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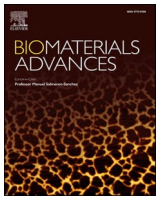

Glioblastoma mechanobiology at multiple length scales

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ABSTRACT

Glioblastoma multiforme (GBM), a primary brain cancer, is one of the most aggressive forms of human cancer, with a very low patient survival rate. A characteristic feature of GBM is the diffuse infiltration of tumor cells into the surrounding brain extracellular matrix (ECM) that provide biophysical, topographical, and biochemical cues. In particular, ECM stiffness and composition is known to play a key role in controlling various GBM cell behaviors including proliferation, migration, invasion, as well as the stem-like state and response to chemotherapies. In this review, we discuss the mechanical characteristics of the GBM microenvironment at multiple length scales, and how biomaterial scaffolds such as polymeric hydrogels, and fibers, as well as microfluidic chip-based platforms have been employed as tissue mimetic models to study GBM mechanobiology. We also highlight how such tissue mimetic models can impact the field of GBM mechanobiology.

1. Introduction

Glioblastoma multiforme (GBM) is one of the most common and malignant primary brain tumors in adults [1,2]. The median survival period is only ~12–14 months from the initial time of diagnosis [3]. Standard treatment consists of surgical resection, followed by chemotherapy and radiotherapy [4]. However, its poor prognosis has not significantly improved despite the development of innovative diagnostic strategies and novel therapies. It is a relentless endeavor to explore better treatment strategies to combat this dreaded disease. To enable this endeavor, it is imperative to understand the subtle and prominent micro-environmental landscape of the GBM tumor. The tumor microenvironment (TME) which provides biophysical, biochemical, and cellular cues has been identified to play a major role in driving tumor progression, recurrence, and chemoresistance [5].

GBM is highly heterogeneous in nature and the unique composition of the TME supports its growth and invasion [6]. The extracellular matrix (ECM) is the non-cellular component of TME that not only provides essential physical scaffolding for the cellular components but also is responsible for providing crucial biochemical and biomechanical cues for GBM tumors [7]. GBM progression is frequently accompanied by ECM deposition and remodeling, which change in composition and architecture owing to increased and altered production of ECM components such as tenascin-C (TNC) and hyaluronic acid (HA) [8,9]. To measure how these biochemical changes might contribute to the mechanical properties of GBM tumors *in vitro* and *in vivo*, various techniques such as magnetic resonance elastography (MRE), atomic force microscopy (AFM), shear wave elastography (SWE), and micro indentation have been utilized [10,11]. It has been observed that lower-grade gliomas and GBMs are progressively stiffer as compared to normal brain tissue, while the higher grade gliomas exhibit lower stiffness than the lower grade gliomas [12,13]. Accordingly, several studies have revealed that the mechanical properties of the cells and the surrounding ECM are also impacted as the neoplastic disease progresses [8,14,15]. These changes in the biomechanical properties of the TME also translate into alterations in cell behaviors such as migration of GBM cells *in vitro* as well as *in vivo* [15–21].

While it is generally appreciated that the biomechanical properties of the TME influences various aspects of GBM tumors, including proliferation, survival, migration, and drug response, the underlying molecular mechanisms are only beginning to be discovered. To study how biomechanical attributes of the TME influence GBM behaviors, a variety of model systems are being employed. While traditional two-dimensional (2D) tissue culture polystyrene (TCPS) based or matrix-free culture models are routinely employed to examine GBM behaviors *in vitro* owing to their ease, they often fail to provide relevant biomechanical cues that are observed *in vivo* (TCPS stiffness ~ 3 GPa) [4,22,23]. In contrast, three-dimensional (3D) culture models based on

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polymeric hydrogels or scaffolds have been employed to study GBM behaviors in a controllable manner in a physiologically relevant *in vitro* setting [24]. Specifically, these models provide a relevant 3D context, including mimicry of biomechanical cues observed *in vivo*, to study the impact of common chemotherapeutic drugs on GBM cells while also considering the effect of ECM stiffness and composition [7]. *In vivo* patient derived xenograft models and genetically engineered murine models offer enormous advantage over *in vitro* models in terms of TME complexity, however, they often lack user control of the TME, and incur high costs [25]. Nonetheless, such *in vivo* models are important for preclinical validation of microenvironmental drivers of GBM progression [26].

This review article focuses on glioblastoma mechanobiology at multiple length scales. We first describe mechanical characteristics of GBM tumors noted *in vitro* and *in vivo* as well as GBM cells *in vitro* through various techniques. We then discuss the impact of biophysical cues of the ECM in terms of stiffness and composition on the behavior of GBM cells focusing on migration/invasion, and proliferation. Finally, we discuss the role of mechanotransduction and composition of ECM on the chemotherapeutic response of GBM cells. The biophysical cues of GBM TME serve a potential pathophysiologic role, and thus targeting these cues by designing mechanobiology inspired therapeutics would present a therapeutic opportunity to improve disease outcomes.

2. Mechanical characterization of GBM tumors

Most malignancies are characterized by increased rigidity compared to the normal tissue, and this atypical stiffening is the key driving force behind their development [27]. Even in GBM, it has been reported that biochemical factors are activated through mechanical signals orchestrated by an abnormal increase in ECM stiffness [10,26]. However, depending on the methods and sample types utilized for measurements, differences in GBM tumor and native tissue mechanical properties have been observed [13,28–31]. In addition, chemotherapeutic drugs and various antibodies utilized for GBM treatment can alter the tumor stiffness as observed in both *in vivo* and *in vitro* systems [32–34]. Quantifying these mechanical changes in GBM tumors can provide new avenues for developing early diagnosis protocols or for targeting GBM tumors. Commonly utilized techniques to measure viscoelastic properties of GBM tumor or tissue specimens include MRE, AFM, SWE, and micro-indentation and are explained in detail elsewhere [10,11]. Here, we discuss the mechanical properties of GBM tumors observed *in vivo* (in human and in mouse models), human/mouse GBM tissue samples, and patient derived cells *in vitro* (Tables 1, 2).

2.1. Mechanical characterization of GBM tumors in vivo and in isolated GBM tissues

MRE is the most commonly employed method to evaluate GBM stiffness quantitatively by directly applying a known frequency of shear

Table 1
Summary of GBM stiffness data from *in vivo* studies.

Cell lines	Human/ mouse	Technique	Quantity measured	Stiffness	Reference
–	Human	MRE	Complex shear modulus (G*)	GBM - 1.32 ± 0.26 kPa Perifocal tissue - 1.38 ± 0.29 kPa Healthy tissue - 1.54 ± 0.27 kPa	Streitberger et al., [35]
–	Human	MRE	Complex shear modulus (G*)	Mean value for all glioma grades - 1.43 ± 0.33 kPa Normal brain tissue - 1.62 ± 0.27 kPa	Reiss-Zimmermann et al., [36]
–	Human	MRE	Complex shear modulus (G*)	Grade II glioma - 2.7 ± 0.7 kPa Grade III glioma - 2.2 ± 0.6 kPa Grade IV glioma - 1.7 ± 0.5 kPa Unaffected white matter - 3.3 ± 0.7 kPa	Pepin et al., [37]
–	Human	MRE	Complex shear modulus (G*)	GBM - 1.10 ± 0.29 kPa Healthy reference tissue of GBM - 1.81 ± 0.23 kPa Meningioma - 1.51 ± 0.34 kPa Healthy reference tissue of meningioma - 1.78 ± 0.25 kPa	Streitberger et al., [28]
U-87 MG human GBM, RG2 rat Glioma and MDA-MB-231	Mouse	MRE	Elastic modulus (G_d) and Viscosity modulus (G_1)	U87 - (G_d - 4.80 ± 0.21 kPa, G_1 - 2.94 ± 0.19 kPa) RG2 - (G_d - 4.22 ± 0.14 kPa, G_1 - 2.41 ± 0.09 kPa) MDA-MB-231 - (G_d - 3.74 ± 0.14 kPa, G_1 - 2.21 ± 0.07 kPa) Healthy tissue - (G_d - 5.89 ± 0.21 kPa, G_1 - 4.36 ± 0.17 kPa)	Jamin et al., [39]
G30 - GBM stem cell line	Mouse	MRE	Viscoelastic modulus	Week 2 - 8.39 ± 0.98 kPa(n = 5) Week 3 - 7.75 ± 0.17 kPa (n = 4) Week 4 - 6.24 ± 0.04 kPa (n = 2) Control - 10.07 ± 0.63 kPa (n = 4)	Schregel et al., [40]
G9 cell line	Mouse	MRE	Complex shear modulus	Untreated: Day 6 - 6.8 ± 0.7 kPa Day 8 - 6.0 ± 0.6 kPa Day 10 - 5.1 ± 0.7 kPa B20 anti-VEGF treated: Day 6 - 7.1 ± 0.7 kPa Day 8 - 7.2 ± 0.3 kPa Day 10 - 6.4 ± 0.9 kPa	Schregel et al., [32]
–	Human	SWE	Young's modulus	Normal brain tissue - 7.3 ± 2.1 kPa Low grade glioma - 23.7 ± 4.9 kPa Meningioma - 33.1 ± 5.9 kPa High grade glioma – 11.4 ± 3.6 kPa Metastasis - 16.7 ± 2.5 kPa	Chauvet et al., [13]

Table 2
Summary of GBM stiffness data from *ex-vivo/in vitro* models.

Cell lines	Tissue/*in vitro* model	Technique	Quantity measured	Stiffness	Reference
–	Fresh human brain tumor tissues	AFM	Young's modulus	Healthy tissue - 61.2 ± 0.4 Pa Meningothelial meningioma - 52.7 ± 0.6 Pa Fibrous meningioma - 69.8 ± 1.7 Pa Hemangiopericytoma - 95 ± 3.1 Pa GBM - 168.8 ± 32.8 Pa GBM adjacent tissue - 43.5 ± 0.1 Pa	Ciesluk et al., [29]
–	Fresh frozen human brain tumor biopsies	AFM	Median Young's modulus	Normal tissue - 10 to 180 Pa Grade II and III - 50 to 1,400 Pa GBM - 70 to 13,500 Pa	Miroshnikova et al., [12]
HK 408	Mouse explanted tissue slices	AFM	Median Young's modulus	Mice 1 Tumor core - 3.7 kPa Tumor Edge - 1.3 kPa Peri tumoral tissue - 0.5 kPa Mice 2 Tumor core - 2.8 kPa Tumor Edge - 1.2 kPa Peri tumoral tissue - 0.3 kPa	A. Sohrabi et al. [43]
U373-MG	*In vitro* model	AFM	Cortical cell stiffness	Control - 2.6 kPa on 2 kPa ECM Control - 6 kPa on 8 kPa ECM siACTN1 - 1.75 kPa on 2 kPa ECM siACTN1 - 2.7 kPa on >18 kPa ECM siACTN4 - 1.5 kPa on 2 kPa ECM siACTN4 - 2.7 kPa on >18 kPa ECM	Sen et al., [52]
U373-MG	*In vitro* model	AFM	Cortical cell stiffness	Control - 4.5 kPa Nocodazole treated - 7 kPa Blebbistatin treated - 2.8 kPa	Sen et al., [33]
SNB-19	*In vitro* model	AFM	Cell stiffness in nuclear region	In CD44 knock down cells - 0.56 ± 0.5 kPa In parent cells - 1.93 ± 2.86 kPa	Maherally et al., [57]
L0, a primary GBM tumor initiating cell line	*In vitro* model	Microfluidic extensional flow device	Shear Modulus	GBM cells - 0.59 ± 0.05 kPa Cytochalasin D treated - 0.22 ± 0.04 kPa Paraformaldehyde treated - 0.73 ± 0.05 kPa	Guillou et al., [34]

waves on tissues. In 2014, Streitberger et al., evaluated complex shear modulus (G*) of GBM in 22 patients using MRE and reported that on average GBM tumors are slightly softer ($|G^*| = 1.32 \pm 0.26$ kPa) than the healthy tissue ($|G^*| = 1.54 \pm 0.27$ kPa) surrounding the tumor [35]. However, they noticed an increase in GBM tumor stiffness among 5 patients and specified that GBM tumors are more heterogeneous. In another study, Streitberger et al., investigated the effect of tissue fluidity on GBM progression by measuring complex shear modulus in 18 patients (9 GBM and 9 meningioma) by MRE. A high water content and accumulation of glycosaminoglycans were observed in GBM tumors (1.10 ± 0.29 kPa) compared to meningiomas (1.51 ± 0.34 kPa), leading to a softer tissue and alterations in tissue fluidity [28]. This trend has been noted in additional studies utilizing MRE wherein GBM tumors were softer than the brain parenchyma and with increase in glioma grades they tend to become even softer [28,36,37]. Among them Pepin et al., further found that glioma tumors ($n = 12$ patients) with mutations in isocitrate dehydrogenase 1 (IDH1) gene (2.5 ± 0.6 kPa) were stiffer compared to wild type IDH1 (1.6 ± 0.3 kPa) ($n = 6$) [37] (Fig. 1A). Recently, researchers have investigated the biomechanical properties and its role in functional changes in 10 GBM patients and 17 healthy subjects *via* MRE. They found that GBM tumors are less stiff compared to healthy white and gray matter in the brain, and necrotic regions are even softer than the swelling regions observed around the tumor (edema) and GBM tumors. They also developed a regression model based on the MRI images to predict differences in cerebral blood flow depending on tissue rigidity, which stated that cerebral blood flow was higher in GBM tumors owing to their reduced rigidity [38]. Compared to MRE, Chauvet et al., utilized SWE to measure stiffness of brain tumors of various grades in 63 patients. They reported an opposite trend comparing GBM (11.4 ± 3.6 kPa) *vs.* normal brain stiffness (7.3 ± 2.1 kPa), but lower grade gliomas expressed higher Young's Modulus (meningioma – 33.1 ± 5. kPa, low grade glioma – 23.7 ± 4.9 kPa) than GBM (high grade glioma) [13] (Fig. 1B).

In addition to measuring GBM mechanical properties directly in patients *via* MRE, researchers have also employed mouse models. For instance, Jamin et al., intracranially injected U87 MG, RG2, and metastatic MDA-MB-231 breast cancer cells into the brain of six week old female athymic mice to develop primary brain and metastatic tumors and measured both elastic and viscosity modulus through MRE, upon the tumor volume reaching 30–40 mm^3. Elasticity modulus for all the three tumors measured indicated that they were softer (U87–4.8 ± 0.21 kPa, RG2–4.22 ± 0.14 kPa, MDA-MB-231–3.74 ± 0.14 kPa) and less elastic than healthy tissue (5.89 ± 0.17 kPa) [39]. To measure the stiffness of GBM tumors over time, Schregel et al., surgically implanted 50,000 GBM stem cells (G30) into 5 nu/nu mice. A week after the implantation, they observed tiny masses in 2 out of 5 animals and utilizing MRE they measured the viscoelastic modulus of surgically implanted tumors which displayed a decrease in tumor viscoelastic modulus over time (Week 2–8.39 ± 0.98 kPa, Week 3–7.75 ± 0.17 kPa, Week 4–6.24 ± 0.04 kPa) compared to healthy tissue (week 1–10.07 ± 0.63 kPa) [40] (Fig. 1C). In recent years, antiangiogenic therapies have shown progress in treating patients with GBM [41]. To examine how anti-angiogenic inhibitors affect GBM tumor stiffness, Schregel et al., surgically implanted 50,000 GBM cells in 28 female athymic nude mice and treated them with B20 anti - vascular endothelial growth factor (VEGF) antibody on day 4, 6 and 8 (10 mg dose was injected intraperitoneally). Mice bearing tumors without the treatment survived only 10 days, compared to 24 days for treated mice. Later, utilizing MRE, they quantified the GBM tumor stiffness on day 6, 8 and 10 for both treated and untreated mice. Interestingly, anti-VEGF (B20) treated mice survived longer by

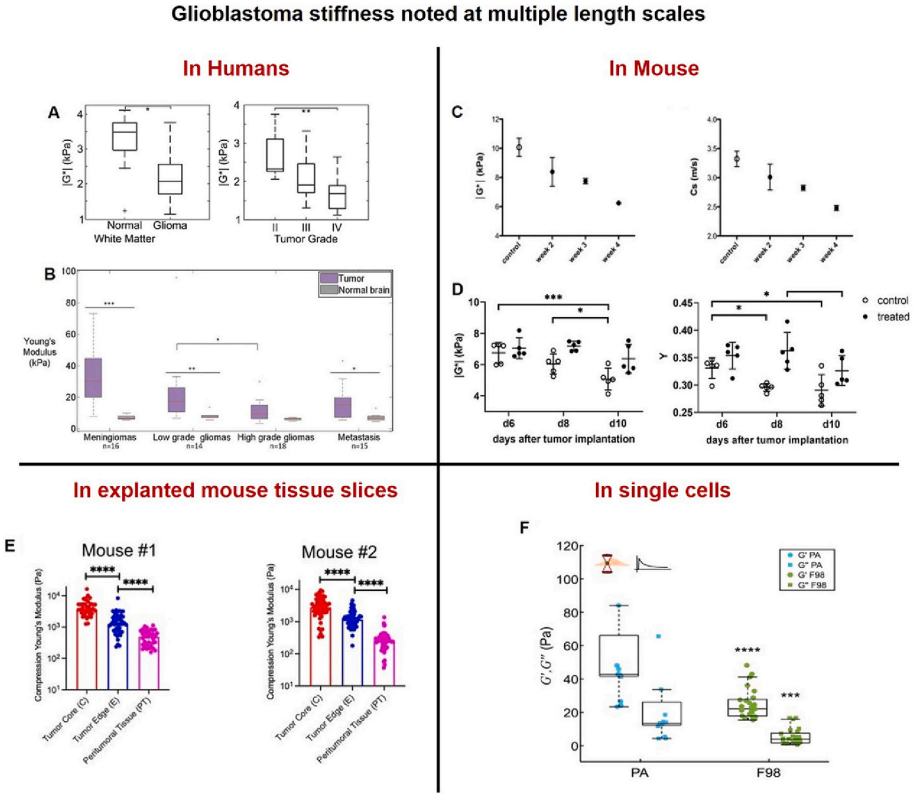


Fig. 1. Heterogeneity in GBM tumor stiffness noted across various length scales. A) MRE measurements in humans show that healthy brain ECM is stiffer than GBM tumor. Figure taken from [37] and reprinted with permission of American Society of Neuroradiology. B) SWE measurements show that GBM tumors are stiffer than healthy brain ECM and contradicts the MRE measurement data. Figure taken from [13] and reprinted with permissions from Georg Thieme Verlag KG. C) MRE measurements of GBM tumors (U-87 MG) in mouse aligns with the measurements in humans *via* MRE. Viscoelastic modulus of GBM tumors is significantly lower than brain parenchyma (control). Figure taken from [40] and reprinted with permission of John Wiley and Sons. D) B-20 an anti-VEGF antibody treatment enhanced the survival of GBM bearing mice by maintaining the tumor rigidity. Figure taken from [32] and reprinted with permission of BioMed Central (Springer Nature). E) GBM tumor stiffness measured by AFM in mouse tissues shows that GBM tumor tissues were stiffer than non-tumor tissues, and supports the SWE data noted in humans. Figure taken from [43] and reprinted with permission of Elsevier. F) At the single cell level, primary rat astrocytes (PA) were stiffer than rat GBM cells (F98). Figure taken from [45] and reprinted with permission of Elsevier.

maintaining nearly similar GBM tumor stiffness (day 6–7.1 ± 0.7 kPa, day 7–7.2 ± 0.3 kPa, day 10–6.4 ± 0.9 kPa) compared to untreated mice (day 6–6.8 ± 0.7 kPa, day 7–6.0 ± 0.6 kPa, day 10–5.1 ± 0.7 kPa). Even though a slight decrease was noted in day 10 for treated mice, the change was not statistically significant (Fig. 1D) [32].

Unlike MRE, AFM is widely used to measure the stiffness of cells or of an excised tissue. Ciesluk et al., quantified mechanics of various freshly removed human gliomas by employing AFM. Results reported in this study were quite contradicting with the results obtained from MRE measurements. In particular, the authors found that Young's modulus of isolated GBM tissues measured at 2 μm depth were stiffer (168.8 ± 32.8 Pa) compared to healthy brain tissue (61.2 ± 0.4 Pa) and other glioma grades [29]. Interestingly, indentations depth (2 μm *vs.* 1 μm) altered the stiffness values (at 1 μm depth, GBM - 115.2 ± 7.2 Pa and healthy brain - 56 ± 0.3 Pa), however the trend remained same. Similar set of stiffness data was obtained in fresh frozen human brain biopsies where Young's modulus of GBM (70 to 13,500 Pa) was significantly higher compared to healthy (non-tumor gliosis (10 to 180 Pa)) and low glioma grades (50 to 1400 Pa) [12]. One more interesting and contradicting finding is that the fresh frozen human brain biopsies of GBM tumors with R132H IDH1 gene mutations were softer compared to wild type IDH1 GBM tumor biopsies [12]. In addition, heterogeneity was noted in necrotic *vs.* non-necrotic regions of GBM tissue samples [42]. In addition to GBM tumor slices isolated from human brain, tumor slices from mouse models have also been employed to measure stiffness. For example, Sohrabi et al., utilized tumor slices from NSG female mouse brain to quantify GBM tumor stiffness [43]. They reported a similar trend to that observed in human GBM slices, where GBM tumor (core) stiffness was significantly higher (mice 1–3689 Pa, mice 2–2781 Pa) compared to healthy or peri tumoral tissue (mice 1–496 Pa, mice 2–263 Pa) as well as that at the tumor edge (mice 1–1260 Pa, mice 2–1171 Pa) [43] (Fig. 1E). In addition to GBM, the mechanical properties of low grade brain tumors have also been measured [31,44]. For example Stewart et al., reported that the steady state modulus of human meningioma samples (3.97 ± 3.66 kPa) were 2 fold higher than freshly isolated mouse brain (1.56 ± 0.75 kPa), and the brain tumors developed in mouse were also significantly stiffer (7.64 ± 4.73 kPa) than the mouse brain [44].

Overall, a lot of variability has been observed in the modulus values reported for GBM tumors depending on the technique (*in situ vs. ex vivo* measurements), the sample type utilized, and possibly measurement location (core *vs.* margin). However, it is difficult to exactly pinpoint where the differences could be coming from due to limited specific information for direct comparison. Even considering tumor core *vs.* margin measurements presented in some studies, differences across techniques are evident. For instance, MRE measurements indicate that the stiffness of perifocal and tumor tissue in GBM patients are nearly similar [35], while in the case of AFM measurements of freshly isolated human tumors and mouse GBM xenografts the adjacent tissue and tumor edge were softer than the GBM tumor core [29,43]. Nonetheless across all of these measurements, (i) In humans, the range of complex shear modulus of GBM tumors is ~1.32 to 1.81 kPa, whereas Young's modulus of GBM tumor is reported to be 11.4 kPa. ii) In mouse models, the measured stiffness of GBM tumors is: elastic modulus - 4.8 kPa, viscosity modulus - 2.9 kPa, visco-elastic modulus - 8.39 kPa and complex shear

modulus - 6.8 kPa. iii) In either fresh or frozen patient GBM tissues, Young's modulus measured is reported to be ~0.07 to 13.5 kPa. iv) In mouse GBM tissues, median Young's modulus is in the range of 2.8 to 3.7 kPa. In some of these measurements, GBM tumors are found to be stiffer than the healthy brain.

2.2. Mechanical characterization of GBM cells in vitro

The tumor initiating cell (TIC) type from which GBM tumors originate is not yet clear and remains as a subject of controversy till date [46]. However, most believe that GBM tumors originate from neuronal stem cells (NSCs) and glial cells [47,48]. Depending on the genetic mutations observed in the GBM tumors and cell specific marker expression, GBM lineage has been traced down to NSCs, NSC-derived astrocytes, and oligodendrocyte precursor cells [47,49,50]. During the process of tumor initiation, TICs undergo multiple genetic mutations, which might alter cell mechanics. Thus, it is crucial to understand these differences in cell mechanics between GBM cells and the cell of origin. Here, in this section, we highlight how GBM cell mechanical properties differ compared to their normal cell counterpart (*i.e.*, astrocytes) and how such properties are influenced *in vitro* in response to targeting the cytoskeleton, cell-ECM interactions, or *via* drug treatment.

To identify differences in mechanical properties at the cellular level between GBM *vs.* astrocytes, Alibert et al., measured whole-cell (*via* single-cell microplate whole-cell rheometer) and intracellular (optical tweezers based) rheology of GBM cells. They found that rat GBM cells were softer at both the whole cell and intracellular level compared to primary rat astrocytes (Fig. 1F). In human glioma cells, grade III glioma cells were rigid compared to grade IV at the whole cell level as they expressed higher levels of cytoskeletal proteins, but at the intracellular level grade IV cells displayed higher modulus [45]. For cells to adhere, migrate, and invade on various ECM surfaces, both focal adhesions and actin binding are key, as they connect the ECM with the cytoskeleton [51]. Studies have shown that by targeting these interactions, cell mechanics is altered. For example, Sen et al., found that U373-MG cell cortical stiffness (cells cultured on $\geq$8 kPa ECM has 6 kPa stiffness) reduced drastically when α-actin isoforms (α-actinin-1 and α-actinin-4) were targeted using siRNA (for both siACTN1, siACTN4 treated cells on >18 kPa ECMs displayed 2.7 kPa stiffness) [52]. A reduction in cell area and cell migration was also noted in targeted cells on different ECM substrates. Even by genetically upregulating the activity of key proteins (RhoA GTPase and myosin light chain kinase (MLCK)) involved in actin reorganization led to an increase in GBM cellular stiffness (310 to 510 Pa) in U373 MG cells and also influenced cell migration [53]. GBM cells are known to express CD44 receptors, which interact with various ECM substrates and alter their growth [54–56]. Accordingly, knockdown of CD44 expression *via* siRNA in SNB-19 GBM cells reduced the nuclear region stiffness (0.56 ± 0.5 kPa) compared to control cells (1.93 ± 2.86 kPa), but no differences were noted in cytoplasmic region [57]. De-adhesive interactions between cells and ECM surfaces after drug treatment can also influence cell mechanics. For instance, U373 MG cells treated with nocodazole increased their cortical stiffness from 4.5 kPa to 7 kPa, whereas blebbistatin reduced cell stiffness to 2.8 kPa [33]. Guillou et al., utilized a microfluidic device to measure the mechanical properties (shear modulus) of GBM tumor initiating cells (L0) (0.59 ± 0.05 kPa) and their response to cytoskeleton targeting pharmacologic agents finding that these agents influence cell mechanical properties (*i.e.*, Cytochalasin D - 0.22 ± 0.04 kPa and Paraformaldehyde – 0.73 ± 0.05 kPa) [34]. Finally, culture on substrates of varying stiffness is also known to alter GBM cell mechanics. For example, the Young's modulus of LN229 GBM cells cultured on polyacrylamide hydrogels increased with an increase in substrate stiffness (stiffness was measured between nucleus and cell edge) [31]. Depending on the substrate stiffness and culture conditions, stiffness of GBM cells varied between *in vitro* studies, however the measured values were generally comparable to that reported in human and mouse studies specifically, shear modulus ~0.59 kPa, cortical cell stiffness ~2.6 to 4.5 kPa and cell stiffness in nuclear region ~1.93 kPa.

3. Tissue mimetic models to study GBM mechanobiology

The GBM TME is a highly heterogeneous niche with a varied range of mechanical gradients. Factors such as density, rigidity, and geometry of the ECM constitute the biomechanical cues that influence cell migration, invasion, and proliferation of GBM cells [58,59]. Simulating these mechanical features in an *in vitro* setting could offer a better understanding of the sophisticated molecular cross talks happening between the cancer cells and its microenvironment. To this end, 3D culture platforms have been employed to study the impact of biomechanical cues on cell invasion, migration, or proliferation, due to its ability to replicate key aspects of the GBM TME [60–65]. These 3D culture systems are developed by utilizing biomaterial based hydrogels or scaffolds. Particularly, hydrogels have been extensively employed due to their wide range of physical and mechanical properties, high water retaining capacity and flexible structure that can better mimic the complex microenvironments of tumors [66–71].

The brain ECM is composed primarily of HA, an anionic glycosaminoglycan, which interacts with tenascins, proteoglycans, and HA-binding proteins forming the scaffold surrounding neuronal and glial cells [24,72,73]. In addition to HA, Collagen IV, Fibronectin, and Laminin, are found in the brain vasculature [74]. Due to their abundance in the brain ECM, hydrogels fabricated from natural polymers are commonly used to mimic brain ECM and also to investigate the role of mechanotransduction in GBM tumors [3,75–79]. However, natural polymer based hydrogels have some limitations, in terms of batch to batch variability, low tunability of mechanical properties, and a higher rate of degradation. To overcome these issues, hydrogels prepared from synthetic or semi-synthetic based materials are being employed to better mimic the GBM tissue architecture more closely in 3D tumor models [26,60,62,80–82].

Further, while hydrogels can mimic several aspects of the brain ECM in 3D, they generally do not provide aligned topographical cues relevant to GBM cells, as they are known to migrate along blood vessels and white matter tracts [83,84]. To address this issue, researchers have utilized nanofiber scaffolds fabricated *via* electrospinning, which are known to provide a large surface area for cells and also provide relevant topographical cues [85]. A wide variety of natural (*e.g.*, HA, gelatin, collagen, chitosan and silk fibroin) and synthetic polymers (*e.g.*, polylactic acid (PLA), poly(ε-caprolactam) (PCL), polyurethane (PU), poly (lactic-*co*-glycolic acid) (PLGA), poly(1-lactide-*co*-ε-caprolactam) (PLLA-CL) and poly (ethylene-*co*-vinyl acetate) (PEVA)) have been employed to fabricate nanofiber scaffolds [86–89]. In this section, we describe the various 3D tissue mimetic models employed to study the mechanobiology of GBM cells (Table 3).

3.1. Mechanical cues impact GBM cell migration

GBM cells are known for their high invasive/migratory behavior, as they aggressively infiltrate into the healthy ECM surrounding the tumor [90]. Thus, tissue mimetic *in vitro* models have been employed to study GBM migration and invasion, and how matrix stiffness governs these processes. For instance, Ulrich et al., utilized Collagen - I and agarose hydrogel system, wherein an increase in the overall agarose concentration increased the hydrogel stiffness (0.01 % - 4 Pa, 1 % - 1 kPa). Encapsulation of U373-MG glioblastoma spheroids in collagen-agarose hydrogels showed that glioblastoma spheroids displayed higher levels of invasion in softer hydrogels (0.01 % - 4 Pa and 0.125 % - 12 Pa) compared to stiffer hydrogels (1 % - 1 kPa) (Fig. 2A) [91]. With an increase in the agarose concentration, the intercalating network between the collagen – I and agarose became denser and created a steric barrier and reduced cell motility. More recently, Sohrabi et al., also found that GBM cell spheroids prefer softer HA matrices (0.34 kPa) to migrate

Table 3
Summary of studies demonstrating the impact of ECM stiffness and its composition on GBM migration/invasion/proliferation.

Cell line/type	Culture configuration	Biomaterial	Biochemical cues	Quantity measured/ stiffness studied	Observations/mechanism of migration/invasion/proliferation	Reference
U373-MG cells	Encapsulated	Collagen, Agarose	–	Elastic modulus/0.003 to 1 kPa	Agarose forms dense networks in Collagen which reduces cell invasion in collagen-agarose matrices.	T.A. Ulrich et al. [91]
L0, L2 and U373-MG cells	Top seeded	Polyacrylamide	Laminin	Elastic modulus/0.08 to 119 kPa	ECM stiffness induced sensitivity was lost in cells upon the inhibition of non-muscle myosin II and ROCK	S.Y. Wong et al. [92]
U373-MG, U87-MG, U251-MG, SNB19 and C6 cells	Top seeded	Polyacrylamide	Fibronectin	Elastic modulus/0.08 to 119 kPa	Increase in substrate rigidity increases cell proliferation/cell migration speed.	T.A. Ulrich et al. [26]
U373-MG and U87-MG cells	Both top seeded and sandwiched between HA and polyacrylamide	HA and Polyacrylamide	Fibronectin	Elastic modulus/$\geq$119 kPa	Interactions between matrix bound HA and CD44 receptors reduces the migration speed.	A.D. Rape et al. [95]
U251MG cells	Encapsulated	Gelatin and HA	–	Elastic modulus/8.8 to 12.8 kPa	GBM cells secreted HA in softer gels, which enhanced cell invasion. Inhibition of HA-CD44 interactions inhibited invasion in soft gels.	J.-W.E. Chen et al. [97]
Patient Derived OSU-2 cells and human astrocytes	Encapsulated	Collagen I/III and HA	–	Elastic modulus/0.3 to 2.1 k Pa	Higher levels of GBM migration were observed in gels containing lower HA wt%.	S.S. Rao et al. [98]
C6 rat glioma cells	Encapsulated	Acid-solubilized (AS) rat tail collagen I, pepsin-treated (PT) bovine hide collagen I, HA and Chondroitin Sulfate	–	Storage modulus/3 to 12 Pa	Presence of chondroitin sulfate significantly reduced cell invasion from spheroid periphery irrespective of collagen –I	Y.L. Yang et al. [99]
DBTRG and U251 cells	Encapsulated	Matrigel, Collagen-I and HA	–	Elastic modulus/23 to 370 Pa	DBTRG cells show higher invasion when compared to U251 cells, irrespective of hydrogel stiffness	S. Wang et al. [100]
Patient Derived OSU-2 cells	Top seeded	Poly(ε-caprolactone), poly (dimethylsiloxane), poly (ethersulfone) and Gelatin	Collagen, HA and Matrigel	Global modulus/2.4 to 33.3 MPa	Fastest cell migration was observed on PCL nanofibers with intermediate modulus	SS. Rao et al. [87]
DBTRG-05MG cells	Top seeded	Polystyrene (PS)	–	Structural stiffness/0.75 to 3.4 Nm^{-1}	Higher migration at lower stiffness	P. Sharma et al. [88]
Glioma Stem Cells	Top seeded	Polyacrylonitrile	–	Young's modulus/3 to 1260 kPa	Migration of GSCs was higher at optimal stiffness (166 kPa)	E. Marhuenda et al. [89]
U87-MG cells	Encapsulated	PEG	MMP-cleavable peptide and RGD peptide	Young's modulus/1 to 26 kPa	Soft hydrogels increased cell proliferation	C. Wang et al. [80]
U87-MG cells	Encapsulated	PEG	MMP-cleavable peptide and RGD peptide	Young's modulus/1.2 to 2 kPa	No change in cell proliferation was observed irrespective of % of MMP-degradable crosslinks	C. Wang et al. [60]
D-270 MG, SU-pcGBM-2, DIPG and U87-MG cells	Encapsulated	PEG	MMP-cleavable peptide and RGD peptide	Young's modulus/0.04 and 1 kPa	Patient derived (adult and pediatric) glioblastoma cells show extensive growth in soft hydrogel compared to U87 cells.	C. Wang et al. [106]
D-270 MG cells	Encapsulated	PEG	MMP-cleavable peptide and RGD peptide	Compressive modulus/0.04 to 1.3 kPa	Soft zones in gradient hydrogels increased proliferation with increased secretion of MMP-1 and 2 compared to stiff gels. Increase in drug resistance to Temozolomide was noted in higher stiffness.	D. Zhu et al. [107]
U373-MG and U87-MG cells	Top seeded	Polyacrylamide	Fibronectin	Elastic modulus/0.08 to 119 kPa	Increase in hydrogel rigidity increased the cell proliferation by activating EGFR pathway	V. Umesh et al. [81]
U373-MG, U87- MG and C6 cells	Top seeded	HA	RGD peptide	Elastic modulus/0.05 to 35 kPa	Cell migration speed increased with increase in hydrogel stiffness in single cells, whereas in 3D spheroids migration was observed in soft hydrogel.	B. Ananthanarayanan et al. [9]
HK 177 and HK 408 cells	Encapsulated	HA and PEG	RGD peptide	Compressive modulus/0.34 and 3.8 kPa	Soft hydrogels increased cell migration in GBM spheroids and they also increased aerobic glycolysis.	A. Sohrabi et al. [43]
U87-MG cells	Top seeded in microfluidic device	Polyacrylamide	Fibronectin	Longitudinal stiffness/1 to 40 kPa	Cell morphology was regulated by increase in hydrogel stiffness.	J. Dou et al., [105]

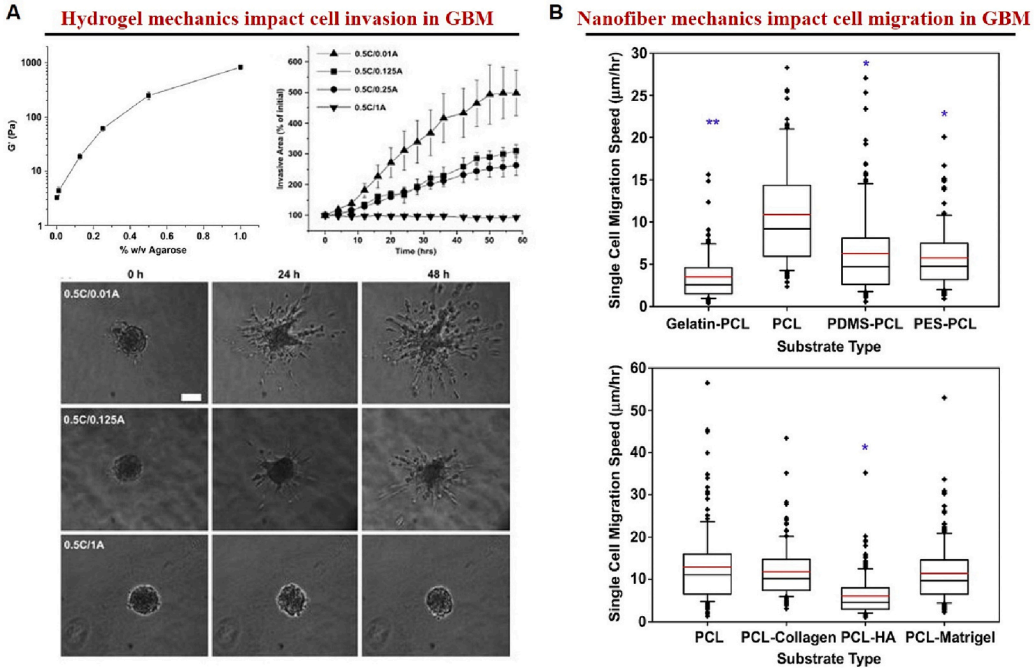


Fig. 2. Hydrogel or nanofiber mechanics influence GBM cell invasion/migration abilities. A) Softer collagen-agarose (0.004 kPa) hydrogels enhance the invasion of encapsulated glioblastoma spheroids compared to stiffer (1 kPa) matrices. Figure adapted from [91] and reprinted with permission of Elsevier. B) Migration speed of GBM cells was maximal in intermediate modulus nanofibers (PCL – 7.9 MPa) compared to softer (Gelatin-PCL – 2.4 MPa) or stiffer modulus nanofibers (PDMS-PCL – 33.3 MPa and PES-PCL – 28.6 MPa). In addition to nanofiber mechanics, brain ECM molecules (*i.e.*, HA) also influence cell migration speed. Figure taken from [87] and reprinted with permission of Elsevier.

compared to stiff HA hydrogels (3.8 kPa) [43]. However, decoupling of matrix stiffness and porosity is typically challenging in 3D cultures. Thus, the sole impact of matrix stiffness is typically studied by seeding cells on top of matrices with varying stiffness. For example, Ulrich et al., studied the impact of ECM stiffness on GBM cells (U373-MG, U87-MG, U251-MG, SNB19, C6) seeded on fibronectin coated polyacrylamide hydrogels of varying stiffness (0.08, 0.8, 19 and 119 kPa). GBM cells were found to be sensitive to ECM rigidity, wherein they strongly adhered to the stiffer matrices (119 kPa) and also exhibited higher migration speed on 119 kPa hydrogels compared to softer hydrogels (0.08 and 0.8 kPa) mimicking native brain ECM [26]. Inhibiting non-muscle myosin II and Rho - associated kinase (ROCK) through pharmacologic agents (blebbistatin, cytochalasin D) disrupted the sensitivity to ECM stiffness in GBM cells. Surprisingly, GBM tumor initiating cells (L0 and L2), isolated from primary human brain tumors cultured on laminin coated polyacrylamide hydrogels (0.08, 0.8 and 119 kPa) did not show any sensitivity to hydrogel stiffness. However, upon the activation of MLCK or ROCK, these cells regained sensitivity especially in softer matrices [92].

GBM cell infiltration occurs along the blood vessels and along white matter tracts [93,94], which provides topographical cues for cell migration. To mimic this process, particularly studying cell migration at tissue interfaces, Rape et al., developed a 3D culture system, wherein the GBM cells were sandwiched between fibronectin-coated polyacrylamide gel (119 kPa) (ventral) and HA methacrylate hydrogel (dorsal) surfaces. Initially GBM cells were seeded on top of Fibronectin-coated polyacrylamide gels and incubated overnight and then HA methacrylate solution was added on top of the cells as an overlay and crosslinked. GBM cells displayed higher migration speeds on fibronectin-coated polyacrylamide gels alone, and the addition of HA hydrogel reduced the migration speed [95]. Reduction in the migration speed in the presence of HA hydrogel was due to the HA-CD44 interactions between the solid matrix and the cells but not due to the presence of soluble HA. Further, they cultured cells between a stiff fibronectin-coated polyacrylamide gel (stiffness > 119 kPa) and HA substrate and noticed a 47 % decline in migration speed compared to no HA overlay culture. However, in between soft fibronectin-coated polyacrylamide gel (119 kPa) and HA substrate, only a 34 % reduction in cell migration speed was noticed, further highlighting the impact of ventral stiffness in controlling migration at tissue interfaces.

Omnipresence of HA in the GBM tumor niche or in the surrounding ECM is associated with the progression of GBM tumors [96]. To study the effect of matrix bound HA on GBM cell invasion, Chen et al., fabricated Gelatin methacrylate (GelMA) hydrogels containing various ratios of HA methacrylate (HAMA) (0 %, 10 % and 15 %). GelMA-HAMA hydrogels were prepared by maintaining the overall concentrations at 4 wt% and 5 wt% respectively. The presence of HA in the hydrogel structure did not have a significant impact on the hydrogel stiffness; however, the elastic modulus of the hydrogel is determined by the concentration of GelMA (4 wt% - 8.8 kPa and 5 wt% - 12.8 kPa). They found that the presence of matrix bound HA did not show any impact on the metabolic activity as well as cell invasion in U251 GBM cells. However, the cells in the softer matrix (4 wt% with 0 % HAMA – 8.8 kPa) exhibited higher invasion. Additionally, CD44 inhibition reduced the GBM cell invasion significantly even in the soft hydrogels. Higher rates of invasion observed in softer matrix lacking matrix bound HA was found to be due to the increased levels of soluble HA secreted by GBM cells [97]. Additionally, Rao et al., investigated the impact of HA composition and matrix stiffness on GBM cell migration finding that with an increase in the concentration of HA (0 to 2 %) in collagen (I/III) hydrogels, the hydrogel stiffness increased from 300 to 2065 Pa. Patient derived GBM cells (OSU-2) displayed low cell spreading area with circular morphology in stiff hydrogels containing 2 % HA compared to soft hydrogels without HA content. With an increase in the matrix stiffness, GBM cell migration reduced from ~9.3 μm/h (in 300 Pa matrix) to no migration in 2065 Pa hydrogel matrix [98]. Similarly, Yang et al. utilized Collagen based hydrogels to study the impact of glycosaminoglycans (chondroitin sulfate (CS) and HA) on GBM cell invasion. Interestingly, they utilized both Acid-solubilized (AS) rat tail collagen-I (with a stiffness of 3 Pa for 1 mg/mL gel) and Pepsin-treated (PT) bovine

collagen-I (with a stiffness of 6 Pa for 1 mg/mL gel). Irrespective of the collagen origin, an increase in the concentration of HA increased the storage modulus of both AS Collagen-I and PT Collagen-I hydrogels, but no change in stiffness was noticed with an increase in CS concentration for both collagen matrices. They encapsulated GBM spheroids in AS Collagen-I, PT Collagen-I, AS Collagen-I/CS, PT Collagen-I/CS, AS Collagen-I/HA and PT Collagen-I/HA hydrogels and found that cell invasion from the periphery of spheroid was significantly lower in AS Collagen-I/CS and PT Collagen-I/CS when compared to pure Collagen-I gels (AS and PT), whereas AS Collagen-I/HA and PT Collagen-I/HA maintained nearly similar levels of invasion as AS and PT Collagen-I gels [99]. Intriguingly, Wang et al., compared the invasive potential of two GBM cell lines (DBTRG and U251) by embedding their spheroids in two different hydrogels, (i) Matrix gel (0.5 mg/mL Collagen I + 3 mg/mL Matrigel) (0.023 kPa) and brain stiffness mimicking matrix gel (0.5 mg/mL Collagen I + 3 mg/mL Matrigel + 3.3 mg/mL HA) (0.37 kPa). They showed that irrespective of the hydrogel stiffness, DBTRG cell spheroids expressed higher invasion distance, invasion area and invasion velocity compared to U251 cell spheroids [100]. Additionally, 2774 differentially expressed genes were noticed in DBTRG cells compared to U251 cells in 2D. Out of them, top 5 differentially expressed genes were associated with invasiveness (WNT7A, VEGFA, EFEMP1, TRPV2, and FOXC2). These studies while demonstrating the impact of matrix stiffness and composition, also highlight differential responses between cell types.

GBM cells migrate directionally through the highly aligned white matter tracts of the healthy brain parenchyma (the diameter of aligned white matter tracts is the range of 500 nm–7 μm) [85,101,102]. While tissue-mimetic hydrogels have been used to study glioma cell migration, including those at tissue interfaces, these hydrogels are fibrous materials in the nano-metric range but are not typically aligned to mimic white matter tracts [9,24,99,103]. Electrospun nanofibers can be utilized to understand the impact of fiber alignment and its topography, along with fiber stiffness on GBM migration. For example, Rao et al., developed aligned core-shell electrospun nanofibers using the co-axial electrospinning technique to mimic the topography of white matter tracts [87]. Using these fabricated nanofibers, they examined the migration of patient-derived GBM (OSU-2) cells. The aligned nanofibers were fabricated with using various core materials such as gelatin, poly(dimethyl siloxane) (PDMS), poly (ether sulfone) (PES), and PCL as the shell (PCL – 7. MPa, gelatin-PCL – 2.4 MPa, PES-PCL – 28.6 MPa and PDMS-PCL – 33.3 MPa). Among these fibers, fastest cell migration speed (11 μm/h) was observed in the PCL nanofibers with intermediate modulus, and the slowest migration (3.5 μm/h) was noticed in gelatin-PCL with lower modulus (Fig. 2B). In another study, aligned parallel single suspended and orthogonally arranged double suspended nanofibers were prepared for studying glioma cell (DBTRG-05MG) migration. The migration speed of glioma cells seeded on single suspended nanofibers was higher when compared to double suspended nanofibers and flat fibers. Furthermore, various lengths (4, 6, and 10 nm) of single suspended nanofibers with altered stiffnesses (3.4, 1.5, and 0.75 Nm^{-1}) were used for examining the migration behavior of glioma cells. Higher migration of glioma cells was observed in lower stiffness nanofibers (0.75 Nm^{-1}) [88]. Electrospun nanofibers have also been employed to examine invasion of glioma stem cells (GSCs) as a function of stiffness. For example, Marhuenda et al., prepared 3D-ex-polyacrylonitrile nanofibers scaffolds with adjustable stiffness by loading multiwall carbon nanotubes and seeded the GSC neurospheres on top of the scaffolds with stiffness of 3, 166, 542, and 1260 kPa [89]. They observed that the GSCs migrate irrespective of fiber stiffness, but at 166 kPa, larger number of GSCs migrated from the neurospheres, meanwhile, the migration was minimal on other nanofibers. These findings suggested that the optimal stiffness of 166 kPa was sufficient to trigger GSC migration [89].

The impact of mechanical cues on GBM migration/invasion has also been examined using *in vitro* microfluidics based organ-on-chip (OOC) or cancer-on-chip (COC) models. Microfluidic devices incorporating matrices with variable stiffness consolidate both fluid dynamics and matrix mechanics and have been profoundly useful in studying the impact of these biophysical cues on GBM phenotype. For instance, Amereh et al., developed microfluidic devices with collagen matrices containing various amount of collagenase with varied stiffness to study GBM migration. Through the peripheral channels of the microfluidic device, collagenase type −1 solution (0.01 and 0.001 mg/mL) was added to the collagen matrices containing tumoroids. They reported that GBM cells exhibited higher invasion as well as growth rates at higher concentrations of collagenase that effectively reduced the stiffness. Their mathematical models coincided with their experimental observations further highlighting the validity of these microfluidic models to study GBM phenotypes [104]. Similarly, Dou et al., showed that matrix stiffness along with biochemical stimulation influences the directional migration of U87-MG GBM cells on a microfluidic chip. By using fibronectin conjugated polyacrylamide gels, they created a matrix with stiffness ranging from 1 kPa to 40 kPa and epidermal growth factor (EGF) gradient by lateral diffusion on a microfluidic device. Their study showed that higher stiffness and EGF concentrations enhanced cell migration with cells exhibiting higher spreading but lower reactive oxygen species (ROS) levels at higher stiffness [105]. Taken together, tissue mimetic scaffold based environments provide a key tool to study the mechanisms associated with GBM migration/invasion mediated by changes in matrix stiffness.

3.2. Mechanical cues impact GBM cell proliferation

In addition to GBM cell migration/invasion, mechanical cues are also known to directly influence cell proliferation. This has been mainly studied using hydrogel based culture substrates. For instance, to investigate the impact of hydrogel stiffness on GBM cell proliferation, Wang et al., utilized a polyethylene glycol (PEG) based hydrogel system, where 8 arm PEG-norbornene was crosslinked with a chemical crosslinker (PEG-dithiol) and matrix metalloproteinase (MMP) -cleavable sequence (1:1). HA was incorporated in the hydrogel as a biochemical cue. Additionally, RGD peptide was covalently crosslinked to the hydrogel. By varying the concentration of PEG from 2 % to 14 % they were able to modulate the hydrogel stiffness (0.5 to 26 kPa). Further, they encapsulated U87 GBM cells in 3 % (1 kPa – soft) and 14 % (26 kPa – stiff) PEG hydrogels and found that soft hydrogels supported the growth of GBM cells compared to stiff hydrogels. Quantification of total DNA content at the end of day 21 confirmed that cell proliferation was higher in soft hydrogels, as the DNA content was 5 fold higher than stiff gels [80] (Fig. 3A). Interestingly, in another study, Wang et al., elucidated the impact of MMP degradable peptide on GBM cell proliferation by formulating 0 % (1.2 kPa), 50 % (1.6 kPa) and 100 % (2.0 kPa) MMP-degradable hydrogels utilizing the PEG hydrogel system. Irrespective of the stiffness and degradability, all the three hydrogels supported growth of GBM cells and no differences in cell proliferation was noted [60]. However, cell spreading was noticed in 50 % and 100 % degradable hydrogels. Depending on the tumor location and age of the patient, tumor biology varies drastically. To understand the impact of ECM stiffness on the growth profile of various brain tumors, Wang et al., utilized 3 patient derived tumor cell lines (adult glioblastoma (aGBM), pediatric glioblastoma (pGBM) and diffuse pontine intrinsic glioma (DIPG)) and immortalized GBM (U87) cells. These cells were encapsulated in 40 Pa PEG hydrogel (soft) and 1000 Pa PEG hydrogel (stiff) and cultured for 3 weeks. Patient derived cell lines displayed robust growth and invasive traits in soft hydrogel whereas immortalized GBM cells exhibited growth in stiff hydrogels. Especially in soft hydrogels, proliferating cells (%Ki67 positive cells) were higher for patient derived cells (aGBM - 33.6 %, pGBM - 21.5 %, DIPG - 11.5 % and U87 - 7.6 %) [106]. These findings further reiterate the heterogeneity in terms of response noted across GBM cells. In addition to single hydrogel systems, gradient hydrogels with varying stiffness have also been utilized. For instance, Zhu et al., developed a gradient hydrogel system to recreate the brain

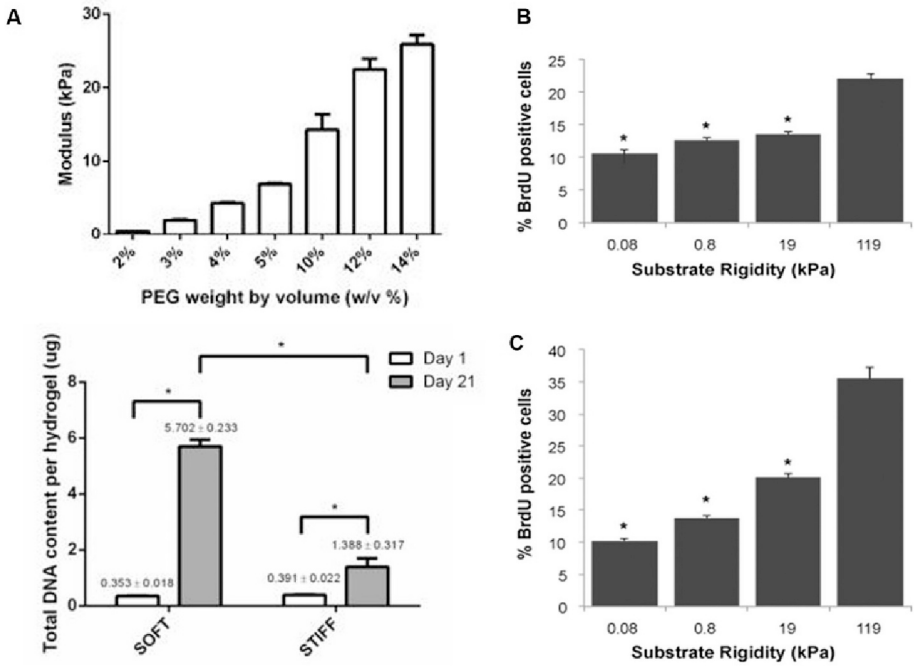


Fig. 3. Matrix stiffness influences cell proliferation in GBM cells. (A) GBM cells encapsulated in soft (1 kPa) polyethylene glycol hydrogels exhibit higher proliferation. Figure adapted from [80] and reprinted with permissions from American Chemical Society. An increase in polyacrylamide gel stiffness led to an increase in GBM cell proliferation tested using B) U373-MG cells and C) U87-MG cells when seeded on top of the hydrogel. Figure taken from [81] and reprinted with permission of PLOS.

ECM more closely, wherein a single hydrogel with five different zones of varying stiffness (40 to 1300 Pa) was prepared. Even in gradient hydrogels, encapsulated D-270 MG cells displayed higher proliferation in the zone 1 with low stiffness (100 Pa) compared to the higher stiffness zone 5 (1300 Pa). Further, the expression of MMP-1 and MMP-2 was significantly upregulated in the softer zones [107].

The impact of matrix stiffness on GBM cell proliferation is also dependent on the culture conditions, similar to that noted for cell migration/invasion. In hydrogel encapsulated cultures, cell proliferation is generally enhanced in soft matrices that are in the range of native brain tissue (<1 kPa) (Fig. 3A). However, in top-seeded hydrogel cultures, cell proliferation generally increases with an increase in matrix stiffness as noted in both synthetic and natural hydrogel systems (Fig. 3B and C). For example, Umesh et al., demonstrated that GBM cell (U373-MG and U87-MG) proliferation increases with an increase in the hydrogel stiffness, when the GBM cells were seeded on the surface of fibronectin-coated polyacrylamide gels (0.08, 0.8, 19 and 119 kPa) [81] (Fig. 3B and C). With an increase in stiffness, changes were also observed in cell cycle distribution, where % of cells in the S phase increased in 119 kPa hydrogel compared to 0.08, 0.8 and 19 kPa matrices. Further, stiff substrates significantly upregulated the levels of phosphorylated epidermal growth factor receptor (pEGFR) leading to increased cell proliferation, with inhibition of EGFR reducing the GBM cell sensitivity to substrate stiffness. Similar findings were also observed in a HA hydrogel system with cell proliferation increasing with an increase in matrix stiffness [9]. Overall, tissue mimetic models provide a useful tool to probe how changes in matrix mechanics influence GBM phenotypes and the underlying mechanisms driving these phenotypes in a variety of culture configurations.

4. Drug response of GBM tumors is modulated by ECM mechanical cues and its composition

Therapeutic approaches employed till date for GBM include a combination of surgery, radiotherapy, and chemotherapy (*i.e.*, Temozolomide, Bevacizumab, Erlotinib) [108]. However, GBM cells are capable of withstanding these therapies by employing various mechanisms including drug efflux, hypoxic areas, cancer stem cells, and oxidative stress [109–111]. In addition, the TME can influence chemotherapeutic drug response in GBM tumors. Delineating ECM induced impact on drug responses would be beneficial for designing better treatment strategies. Here, we discuss how ECM stiffness and its composition impact drug response/resistance in GBM cells (Table 4).

To study the impact of ECM stiffness on Temozolomide resistance in GBM, Erickson et al., employed a 3D scaffold composed of Chitosan-HA. By altering the concentration of Chitosan, three different scaffolds with multiple stiffness were prepared (2 % - 1.41 kPa, 4 % - 5 kPa and 8 % - 27.7 kPa). In this system, they demonstrated that even though no differences were noted in GBM cell metabolism across the scaffolds, GBM cells cultured on stiff chitosan-HA scaffold (27.7 kPa) exhibited higher ED_{50} value (3840 μM) compared to soft scaffold (1.41 kPa) (255 μM) for the chemotherapeutic drug Temozolomide [112] (Fig. 4). Additionally, upregulation of genes related to drug resistance (ABCG2) and invasion (CD44 and MMP-2) were observed in stiff chitosan-HA scaffold (27.7 kPa) compared to both 1.41 and 5 kPa chitosan-HA scaffolds. Similarly, Wang et al., employed a PEG based hydrogel system to investigate the impact of hydrogel stiffness on chemotherapeutic (Temozolomide) resistance in patient derived GBM cells. By varying the concentration of PEG monomer, they formulated five different hydrogels with stiffness ranging from 0.04 to 26.6 kPa. Here, they encapsulated cells in the hydrogels and cultured them for 10 days before adding Temozolomide. They found that an increase in hydrogel stiffness also increased drug resistance in patient derived GBM cells. Specifically, at 30 μM temozolomide concentration, 93 % cells were viable in 26.6 kPa hydrogel compared to 58 % cell viability in 0.04 kPa hydrogel [62]. Additionally, researchers have also studied the impact of chemotherapeutic drugs on GBM cells by culturing them in softer hydrogel substrates *vs.* traditional tissue culture polystyrene based 2D cultures and reported that IC50 values for Temozolomide and Carmustine were significantly higher in hydrogel substrates [113,114]. However, in contrast, Bruns et al., reported that irrespective of PEG hydrogel stiffness (0.93 *vs.* 6.7 kPa), no differences were noted in the IC_{50} values for GBM cell spheroids (U87)

Table 4
Summary of studies demonstrating the impact of ECM stiffness and its composition on GBM drug resistance.

Cell lines	Culture configuration	Chemotherapy drugs tested	Biomaterial	Biochemical cues	Quantity measured/ stiffness studied	Observations/mechanism of drug resistance	Reference
U87 MG	Top seeded	Temozolomide	Chitosan and HA	–	Compressive young's modulus/1.41 to 27.7 kPa	With an increase in stiffness, an increase in the expression of ATP-binding cassette gene (ABCG2) was observed	Erickson et al., [112]
D-270 MG	Encapsulated	Temozolomide	PEG-norbornene	RGD peptide and MMP-cleavable peptide	Young's modulus/ 0.04 to 26.6 kPa	Increase in hydrogel stiffness enhanced drug resistance	Wang et al., [62]
GBM39, HK301, HK423	Encapsulated	Erlotinib	HA and PEG-malemide	RGD peptide and L. Cysteine	Compressive modulus/1 and 2 kPa	1. Activation of EGFR by increasing p-AKT levels. 2. Activation of p-FAK levels through integrin signaling	Xiao et al., [124]
HK301, GBM6, GS024, GS025	Encapsulated	Temozolomide, Carmustine	HA and PEG-malemide	RGD peptide and L. Cysteine	Compressive modulus/1 kPa	Inhibiting BCL-2 family apoptotic factors by activation of Src signaling through CD44 receptors and integrin α_v.	Xiao et al., [126]
U87, G-10	Encapsulated	Temozolomide	PEG-acrylate and PEG-dithiol	RGD peptide	Young's modulus/ 0.93 and 6.7 kPa	No differences were noted in IC_{50} values of GBM spheroids irrespective of hydrogel stiffness	Bruns et al., [115]
GBM6, GBM12	Encapsulated	Erlotinib	Gelatin and HA	–	Compression modulus/15.3 and 17.5 kPa	EGFR-CD44 activation by p-STAT3	Pedron et al., [125]

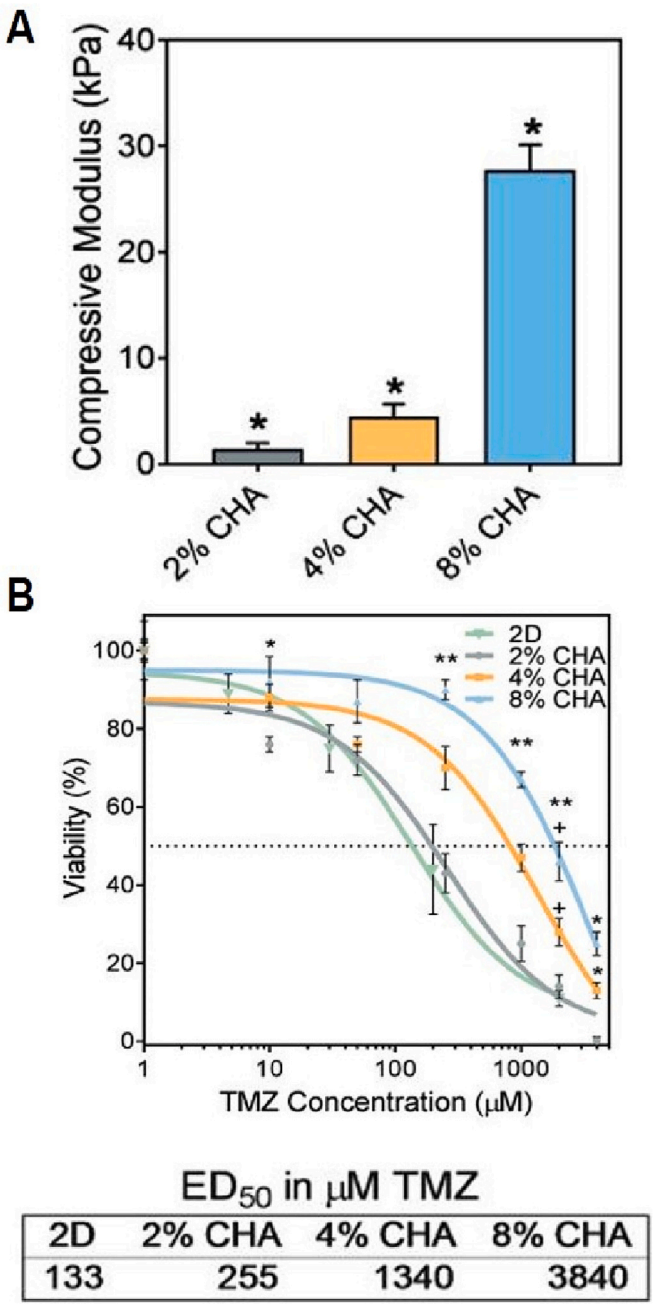
upon exposure to Temozolomide. However, in the presence of 2 mM dosage of temozolomide, a reduction in the spheroid diameter and cell viability was observed in both hydrogel conditions. Surprisingly, in soft hydrogels, the cell viability was higher in the core (80.1 %) compared to periphery of the spheroid (48.1 %), whereas in stiff hydrogels, cells were more viable in periphery (66.2 %) compared to core region (47.1 %) [115]. Even in patient derived xenoline G-10 cells, 2 mM dosage reduced the cell viability by half in both soft and stiff hydrogels [115]. These studies indicate that the ECM stiffness plays a key role in modulating the GBM drug response.

In addition to the sole effect of ECM stiffness, combination of ECM stiffness and its composition has also been shown to influence drug resistance in GBM through CD44 expression. CD44 is a transmembrane molecule, which is highly expressed on the surface of several cancer cells including GBM [116–118]. Increase in CD44 expression is specifically associated with increase in GBM growth, stemness and drug resistance [116,119]. Also, HA binds to CD44 receptors at the extracellular domain, which activates several downstream signaling pathways (PI3K/AKT, RhoGTPases, Hippo signaling) known for cell survival, growth, and drug resistance [120–123]. To this end, Xiao et al., demonstrated that GBM tumors developed by injecting GBM39 or HK301 cells intracranially in female nod - severe combined immune deficiency gamma (SCID-γ) mice, exhibited resistance to chemotherapeutic drug Erlotinib with increased levels of pEGFR, which is related to omnipresence of HA in both the tumors and also in the neighboring ECM. To further delineate the impact of HA concentration and ECM stiffness on chemotherapeutic resistance, they utilized an *in vitro* biomimetic PEG hydrogel system containing HA (1 kPa with 0.1 and 0.5 % HA and 2 kPa with 0.5 % HA) [124]. Initially, they cultured patient derived GBM cells as glioma-spheres, and these spheres were susceptible to Erlotinib treatment. To investigate the impact of ECM stiffness and composition on Erlotinib resistance, they dissociated spheres into single cells and encapsulated them into hydrogels. GBM cells cultured in the presence of high HA concentration (0.5 %) showed a significant increase in CD44 expression irrespective of the hydrogel stiffness and differences were noted in 1 kPa hydrogel (0.1 % HA) with minimal CD44 expression. Upon treatment with 1 μmol/L erlotinib, cells cultured in 1 kPa and 2 kPa hydrogels containing 0.5 % HA expressed resistance when compared to 1 kPa hydrogel with 0.1 % HA. An increase in the percentage of apoptotic cells was observed in treated cultures compared to untreated cultures in 1 kPa hydrogel with 0.1 % HA. Interestingly, in hydrogels with 0.5 % HA GBM cells attained drug resistance by day 9 on softer modulus (1 kPa) compared to day 12 on stiffer modulus hydrogels (2 kPa). GBM cells cultured in 1 kPa PEG hydrogels containing 0.5 % HA expressed resistance to Erlotinib treatment by upregulating p-AKT levels (a downstream pathway of EGFR) when compared to glioma spheres [124]. In a similar study, Pedron et al., reported that HA composition can influence the GBM cell behavior and response to Erlotinib independently based on their EGFR status ($EGFR^{+}$ and $EGFR^{vIII}$), when cultured in GelMA hydrogels (GelMA – 15.3 kPa, GelMA with 1 % HA – 17.5 kPa). Upon the incorporation of 1 wt% of HAMA into GelMA hydrogels, $EGFR^{+}$ GBM cells survived double doses of 10 μM Erlotinib with a gap of 4 days by increasing the p-STAT 3 levels through CD44 signaling [125].

The sole impact of ECM composition on drug resistance has also been investigated. For instance, a follow up study by Xiao et al., investigated how the presence of ECM composition and peptides impact alkylating chemotherapy efficacy in patient derived GBM cells by utilizing 1 kPa PEG hydrogels [126]. GBM cells in hydrogels containing high HA (0.5 %) concentration with RGD peptide exhibited more resistance to alkylating chemotherapeutic drugs (Temozolomide and Carmustine) compared to those in low (0.1 %) HA concentrations. GBM cells were able to interact with HA and RGD through CD44 and integrin-αv, and these interactions lead to the activation of Src signaling pathway, which increased chemotherapeutic resistance in GBM cells. HA and RGD interactions also reduced the expression of pro-apoptotic factors of BCL-2 family in GBM cells [126]. In a recent study, Hill et al., studied the impact of ECM composition on U87 cell spheroids growth and drug resistance by maintaining similar stiffness (1.2 to 1.75 kPa), wherein these spheroids encapsulated in two different degradable hydrogel formulations had more number of viable cells compared to non-degradable hydrogel formulation upon treatment with 2 mM Temozolomide [127]. Taken together, these studies demonstrate that ECM stiffness along with composition influences response to therapy in GBM tumors.

5. Conclusions and perspectives

There is immense heterogeneity in terms of mechanical properties, ECM constituents and cellular composition in the GBM TME [128]. Our current understanding pertaining to the impact of mechanical properties of GBM TME on cancer hallmarks is still evolving. In this review, we examined the mechanical properties, particularly the stiffness of GBM tumors *vs.* normal tissue reported across the literature. Evidently, the stiffness of GBM tumors varies profoundly with respect to grade and the host. However, no consensus exists regarding the differences in the



ED_{50} in μM TMZ

2D	2% CHA	4% CHA	8% CHA
133	255	1340	3840

Fig. 4. ECM stiffness modulates chemotherapeutic drug resistance in GBM. A) Increase in the weight% of chitosan utilized to fabricate chitosan - HA scaffolds increased the scaffold stiffness. B) GBM cells cultured on stiffer matrices exhibited resistance to Temozolomide with higher ED_{50} values. Figure adapted from [112] and reprinted with permission of John Wiley and Sons.

stiffness between GBM tumors and adjacent healthy tissue leading to profound variability in the characterization of GBM phenotype. The disparity in the reported mechanical properties might have arisen from the usage of different techniques to measure these mechanical properties along with the heterogeneity in the genetic makeup and tumor grade as well as the sample type. In addition, not all studies have measured the same quantities while characterizing the mechanical properties. To alleviate these issues concerning the biomechanical attributes of GBM, development of guidelines for standardization across studies is imperative.

The compositional and structural complexity of the brain tissue renders it incredibly challenging to assign one specific method to define the mechanical properties of GBM [129]. Essentially, measuring the stiffness by one technique may not effectively characterize GBM mechanics, owing to the plethora of techniques with its own advantages and limitations [11,128,129]. The disparity in the mechanical characteristics of GBM could possibly be reduced by establishing a standardized set of parameters for reporting the mechanical attributes of GBM. Combining multiple techniques such as MRE and SWE coupled with *ex vivo* indentation and AFM methods may provide a better representation of the mechanical attributes of GBM. The detailed and full disclosure of sample criteria including tissue type (*e.g.*, white matter *vs.* gray matter), measurement area and location (*e.g.*, tumor core *vs.* margin), time scale, sample storage conditions, experimental conditions, should be made available with the reported values as these factors significantly affect the measurements being recorded. Implementing these guidelines pertaining to the quantification of measurements at the global scale would result in data sets that can be compared across studies. Consequently, this will assist in better characterization of GBM, which, in turn, could be useful for designing better therapeutic strategies.

Intricate crosstalk between cytoskeletal components and the ECM facilitates the mechanotransduction of external stimuli that dictates the biomechanical properties of GBM [12,130,131]. An appreciation of the impact that these external factors have on GBM attributes have led to the evolution of traditional 2D cultures into 3D scaffold based approaches fabricated out of synthetic or natural polymers that partially reflect the *in vivo* microenvironment. Particularly, the stiffness of hydrogel scaffolds has been found to significantly impact the biomechanical properties of GBM cells with stiffer substrates inducing higher stiffness in GBM cells [52]. By exploiting the mechanical and chemical tunability of hydrogels and nanofiber scaffolds, the role of substrate stiffness on GBM phenotypes have been studied in greater detail leading to better understanding of GBM mechanobiology. However, further studies will be necessary for deeper understanding of underlying mechanisms through which mechanical and chemical properties of ECM collectively modulate the fate of GBM cells.

Taking cues from the profound impact of biomechanical and biochemical properties of ECM on GBM cell behavior, several studies have highlighted the impact of microenvironment on the response of GBM cells towards therapeutic agents. Depending upon the hydrogel stiffness and chemical composition, GBM cells acquired resistance to therapeutic agents through activation of drug resistance and mitogenic signaling pathways. Notably, the direct proportionality in acquired resistance to therapeutic agents with respect to the ECM stiffness *in vitro* was observed to be more prominent in GBM single cells as compared to GBM spheroids. The differential response of GBM single cells or a group of cells as spheroids to therapeutic challenges in an ECM stiffness dependent manner may be an important factor to be considered in the development of therapeutic strategies. It may be worth noting that under *in vivo* conditions, disseminated single cells from the primary GBM tumor may survive therapeutic treatments leading to recurrence [132]. In addition to stiffness, chemical composition of ECM is a major determinant of GBM drug response. The brain prevalent HA imparted drug resistance to GBM through activation of its cognate receptor CD44 in which the stiffness of the scaffold also played a significant role. Essentially, HA mediated resistance to various clinically relevant drugs poses a major challenge in the treatment of GBM [112,114,124–126]. Possibly, therapeutic targeting of HA and its interactions with CD44 could potentially alleviate HA mediated pro-tumorigenic and drug resistant properties in GBM [96,133,134].

In advancing tissue mimetic models, utilization of patient-derived cells as well as understanding how results from tissue mimetic models connect to patient outcomes would be important. Efforts have already been made in this direction. For example, Xiao et al., employed a microvasculature on chip model and found that patient derived brain tumor stem like cells preferentially localize to the perivascular niche and possibly these microvascular tracks are utilized by these cells for movement. Additionally, single cell transcriptomics of patient derived

tumor cells revealed that gene signature profiles strongly correlated with homing of tumor cells to the perivascular niche. Their study highlights the ability of *in vitro* microfluidic models in capturing *in vivo* GBM heterogeneity and subtypes [135]. In a similar vein, perivascular niche on chip microfluidic device developed by Gerigk et al., was able to maintain the stemness of patient derived glioblastoma stem like cells in a 3D environment [136]. Studying GBM migration *in vitro*, Smith et al., employed 1D fibrillar surfaces that mimics the features of 3D environment at the nanometer scale found in the brain tissue ECM. They reported that single cell migration on this surface mimicked migration *in vivo*. Interestingly, they found that migratory phenotype of patient derived cells in response to platelet-derived growth factor could predict the location of tumor cells as well as future recurrence. Their findings in relation to migratory phenotypic classifiers have patient specific prognostic and diagnostic implications [137]. Microfluidic based assays to study GBM cell migration have also enabled categorization of patients according to progression free survival with high accuracy, particularly, high motility of GBM cells correlating with poor prognosis of the disease [138]. In the future, such models may inform the design of therapeutic approaches against GBM.

In sum, while the impact of ECM on GBM cancer hallmarks and its response to therapeutic challenges is established, the underlying molecular mechanisms are only beginning to be discovered. While there is no proper consensus on the mechanical properties (*i.e.*, stiffness) of GBM tumors, these have still served as a guide for development of 3D culture models to study GBM mechanobiology. Nonetheless, future model development should consider measurements of mechanical properties in humans and/or *ex vivo* samples and in 3D models using identical techniques. In this way, a direct comparison can be made while simultaneously validating the 3D biomimetic model. Additionally, the impact of the rich cellular heterogeneity and how this influences GBM mechanics remains elusive. Future studies should consider examining how the cellular composition influences the biomechanical attributes of GBM for better understanding of the GBM TME. Inclusion of relevant cell types in 3D *in vitro* models with proper considerations for both chemical and physical characteristics of ECM components may yield more appropriate models and better understanding of GBM mechanobiology, including response to therapy, ultimately leading to development of superior therapeutic strategies with the potential to impact survival outcomes.

CRediT authorship contribution statement

Raghu Vamsi Kondapaneni: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Sumiran Kumar Gurung:** Writing – review & editing, Writing – original draft. **Pinaki S. Nakod:** Writing – original draft. **Kasra Goodarzi:** Writing – original draft. **Venu Yakati:** Writing – original draft. **Nicholas A. Lenart:** Writing – original draft. **Shreyas S. Rao:** Writing – review & editing, Visualization, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Not applicable as this is a review article.

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