO₂. At the end of the culture period, cells encapsulated in the hydrogel were collected from the hydrogel by adding 10k U/ml hyaluronidase (Sigma-Aldrich, catalog no. H3884) to dissociate the HA hydrogel. Total mRNA was then isolated using TRIzol Reagent (Invitrogen, catalog no. 15596018) according to manufacturer’s recommendations protocol. cDNA was synthesized from the isolated mRNA using IscriptTM cDNA Synthesis Kit (Bio-Rad, catalog no. 1708891). qPCR was performed using Power up Sybr green master mix (Fisher Scientific, catalog no. A25777) for 40 cycles in a CFX real-time PCR detection system (Bio-Rad). To quantify relative fold change in the level of genes, qPCR data were analyzed by calculating $\Delta\Delta$ Ct with respect to RSP9 as the housekeeping gene. The primer sequences used in this study are listed in Supplemental Table 1.

TCGA and Rembrandt Transcriptomic analysis

Human glioma transcriptomic data was obtained from the Rembrandt and The Cancer Genome Atlas (TCGA) patient databases via the GlioVis data portal (<http://gliovis.bioinfo.cnio.es/>). Using the Rembrandt database, mRNA expression of TGFBR1, TGFBR2, TGFB1, and TGFB2 from non-tumor, oligodendroglioma, astrocytoma, and glioblastoma tissue was analyzed. mRNA expression levels in each of the glioma tissue was normalized to non-tumor tissue and the data was presented as log₂(fold change). TCGA was used to extract mRNA expression of TGFBR1, TGFBR2, TGFB1, and TGFB2 from mesenchymal and proneural GBMs subtypes. Additionally, mRNA expression of TGF- β target genes for mesenchymal and proneural subtypes were also extracted from TCGA. mRNA expression of proneural tissues were used to normalize when comparing mRNA expression of mesenchymal and proneural, and the data was presented as log₂(fold change).

Western blot analysis

Cells were cultured for 48 hours with or without TGF- β in the 24-well STEM CELL Technology microwell culture plate. A total of 6 samples were used where 3 samples were treated with TGF- β and 3 samples were left untreated. After 48hrs, cells were then harvested and lysed in RIPA buffer (Sigma-Aldrich, catalog no. R0278) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, catalog no. 78442) for protein. Equal masses of protein (~20 μ g) were loaded into each well of a 4% to 12% gradient bis-tris gel (Thermo Fisher Scientific), and then blotted onto a nitrocellulose membrane (Licor, catalog no. 926-31092). Membranes were probed with rabbit anti-SMAD2 antibody (Cell Signaling Technology, catalog no. 5339) or rabbit anti-pSMAD2 (Cell Signaling Technology, catalog no. 18338T), and mouse anti- β -actin (Sigma-Aldrich, catalog no. A2228). Blots were then probed with Alexa Fluor 488 goat anti-mouse (Thermo Fisher Scientific, catalog no A-11001) or Alexa Fluor 647 goat anti-rabbit (Thermo Fisher Scientific, catalog no. A-21245a) secondary antibodies. Primary antibodies were diluted at 1:1000, except mouse anti- β -actin (1:15000). Secondary antibodies were diluted at 1:10,000. Membranes were imaged using iBright imaging system (Invitrogen).

Statistical analysis

Graphical representation and statistical analyses of the data from this study were generated using GraphPad Prism 9. Details of replicates and statistical tests are described in the appropriate figure legends.

2.7 Acknowledgements

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Chapter 3: Influence of geometric confinement on emergent migration dynamics of glioma stem cells

3.1 Abstract

Epithelial-derived cancer cell models are frequently used to understand the impact of the physical tumor microenvironment on collective cancer cell migration. As a result, studies involving non-epithelial tumors are scarce, and little is known about how physical features of the microenvironment, such as geometric confinement, control collective migration dynamics in non-epithelial tumors. Here, we use glioblastoma (GBM), a highly aggressive brain tumor that is marked by rapid confined migration along blood vessels, as a non-epithelial tumor model to study collective migration under confinement. We first employ a spheroid dispersion migration assay to characterize the emergent migration phenotype of three glioma stem cell (GSC) lines, GSC11, GSC268, and GSC295. We find that all three GSC lines exhibit a stream-like emergent migration phenotype with similar dispersed migration distances and cell migration speeds but differences in their ability to migrate persistently. GSC11s migrate less persistently than GSC268s and GSC295s. Surprisingly, we found that GSC11s are more persistent and migrate faster when seeded randomly as single cells compared to when emerging from spheroids. In contrast, directionality was decreased in GSC268s and GSC295s with no observed difference in migration speed when these lines were seeded as single cells. Next, we subjected GSC11s and GSC268s spheroids to confined culture conditions using protein micropatterned lines (microlines) corresponding to the vessel diameters that GBM cells are associated with *in vivo*. Microline-induced confinement had the greatest effect on migration speed and a marginal increase in persistence for the GSC268s, whereas in GSC11s, microlines had the greatest effect on persistence and a marginal effect on migration speed. Together, we find that confinement differentially influences collective migration dynamics in GSCs. This study lays down the foundation to further dissect biophysical and molecular contributors that regulate the differential impact of confinement on collective cell migration speed and persistence in GBM.

3.2 Introduction

Recent studies have supported the notion that collective migration drives tumor progression.^{281–283} This has led many groups to seek to understand the biophysical and molecular elements that regulate this mode of motility in tumors.^{283–285} In addition to other variables, such as cell-cell contact, cell-matrix adhesion, and epithelial-mesenchymal transition, the physical tumor microenvironment has also been identified as a contributing factor in driving collective migration in cancer.²⁸¹ Notably, confinement induced by the extracellular matrix and stromal components of the tumor microenvironment can modulate migration dynamics such as speed and persistence, control collective movement irrespective of cell-cell junctions, and dictate conditions in which tumor cells can disseminate in a collective manner.^{68,286–288} While these findings have helped advance the field's understanding on the role of confinement in tumor tissues, epithelial-derived cancer cells have been largely used in these studies. As a result, little is known about how the findings hold in non-epithelial tumor models such as glioblastoma (GBM), where evidence of collective migration has been reported.^{94,289,290}

In GBM, collective cancer cell migration can occur as epithelial or filamentous connections, microtube-mediated connections, and collective oncostreams.^{289–293} Additionally, a hallmark of GBM is rapid confined migration along vascular tracks through vessel co-option.⁹³ Migration along vessels is one of the fastest migration routes for GBM cells and migration along the vessels can occur as collective migration, suggesting a potential influence of vessel mechanics.^{96,294} Moreover, GBM cells are found migrating along vessels of varying diameters (2 μm – >35 μm), but it remains unclear if vessel geometry can regulate GBM cell migration dynamics.^{20,96}

Here, we utilized protein micropatterned lines (microlines) with glioma stem cells (GSCs) to investigate how vessel-mimetic geometric confinement controls emergent migration dynamics of GSCs. We found three GSCs that exhibit similar migration dispersion distances and speeds, but differ in their ability to migrate persistently. We next subjected the most persistent and least persistent GSCs to microlines. Overall, we found that the microlines did not have a uniform influence on the migration speed and persistence of the GSCs. We observed that microlines had the greatest influence on migration speed with minimal effect on persistence of the most persistent GSC line. However, the opposite was observed for the GSC line that was least persistent, where microlines had the greatest influence on persistence with minimal impact on migration speed. Taken together, this study establishes a foundation to better understand how vessel geometry can control migration dynamics of GBM cells and non-epithelial-derived tumor cells at large.

3.3 Results

Stream-like collective migratory GSCs exhibit differences in migration persistence

We first employed a spheroid migration assay to screen through a panel of GSCs and identified three GSCs (GSC11, GSC268, GSC295) with similar stream-like collective migration phenotype (**Fig. 1A**). Given recent *in vivo* findings of collective oncostreams in GBM, we decided to use these three cell lines for our study.^{289,295} To characterize the migration phenotype of these GSCs, we first measured migration dispersion distances displayed by these GSCs and found that they dispersed similarly (**Fig. 1A**). However, we noticed differences in coordination as the cells emerge from the spheroid. Specifically, cells from the GSC11 spheroids emerged through an uncoordinated random motion. We observed events of single cell emergence and random cell motility, such as a single cells independently traveling out of sync with neighboring cells and cells that had emerged from the spheroid changing direction to move back towards the spheroid. On the other hand, cells from GSC268 and GSC295 spheroids emerged in a more coordinated and directional fashion, although these cells seemed to enter a regime of random motility towards the end of the timelapse. To gauge coordination and collectiveness across these three cell lines, we measured the number of cells found more than 50 μm apart from any neighboring cell at the end of our 10-hour timelapse. We found that GSC11s had a significantly higher number of single cells found in isolation compared to GSC295s, but not GSC268s (**Fig. 1B**). This preliminary single cell analysis in combination with our qualitative observation suggests that cells from GSC11s can emerge from the spheroid using random single cell motility.

Findings from a recent study suggested that OLIG2 status in GBM tumors could correlate with migration phenotype. In the study, OLIG2-positive tumor cells were reported to employ single cell migration along blood vessels and OLIG2-negative tumors displayed a collective migration phenotype.²⁹⁴ To see if a similar correlation could be observed in our findings between GSC11s, GSC268s, and GSC295s, we compared OLIG2 gene and protein expression in the GSC lines. While the three GSC lines displayed modest differences in OLIG2 gene expression (**Fig. 1C**), OLIG2 protein expression was significantly higher in GSC11s compared to GSC268s and GSC295s (**Fig. 1D**), which correlated with our qualitative assessment and single cell quantification (**Fig. 1B**). Additional experiments are needed to better understand how OLIG2 signature could regulate the observed migration differences.

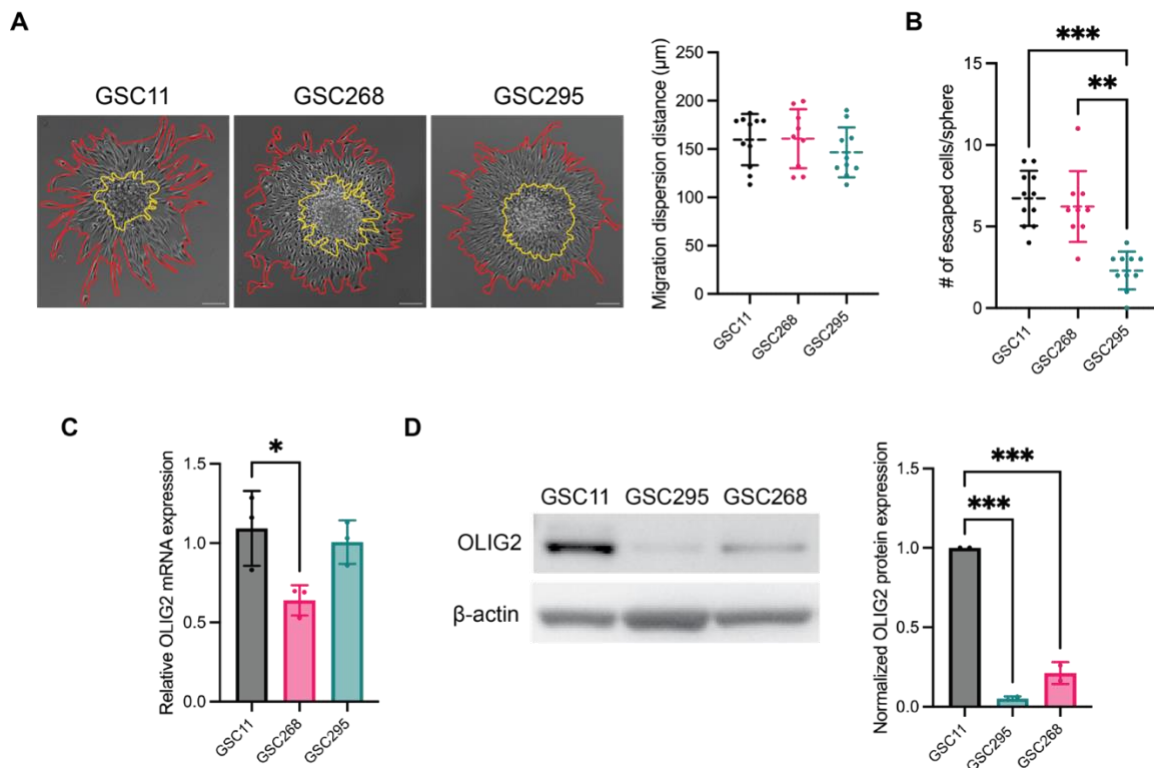


Figure 1. Stream-like migratory GSCs exhibit similar migration dispersion distances but demonstrate differences in their collective motion. (A) Representative images and quantification of migration distance of GSC11, GSC268, and GSC295 after 10hrs of culture on laminin-coated plates. Yellow outlines represent initial spheroid area. To quantify migration dispersion distance, we first outlined the spheroid area within the first frame (outline in yellow in the representative image) and then outlined the total migration area in the final frame (outline in red in the representative image) at 10 h. We then calculated migration dispersion distance by applying the equation reported in the materials and methods section under spheroid cell migration timelapse and quantification. Data represent n = 11 (GSC11), n=9 (GSC268), and n=10 (GSC295). Values display mean $\pm$ standard deviation (SD). Scale bar: 100μm. (B) Counts of the number of cells that were found >50 μm away from neighboring cells. Data represent n = 11 (GSC11), n=9 (GSC268), and n=10 (GSC295). Values display mean $\pm$ SD. (C) Relative OLIG2 mRNA expression in GSC11, GSC268, and GSC295. Measured by qPCR. Data represent n = 3 (GSC11), n=3 (GSC268), and n=3 (GSC295). Values display mean $\pm$ SD. (D) Western blot probing OLIG2 expression in GSC11, GSC268, and GSC295. Data represent n = 2(GSC11), n=2 (GSC268), and n=2 (GSC295). Values display mean $\pm$ SD.

Next, we investigated how the individual cells within GSC11, GSC268 and GSC295 spheroids contribute to the migration dispersion distances measured in each cell line. To do so, we measured migration speed and persistence of the individual cells within the emerging cell population. Surprisingly, we found no difference in migration speed across the three GSCs. However, cells from GSC268s and GSC295s migrated in a more persistent manner than GSC11s. Persistence was two-fold higher in GSC268s and GSC295s compared to GSC11s (**Fig. 2C, 2D**). There was no difference in persistence between GSC268s and GSC295s. We then asked whether the cell migration speed and persistence of cells migrating from spheroids were unique to the spheroid emergence paradigm. To answer this question, we seeded each cell line as single cells to measure migration and persistence. Across all three cell lines, the migration tracks of the seeded single cells were shorter than tracks of cells migrating from spheroids (**Fig. 2A, 2B**). We then compared migration speed and persistence of seeded single cells and cells emerging from tumor spheroids. We found a modest increase in migration speed and a significant decrease in migration persistence for GSC295s and GSC268s (**Fig. 2G, 2H**). In contrast, single cell seeded GSC11s migrated slower and more persistently compared to cells that migrated from the spheroids (**Fig. 2G, 2H**). Surprisingly when comparing migration speed and persistence of GSC11s, GSC268s, and GSC295s seeded as single cells, we saw that GSC11s migrated slower, but more persistently. This contrasts GSCs emerging from spheroids where cells from GSC11 spheroids were less persistent but had similar migration speed to cells from GSC268 and GSC295 spheroids. Together, this suggests that migration dynamics of seeded single cells differ from dynamics of cells emerging from spheroids as a collective cell population in GSCs.

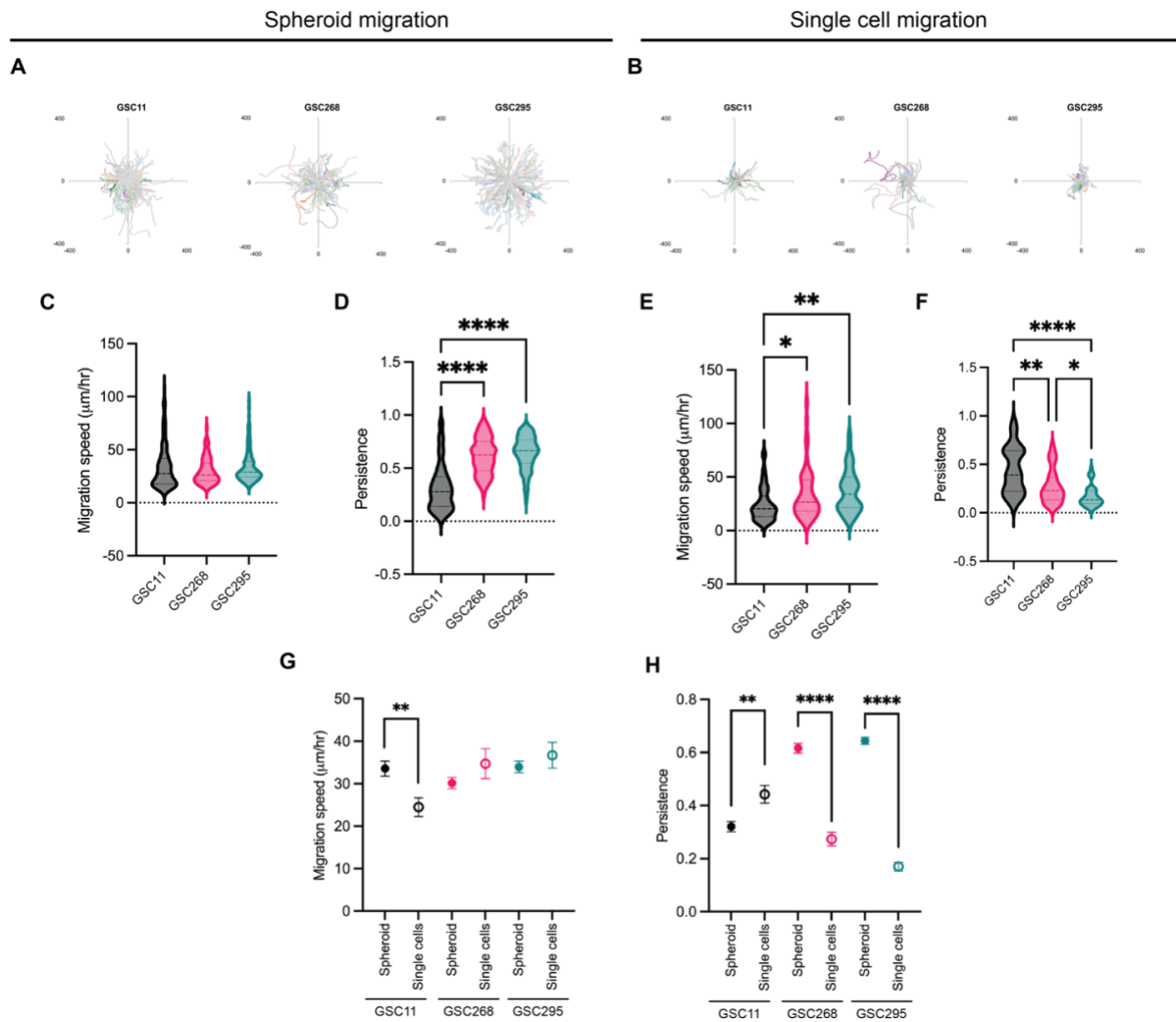


Figure 2. GSCs migrating from spheroids exhibit similar migration speeds but differ in persistence compared to their single cell counterparts. (A) Migration tracks of GSC11, GSC268, and GSC295 migrating from spheroids. (B) Migration tracks of GSC11, GSC268, and GSC295 seeded as single cells. (C) Migration speed of GSC11, GSC268, and GSC295 migrating from spheroids. Data represent $n=131$ (GSC11), $n=84$ (GSC268), $n=129$ (GSC295). (D) Persistence of GSC11, GSC268, and GSC295 migrating from spheroids. Data represent $n=131$ (GSC11), $n=84$ (GSC268), $n=129$ (GSC295). (E) Migration speed of sparsely seeded GSC11, GSC268, and GSC295. Data represent $n=52$ (GSC11), $n=48$ (GSC268), $n=42$ (GSC295). (F) Persistence comparison between cells migrating from spheroids and single seeded cells. Data representation was mentioned in D and F. Values display mean $\pm$ SEM. (G) Migration speed comparison between cells migrating from spheroids and single seeded cells. Data representation was mentioned in C and E. Values display mean $\pm$ standard error of the mean (SEM). (H) Persistence comparison between cells migrating from spheroids and single seeded cells. Data representation was mentioned in D and F. Values display mean $\pm$ SEM.

Confinement induced by vessel-like geometries enhances migration speed and persistence of GSCs

We then sought to understand how these migration dynamics are influenced under geometric confinement. To do so, we designed protein micropatterned lines with widths representative of vessel diameters reported to be used by GBM tumor cells for vessel co-option (**Fig. 3A**). We implemented these protein patterns in our spheroid migration assay

(**Fig. 3B**). For this study, we focused on GSC11s and GSC268s because earlier analysis of GSC268s and GSC295s demonstrated that these two cell lines share similar migration phenotypes. GSC11s and GSC268s cultured on microlines migrated faster and more persistently compared to when they were cultured on an unpatterned surface (no microline) (**Fig. 3C, 3F, 3D, 3G**). There was no clear correlation between microline width and migration speed or persistence for both cell lines (**Fig. 3C, 3F, 3D, 3G**). Interestingly, microline culture exhibited the greatest influence on persistence for GSC11s (**Fig. 3C, 3F**), whereas the greatest influence on migration speed was observed for GSC268s (**Fig. 3D, 3G**). Interestingly, migration speed for GSC11s was only significantly faster when cultured on the 50 μm and 35 μm microline width when compared to no microline condition (**Fig. 3C**). Additionally, persistence was significantly higher across all microline widths compared to the no microline condition for the GSC11s, with the highest persistence value measured on 50 μm microline width (**Fig. 3D**). There was no statistically significant difference in persistence and speed when comparing between the individual microline widths for GSC11s. For GSC268s, persistence was only significantly higher at the 7 μm and 15 μm microline widths compared to the no microline condition (**Fig. 3G**). Additionally, GSC268s migrated faster on all microline widths compared to the no microline condition (**Fig. 3D**). The fastest speed was measured in 35 μm microline widths, with no significant differences in speed between the 7, 15, and 35 μm widths.

When comparing migration speed and persistence between GSC11s and GSC268s, both cell lines displayed similar migration speed on 50 μm microline width (**Fig. 3E**). However, GSC268s migrated faster than GSC11s with decreasing microline width (**Fig. 3E**). In terms of persistence, GSC11s exhibited greater directional migration across the different microline widths tested (**Fig. 3H**). The greatest observed difference in persistence was with the 50 μm microline width with a modest increase observed at the 7 μm , 15 μm , and 35 μm widths (**Fig. 3E**). This data suggested that while microline-induced confinement increases migration speed and persistence of GSCs, the magnitude of influence per metric varies across cell lines.

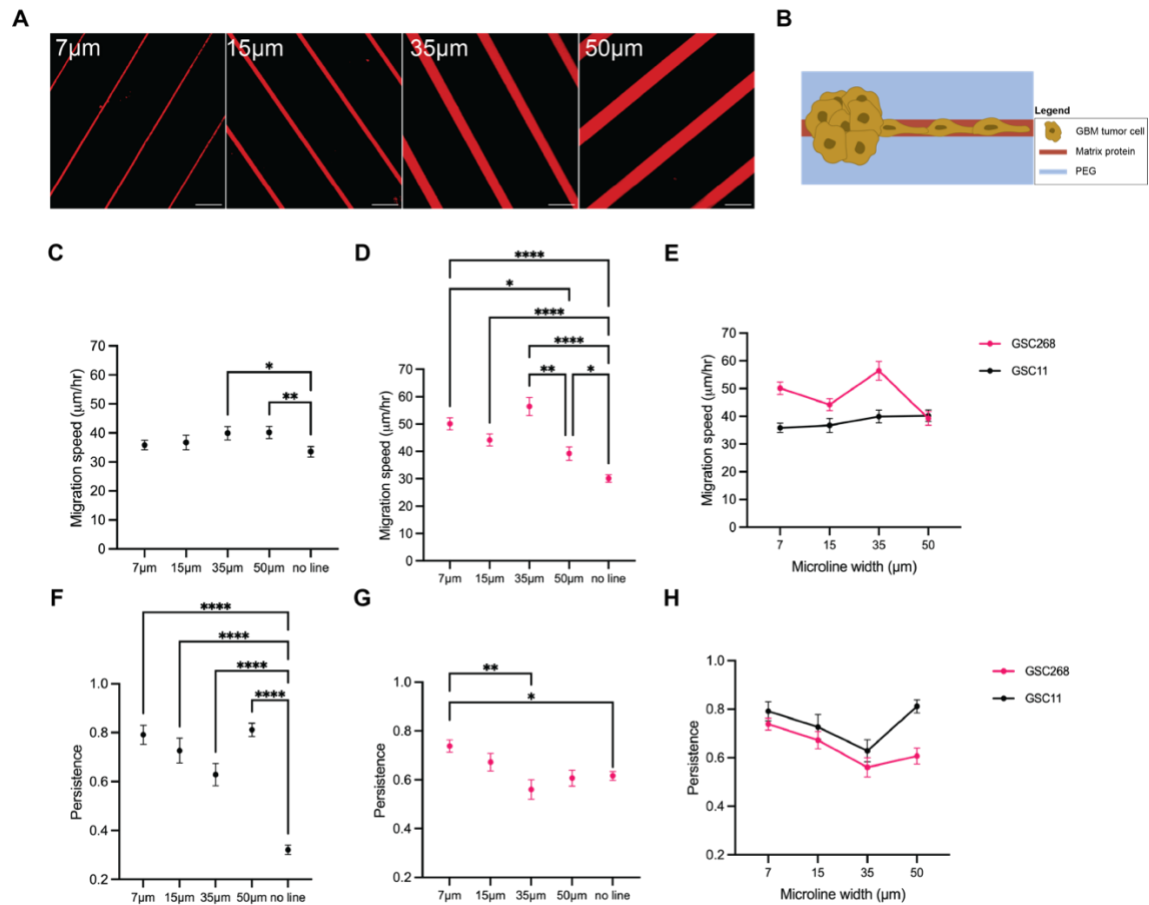


Figure 3. GSCs migrate faster and more directionally under geometric confinement. (A) representative images of protein micropatterned lines for the various distances used, 7 μm , 15 μm , 35 μm , and 50 μm . In red is laminin labelled with sulfo-Cyanine5 succinimidyl ester. Scale bar 100 μm . (B) Schematic of microline spheroid migration assay. (C) Migration speed of GSC11 on different microline widths, including 7 μm , 15 μm , 35 μm , and 50 μm . Data represents $n=131$ (no line), $n=35$ cells (50 μm), $n=35$ cells (35 μm), $n=27$ cells (15 μm), $n=44$ cells (7 μm). Values display mean $\pm$ SEM. (D) Migration speed of GSC268 on different microline width, including 7 μm , 15 μm , 35 μm , and 50 μm . Data represents $n=84$ cells (no line), $n=39$ cells (50 μm), $n=33$ cells (35 μm), $n=37$ cells (15 μm), $n=36$ cells (7 μm). Values display mean $\pm$ SEM. (E) Migration speed comparison of GSC11 and GSC268 on different microline width, including 7 μm , 15 μm , 35 μm , and 50 μm . Data presented here are replots of panel C and D combined without the no line condition. Values display mean $\pm$ SEM. (F) Persistence of GSC11 on different microline width, including 7 μm , 15 μm , 35 μm , and 50 μm . Data represents $n=131$ cells (no line), $n=35$ cells (50 μm), $n=35$ cells (35 μm), $n=27$ cells (15 μm), $n=44$ cells (7 μm). Values display mean $\pm$ SEM. (G) Migration speed of GSC268 on different microline width, including 7 μm , 15 μm , 35 μm , and 50 μm . Data represents $n=84$ cells (no line), $n=39$ cells (50 μm), $n=33$ cells (35 μm), $n=37$ cells (15 μm), $n=36$ cells (7 μm). Values display mean $\pm$ SEM. (H) Persistence comparison of GSC11 and GSC268 on different microline width, including 7 μm , 15 μm , 35 μm , and 50 μm . Data presented here are replots of panel F and G combined without the no line condition. Values display mean $\pm$ SEM.

3.4 Discussion

The physical tumor microenvironment plays an influential role in collective cancer cell migration. Specifically, confinement induced by the matrix and stroma has been well established in epithelial-derived cancers to give rise to collective cancer cell migration.

While in vivo evidence suggests that collective migration can occur in non-epithelial-derived cancer tissue, little is known about how the physical microenvironment can influence collective migration dynamics in non-epithelial-derived tumor cells. To fill this knowledge gap, we combined vessel-inspired protein micropattern lines and GSCs to investigate how vessel-like geometric confinement found in GBM tumors influence collective cell migration dynamics of GBM cells. We found that confinement induced by microline culture led to faster migration speed and greater persistence in GSCs. However, the magnitude at which the microline influenced faster migration speed and improved persistence was tied with the inherent migratory property of the GBM cell.

In this study, we first characterized the migration phenotype of three GSCs (GSC11, GSC268, and GSC295) that employed a stream-like collective migration phenotype. Interestingly, we found that GSC11s migrated less persistently than GSC268s and GSC295s (**Fig. 2D**). Despite difference in persistence, GSC11 spheroids dispersed at similar migration distances (**Fig. 1A**) and employed similar migration speeds (**Fig. 2C**) as GSC268 and GSC295 spheroids. Surprisingly, GSC11 cells migrated in a more persistent manner when the GSCs were seeded as single cells in isolation (**Fig. 2H**). Coupled with our finding that GSC11s had more single cells found isolated away from the spheroid (**Fig. 1B**), it is conceivable that these few single cells in the GSC11s are displaying a more persistent behavior which increases the migration front leading to displacement values that compared to GSC295s and GSC268s.

Moreover, when GSC11s and GSC268s were subjected to microlines, cells emerging from GSC11 spheroids migrated more persistently than GSC268s, although the differences were nuanced across the 7 μm , 15 μm , and 35 μm line widths (**Fig. 3H**). Overall, cells emerging from GSC11 spheroids employed greater persistent migration phenotype on all microline widths compared to unpattern surface (**Fig. 3F**). On the other hand, persistence was only significantly increased on 7 μm and 15 μm microline widths for GSC268s compared to the unpatterned surface (**Fig. 3G**). Since cells emerging from the GSC268s already employed a more persistent form of migration, it is plausible that geometric confinement has a limited dynamic range for improving persistence in this cell line.

The difference between how microlines influenced GSC11 and GSC268 migration was surprising. However, it does support previous findings suggesting that differences in glioma migration pattern might be dictated by intrinsic glioma cell autonomous mechanisms.^{296,297} For example, a recent study identified differences in laminin A/C expression as being able to explain differences in migration dynamics in narrow spaces in glioma.²⁹⁶ Lamin A/C expression affects nuclear mechanics, including stiffness, which can influence the ability of a cell to squeeze through narrow spaces which effectively influence migration phenotype in these confined microenvironments.²⁹⁸ Our study could be extended to investigate if laminin A/C does have an influence in our system.

Furthermore, GBM cells are most found in association with vessel diameters typical of capillaries.^{20,96,299} For example, one study found that out of the total vessel area covered by GBM cells, 47.5% were found in association with capillaries that were less than 7 μm

in diameter.²⁰ Additionally, GBM tumor cells migrating on vessels decrease vessel size through vasoconstriction.²⁰ Together, this suggests that GBM tumor cells may have a preference for smaller sized vessels. However, it is not well understood how vessel diameter supports GBM migration. In our studies, we observed enhanced persistent migration of the GSC11s and GSC268s on microlines with widths on the order of capillary diameters (7 μm and 15 μm) (**Fig. 3H**). Surprisingly, migration speed was not significantly enhanced in both GSCs on 7 μm and 15 μm microlines (**Fig. 3E**) suggesting that GSCs may exhibit differences in migration efficiency on capillary-like geometries. This raises the question of whether migration efficiency dictates the likelihood GBM cells employ vessel co-option when invading. Indeed, not all GBM cells perform vascular co-option as other routes such as the parenchyma and white matter are also routes of GBM invasion.^{15,180,296} Given differences in migration phenotype on capillary geometries, our study could be extended *in vivo* to investigate migration routes employed by GSC11s and GSC268s using patient derived xenograft models. More recent work has demonstrated that migration metrics, such as migration speed and persistence, measured in physiologically representative physical culture models better recapitulate *in vivo* findings.³⁰⁰ Therefore, it is likely that the differences measured in our microline platform would translate *in vivo* and help to investigate how GBM cell migration speed and persistent influence invasion routes in GBM.

Our findings from this study help to provide a foundation for further exploration of how geometric confinement influences the collective cell migration of GBM tumor cells. In this study we used simple linear micropattern features although the microvascular architectures found *in vivo* are quite complex.^{20,96} As a result, an important next step to this study is to investigate how our findings hold in more physiologically representative geometries. Finally, it has become increasingly clear that tumor cell invasion is a metabolically demanding process.^{301,302} It is conceivable that some of the findings here could be explained by differences in energetics across the different conditions and GSC cell lines used. In conclusion, this work contributes to the growing, and crucial, body of work in studying regulation of collective cancer cell migration by the physical tumor microenvironment in non-epithelial-derived tumors.

3.5 Materials and Methods

Cell culture

GSC20, GSC28, GSC262, and GSC295 cells were obtained from MD Anderson Cancer Center and propagated as neurospheres in DMEM/F12 basal medium (Corning, catalog no. 10-090-CV) supplemented with 2% (vol/vol) B-27 supplement (Gibco, 17504-044), 20 ng/mL EGF (R&D Systems, 236-EG-01M), and 20 ng/mL FGF (R&D Systems, 233-FB-025/CF). For migration experiments, the medium was supplemented with 0.1% penicillin/streptomycin (Thermo Fisher Scientific, catalog no.15140-122). Cells were screened for mycoplasma and validated by Short Tandem Repeat (STR) analysis every six months.

Generation of spheroids

Glioma stem cell (GSC) spheroids were generated using AggreWell Microwell Plates (the STEMCELL Technologies, catalog no. 34415). Briefly, the wells of the microwells were treated with non-adherent rinsing solution (STEMCELL Technologies, catalog no. 07010). GSCs were then seeded as single cells at ~350k per well for 2-3 days to allow the cells to aggregate and form uniform tumor spheroids. The spheroids were then used for spheroid migration assay.

Spheroid cell migration timelapse and quantification

SPY555-DNA (Cytoskeleton, CY-SC201) live cell fluorescence probe was added to the microwell of spheroids the night before the migration assay at the recommended manufacturer's concentration (1:2000). Glioma cell spheroids stained with SPY555-DNA were seeded either in a 24 well glass bottom plate pre-coated with 100 µg/ml of laminin (Gibco Laminin Mouse Protein, Catalog No. 23-017-015) or protein micropattern lines that is secured in a 6-well culture plate. Time-lapse imaging was performed using Nikon Ti2 (objective specs: 10x/0.3NA), equipped with a heating (37°C) and 5% CO₂ chamber, to track the migration of single cells emerging from the spheroids. An image was taken every 10mins for 10 hours.

To quantify migration speed of individual cells that emerge from the spheroids, tracks consisting of individual cell positions were generated using the Fiji plugin Trackmate.³⁰³ A custom MATLAB script was used to analyze each track to quantify migration speed of each cell. Migration speed was calculated by dividing the duration of time the cell was tracked over the total step distance the cell traveled. Migration tracks were generated using DiPer.³⁰⁴

To quantify spheroid dispersion migration distance, we first outline the spheroid area within the first frame and then measure the total migration area in the final frame at 10hrs. We then apply the following equation to calculate migration distance.

$$\text{Migration distance} = \sqrt{\frac{\text{Total migration area}}{\pi}} - \sqrt{\frac{\text{Initial spheroid area}}{\pi}}$$

To identify the number of single cells/spheroid, we determine cells that are more than 50 μm away from neighboring cells and are not in contact with any neighboring cells in the final frame at 10hrs. Neighbor-neighbor distance measurements were done by using the line tool in Imagej to measure distance from the cell's nucleus.

Protein micropatterned line fabrication

Micropatterns were made as described elsewhere.^{188,305} Briefly, plasma-treated coverslips were coated with 100 $\mu\text{g}/\text{ml}$ poly-L-lysine grafted to polyethylene glycol (PLL-g-PEG; SuSoS) in 10 mM HEPES, pH 7.4, for 1 h at room temperature before being illuminated under 180 nm UV (Jelight) light for 15 min through a quartz-chrome mask bearing the micropattern features designed using AutoCAD (Autodesk). Photomask was manufactured by frontrange-photomask. Coverslips were then incubated with 100 $\mu\text{g}/\text{ml}$ laminin (Invitrogen, catalog no.23017-015) in 100 mM sodium bicarbonate (pH 8.5) overnight at 4°C and washed with phosphate-buffered saline (PBS) prior to spheroid seeding. For unpatterned surfaces, PLL was precoated in the wells of a glass bottom plate, washed with PBS, and then coated with 100 $\mu\text{g}/\text{ml}$ laminin (Invitrogen, catalog no.23017-015) in 100 mM sodium bicarbonate (pH 8.5) overnight at 4°C.

Quantitative real time polymerase chain reaction

Cells were cultured in the 24-well STEM CELL Technology microwell culture plate. A total of 3 samples per cell line were used. Total mRNA was then isolated using Rneasy Plus Micro Kit (Qiagen, catalog no. 74134) according to manufacturer's recommendations protocol. cDNA was synthesized from the isolated mRNA using Iscript™ cDNA Synthesis Kit (Bio-Rad, catalog no. 1708891). qPCR was performed using Power up Sybr green master mix (Fisher Scientific, catalog no. A25777) for 40 cycles in a CFX real-time PCR detection system (Bio-Rad). To quantify relative fold change in the level of genes, qPCR data were analyzed by calculating $\Delta\Delta\text{Ct}$ with respect to RPS9 as the housekeeping gene. The primer sequences used in this study are listed below in the table below.

Gene	Forward Primer	Reverse Primer
RPS9	GACTCCGGAACAAACGTGAGGT	CTTCATCTTGCCCTCGTCCA
OLIG2	TGGCTTCAAGTCATCCTCGTC	ATGGCGATGTTGAGGTCGTG

Western blot analysis

Cells from each GSC cell line was cultured in 24-well STEM CELL Technology microwell culture plate for 3-5 days and were then harvested and lysed in RIPA buffer (Sigma-Aldrich, catalog no. R0278) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, catalog no. 78442) for protein. Equal masses of protein (~20 μg) were loaded into each well of a 4% to 12% gradient bis-tris gel (Thermo Fisher Scientific), and then blotted onto a nitrocellulose membrane (Licor, catalog no. 926-31092). Membranes were probed with rabbit anti-OLIG2 antibody (Cell Signaling Technology, catalog no. MA5-15810) and mouse anti- β -actin (Sigma-Aldrich, catalog no. A2228). Blots were then probed with IRDye 680RD Goat anti-Rabbit IgG (H + L), 0.1 mg (LI-COR Biosciences, catalog no 925-68071) or IRDye® 800CW Goat anti-Mouse IgG

Secondary (LI-COR Biosciences, catalog no 925-32210) secondary antibodies. Primary antibodies were diluted at 1:1000, except mouse anti- β -actin (1:15000). Secondary antibodies were diluted at 1:10,000. Membranes were imaged using iBright imaging system (Invitrogen).

Statistical analysis

Graphical representation and statistical analyses of the data from this study were generated using GraphPad Prism 9. Details of replicates and statistical tests are described in the appropriate figure legends.

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Micropatterns were fabricated at the QB3 Biomolecular Nanotechnology Center. Western blots were imaged using equipment owned by the Amy Herr lab. We thank Paul Lum, Cameron Morley, PhD and Stacey Lee, PhD for training and/or assistance.

Chapter 4: Conclusions

The work described in this dissertation demonstrates the application of engineered cell culture models to study how key aspects of the physical GBM microenvironment influence glioma stem cell motility. In Chapter 1, we reviewed shared common physical elements of the tumor microenvironment that serve as universal regulators of malignancy. We use two distinct tumor types, breast cancer and glioblastoma, as instructive examples to highlight how invasion in both diseases is driven by structural guidance cues, heterotypic cell-cell contact with stromal cells, and elevated interstitial fluid pressure and flow. We also briefly summarized engineering strategies used to measure and model these physical properties for mechanistic studies.

In GBM, tumor cells invade through distinct microenvironments including an HA-rich parenchyma and along microvasculature. These unique microenvironments can be modeled using engineered culture strategies to systematically study how the physical tumor microenvironment influences motility. In Chapter 2, we applied a previously described an engineered 3D HA-based hydrogel to model brain parenchyma matrix and understand how biophysical and biochemical factors drive invasion of glioma stem cells.¹⁷⁹ Specifically, we investigated how the pleiotropic factor, TGF- β , supports invasion of GSCs. We find that HA uniquely recapitulated TGF- β induced invasion and that TGF- β stimulated GSCs differ in their ability to invade HA in a way that can be predicted by TGF- β receptor 2 expression and SMAD2 phosphorylation. Surprisingly, GSC invasion was strongly dependent on the presence of RGD peptides on the HA backbone but was independent of matrix metalloprotease degradability. Our findings demonstrate the capability of HA to serve as a model to systematically dissect contributions of candidate growth factors and other cytokines to GSC invasion.

In Chapter 3, we focused on microvascular migration. In GBM, tumor cells are often found co-opted on vessels of varying diameters and this association leads to rapid migration. It remains unclear if and how vessel geometry (e.g vessel diameter) can influence tumor cell migration dynamics in GBM. Here, we began to explore how vessel-mimetic features influence migration dynamics of GSCs using protein micropattern cell culture paradigm. We found that GSCs with stream-like collective migration phenotype exhibit similar migration distances and speeds but differ in their migration persistence. When subjected to vessel-like geometries, the GSCs exhibited faster migration speed with improved persistence. Interestingly, we found that vessel-like geometries improved either migration speed or persistence, but not both speed and persistence in any individual GSC used. Taken together, we find that confinement differentially influence collective migration dynamics in GSC.

Overall, our work demonstrates the utility of engineered cell culture models to systematically study invasion in different parts of the tumor microenvironment. Additionally, our findings add to the growing body of work elucidating how the physical tumor microenvironment inform tumor cell migration dynamics. However, additional questions remain unanswered. First, our results in Chapter 2 show that TGF- β stimulated invasion is strongly dependent on the presence of RGD on the HA backbone suggesting that integrin adhesions were more important in driving this process than HA adhesions. This raises the question whether HA adhesions play an important role in supporting TGF-

β stimulated GSC invasion in HA matrices. A systematic study investigating TGF- β stimulated GSC invasion of GSCs that have lost HA adhesion receptor expression through CRISPR-mediated knockout or shRNA mediated knockdown would provide further insight into the functional role of HA adhesions in this process. In Chapter 3, we used simple line patterns to study how microline width influence migration dynamics of GSCs. Since vasculature naturally consists of branching networks, it would be important to ask whether our findings on linear lines holds true in more complex geometries that are inspired by microvascular networks. Additionally, the brain perivascular niche (PVN) consists of heterogeneous extracellular matrix (ECM) composition and stromal cells that support vessel integrity. It will be important to investigate how cell adhesions informed by the ECM composition and heterotypic interactions between stromal and tumor cells in the PVN influence GBM cell migration behaviors. Finding from such investigations can inform engineering culture platforms that enables systematic integration of these variables to gain a greater understanding of GBM vascular migration mechanics.

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