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Extracellular Hyaluronic Acid Influences the Efficacy of EGFR Tyrosine Kinase Inhibitors in a Biomaterial Model of Glioblastoma

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Abstract

3D biomaterial models have potential to explore the influence of the tumor microenvironment on aberrant signaling pathways and compensatory signals using patient-derived cells. Glioblastoma (GBM) tumors are highly heterogeneous, with both cell composition and extracellular matrix biophysical factors seen as key regulators of malignant phenotype and treatment outcomes. Amplification, overexpression, and mutation of the epidermal growth factor receptor (EGFR) tyrosine kinase have been identified in 50% of GBM patients. Here, hyaluronic acid (HA) decorated methacrylamide-functionalized gelatin (GelMA) hydrogels are used to examine the synergies between microenvironmental factors and a model EGFR tyrosine kinase inhibitor (TKI) using patient-derived xenograft cells. The in vitro behavior of 3 patient-derived xenografts that reflect a clinically relevant range of EGFR variants is characterized: GBM10 (EGFR, wild type),

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Conflict of Interest

The authors declare no conflict of interest.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

GBM12 (EGFR+), and GBM6 (EGFRvIII). GelMA hydrogels support xenograft culture; cells remain viable, active, respond to matrix-immobilized HA, and upregulate genes associated with matrix remodeling and tumor growth. Interestingly, matrix-immobilized HA alters the response of GBM cells to a model tyrosine kinase inhibitor, erlotinib. While constitutively activated EGFRvIII cells are sensitive to TKI in gelatin hydrogels, hyaluronic acid mediated adhesive signaling interacts with EGFRvIII signaling to increase cell metabolic activity, increase soluble hyaluronic acid synthesis, and modify response to erlotinib exposure.

Keywords

EGFR; glioblastoma; hyaluronan; patient-derived tumor cells; tumor microenvironment

1. Introduction

Glioblastoma (GBM) is one of the most common and difficult to treat forms of brain cancer. Every year, $\approx 12\,000$ new GBM cases are diagnosed in the U.S.,^[1] with patients showing 2-year survival rates of $\approx 27\%$ with current forms of therapy.^[2] Recurrent genetic alterations in GBM have been identified, with frequent amplification of several receptor-tyrosine kinases (RTK) including epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and tyrosine kinase receptor MET.^[3] As the most frequent RTK amplification in glioblastoma, EGFR is amplified in about half of all primary glioblastoma and in 95% of the classical subtype.^[4] Tumor microenvironmental parameters such as proteases, hyaluronan,^[5] and stiffness^[6] influence EGFR-dependent signaling in controlling cell cycle and proliferation. The EGFR has been identified as a molecular target in GBM associated with poor clinical outcomes. Gene amplification and mutations of the EGFR lead to increased proliferation, angiogenesis and therapeutic resistance. These variations are also common in epithelial cancers;^[7] however, while molecular subtype therapies have been effective for those tumors,^[8] they exhibited poor performance against GBM. So, while EGFR-specific inhibitors such as erlotinib or gefitinib are currently used for treatment of non-small cell lung carcinoma (NSCLC), these therapies have shown poor efficacy against GBM.^[9] Reasons associated with failure of these therapies have been mostly suggested to be due to poor tissue penetration and the existence of compensatory signaling pathways in GBM that reduce efficacy.^[10]

The genetic aberration of EGFR (EGFR+) often comes with the overexpression of the mutant EGFR variant III (EGFRvIII). EGFRvIII is a functional and permanently activated mutation of EGFR, found in only a minority of the cancer cells. While ligand independent, the addition of EGF ligand results in increased phosphorylation of EGFRvIII in cells expressing both EGFR and EGFRvIII, suggesting EGFRvIII is a substrate of EGFR.^[11] Alternatively to in vitro approaches, patient-derived xenograft (PDX) models have been used to maintain EGFR amplification present in the original tumor^[12] but culture and analysis are laborious and time-consuming. Further, mechanistic studies of therapeutic resistance are largely intractable in vivo due to the complex tumor microenvironment. Increasingly, biomimetic biomaterial models of the tumor microenvironment are increasingly being exploited to examine mechanisms of GBM expansion and invasion.^[13] Hydrogel-based

models offer the flexibility to systematically explore individual attributes of the tumor microenvironment, as well as synergies between signals, on tumor cell activation and therapeutic response.^[14] Hyaluronan containing hydrogels have increasingly been used to assess migration capacity^[15] and mechanisms,^[16] finding that GBM cell invasion modes depend on the hyaluronic acid (HA) present in the matrix, directly associated to HA synthase. Additionally, the concomitant presence of elastin in the GBM microenvironment have been associated with increased ECM degradation and invasion potential.^[17] Moreover, sensitivity of GBM patient-derived xenograft cells within HA based hydrogels to radiation and chemotherapy have been tested previously,^[18] finding similar differences in therapeutic response, among the various tumor samples, between in vivo and ex vivo systems. Therefore HA-based hydrogels have great potential for exploring the mechanisms of action of anticancer drugs in glioblastoma, an area that has been underexplored.

The glycosaminoglycan HA is a principal component of the extracellular matrix (ECM) within the brain. Recently it has been implicated as a potential regulator of tumor growth and resistance to chemotherapy.^[19] It has been reported that GBM tumors contain larger amounts of HA than the surrounding brain matrix, and may contribute to poorer prognosis.^[20] Cells interact with HA content in the ECM via the cell surface receptor CD44. And while normal brain tissue show no or weak CD44 expression, a higher percentage and intensity of CD44-positive tumor cells have been observed in high-grade astrocytomas such as GBM compared to low-grade astrocytomas.^[21] Further, CD44 is a costimulator of ErbB and c-Met RTK signaling pathways,^[22] while CD44 knockdown can inhibit GBM proliferation in vivo.^[23] Interestingly, CD44 constitutively colocalizes and coimmunoprecipitates with EGFR in GBM, suggesting extracellular HA may mediate the formation of a complex including CD44 and EGFR, leading to therapeutic resistance.^[24] Previous studies have shown that when EGFR is overexpressed it becomes tyrosine phosphorylated and constitutively activated.^[25] However, phosphorylation of Akt and ERK only happens with EGF present. Further, EGFR oscillates between alternative programs of signaling depending on the availability of the ligand, while the AKT-PI3K pathway is activated independently of EGFR-receptor activation in cells with suppressed or low wtPTEN.^[11] PI3K signaling can also be activated by the loss of the tumor suppressor phosphatase and tensin homolog (PTEN),^[26] and is associated with increased cell survival and tumor dissemination in GBM.^[27] Erlotinib is FDA approved highly specific reversible EGFR kinase inhibitor, and is known to be more effective in PTEN-deficient glioblastoma tumors where EGFR signaling is activated.^[28] The use of tyrosine kinase inhibitors (TKIs) such as erlotinib have the potential to inhibit PI3K signaling in GBM, and therefore reduce tumor invasion and growth.^[29] Potential explanations for the reduced efficacy of TKIs as single therapy agents in GBM include that PI3K signaling may be uncoupled from upstream control by inactivation of PTEN or that interactions between EGFR and HA-mediated signaling paths may lead to unexpected biological outcomes that are difficult to monitor in vivo. As a result, biomaterial models of the tumor microenvironment provide a platform to explore the functional importance of microenvironmental ligands such as HA (Figure 1) in the intrinsic resistance of GBM cells to TKI, such as erlotinib. These engineered hydrogel matrices offer unique capabilities to help elucidate why this very promising candidate drug does not perform in clinical assays.

The foundation of our effort is our recent development of a suite of engineered glioma biomaterials based on methacrylamide-functionalized gelatin (GelMA) hydrogels. We have demonstrated microfluidic forming tools to generate combinatorial platforms including biophysical and biomolecular properties found in the GBM tumor microenvironment.^[14] Using this model, we identified ranges of biophysical signals that alter expression profiles of invasion associated genes in U87 and U251 GBM cell lines.^[30] We also showed matrix-bound HA promotes an invasive phenotype and EGFR-dependent signaling to regulate proliferation and gene expression in U87 cell lines in a nonlinear manner.^[13a] Our ability to recover cell lysates from defined regions of these 3D cultures allows for downstream proteomic analysis by immunoblotting. In this manuscript, we describe a biomaterial approach to monitor the response of three patient-derived xenograft cell populations with disparate EGFR and PTEN status: GBM10 (EGFR⁻/PTEN⁻; poorly invasive in vivo), GBM12 (EGFR⁺/PTEN⁺), and the constitutively activated vIII mutant GBM6 (EGFR^{vIII}/PTEN⁺; highly invasive in vivo). We examine PDX cell response to erlotinib within the GelMA hydrogel platform as a function matrix-immobilized HA as well as in the presence and absence of inhibition of HA-CD44 interactions. In so doing we begin to address the question of how the tumor matrix microenvironment provides a biophysical context that may alter how cohorts of GBM cells respond to molecular inhibitors such as erlotinib.

2. Results

2.1. HA-Decorated GelMA Hydrogels Alter Xenograft Bioactivity in an HA-Dose Dependent Manner

We first examined GBM proliferation, metabolic activity, and the expression of a series of matrix remodeling (MMP-2, MMP-9, Fibronectin) and proangiogenic (HIF-1 and VEGF) genes as a function of matrix-immobilized HA-content (0–2 wt%) within the GelMA hydrogel. This range of matrix-immobilized HA (0–2 wt%) was chosen from one previously shown to alter activity of U87MG GBM cell lines,^[31] and where HA wt% could be altered without influencing the elastic moduli of the GelMA hydrogels (14 kPa: ranging from 13 ± 3 to 14.7 ± 1.8 kPa, unpublished data). Interestingly, gene expression levels were altered significantly by the combination of HA-matrix content and PDX EGFR status. Poorly invasive EGFR GBM10 showed heightened expression profiles of both matrix remodeling and proangiogenic genes at low (0.3–0.5 wt%) HA content hydrogels. EGFR⁺ GBM12 showed heightened expression profiles at higher (1–2 wt%) HA content. Further, constitutively active EGFR^{vIII} GBM6 showed smaller variations in gene expression profiles as a function of matrix-immobilized HA (Figure 2a). We subsequently examined the metabolic activity, proliferation rate, and PI3K expression for a subset of HA-containing hydrogels that displayed the widest disparity in gene expression results (0, 0.5, 1.0 wt%). Here, the metabolic activity of GBM12 (EGFR⁺) and GBM6 (EGFR^{vIII}) increased significantly with matrix-immobilized HA content (Figure 2b). Matrix-immobilized HA did not influence the proliferative expansion of EGFR^{vIII} GBM6 and inhibited proliferation of EGFR⁺ GBM12 (Figure 2c), but the dramatic increase in the metabolic activity of these PDX variants with matrix-bound HA indicates matrix-bound HA may activate other pathways. Consistent with proliferation results, PI3K signaling was largely unaffected by matrix-bound HA for EGFR GBM10 and EGFR^{vIII} GBM6 variants. However, EGFR⁺

GBM12 showed significantly upregulated PI3K activity in HA-free GelMA hydrogels that was lost with the addition of matrix-bound HA (Figure 2d).

2.2. Xenograft Mediated Biosynthesis of HA is Affected by EGFR Expression and the Presence of Matrix-Bound HA

Immunohistochemical analyses of tissue sections taken from GBM10, GBM12, and GBM6 intracranial xenograft tumors show the presence of significant intracellular HA content (Figure S4, Supporting Information). As a result, and due to the influence of matrix-bound HA on PDX cell activity in GelMA hydrogels, we subsequently examined the capacity for GBM PDX cells to secrete HA in culture. In conventional 2D culture, GBM PDX lines demonstrate the capacity to secrete soluble HA (Figure S3a, Supporting Information). We subsequently examined the degree by which the presence of matrix-bound HA alters PDX HA biosynthesis. HA-biosynthesis largely increased with culture time (up to day 10), with EGFR GBM10 and EGFR^{vIII} GBM6 variants secreting significantly greater levels of soluble HA production in GelMA hydrogels than EGFR⁺ GBM12. Further, the presence of matrix-bound HA significantly reduced HA secretion in GBM10 and GBM6 (Figure S3b,d, Supporting Information), while EGFR⁺ GBM12 showed no significant change in HA biosynthesis in the presence of matrix-bound HA (Figure S3c, Supporting Information).

2.3. Xenografts Demonstrate a Dose–Response to Erlotinib Within GelMA Hydrogels

We subsequently examined the influence of erlotinib (2×10^{-6} , 10×10^{-6} , 100×10^{-6} M) on GBM variants within HA-decorated GelMA hydrogels, with results normalized against dimethyl sulfoxide (DMSO) only culture. Live dead (L/D) images (Figure 3a) do not show qualitative evidence of a direct cytotoxic effect. Rather, erlotinib treated cells show less cell aggregation, most dramatically for GBM6. Cell aggregation was also reduced in HA-decorated GelMA hydrogels compared to GelMA hydrogels (Figure S1, Supporting Information).

Changes in metabolic activity of cell cultures, as determined by MTT assay, suggest EGFR and matrix-bound HA dependent responses of xenograft cells to erlotinib. GBM10 (EGFR) xenografts were relatively erlotinib insensitive, showing minimal response to erlotinib in both GelMA and GelMA-HA (1.0 wt%) hydrogels (Figure 3b,c). EGFR⁺ GBM 12 cells showed dose-dependent decreases in metabolic activity to erlotinib in GelMA or HA-modified GelMA hydrogels (Figure 3b,c). However, while EGFR^{vIII} GBM6 cells show decreased, but still erlotinib dose-sensitive responses in GelMA hydrogels, they show significantly enhanced metabolic activity in HA-decorated GelMA hydrogels even in the presence erlotinib dosages up to 10 μ M. Together, these data show that, while EGFR⁺ cells (GBM12) are initially sensitive to TKI in HA-decorated GelMA hydrogels, the presence of matrix-bound HA alters the response of EGFR^{vIII} PDX specimen signaling to significantly enhance cell activity (GBM6). Given the differential phenotypic response exhibited between GBM10, 12, and 6, subsequent experiments employed 10 μ M erlotinib to examine potential interactions in the CD44 and EGFR pathways.

2.4. Matrix-Immobilized HA Alters CD44-EGFR Interactions

We hypothesize that hyaluronan binding to CD44 promotes its dimerization with EGFR, leading to EGFR phosphorylation, and resulting in cancer progression. We subsequently examined the combined effect of anti-CD44 ($3 \mu\text{g mL}^{-1}$ in media, EMD Mil-lipore clone A020) with the TKI erlotinib ($10 \mu\text{M}$) on xenograft cell response in GelMA versus GelMA-HA (1.0 wt%) hydrogels (Figure 4). The metabolic activity of GBM10 was not affected by matrix-bound HA in response to erlotinib; while blocking CD44 did not affect metabolic activity in GelMA only hydrogels, metabolic activity decreased significantly when blocking CD44 activity in the presence of matrix-immobilized HA. Further, inclusion of anti-CD44 leads to significantly poorer metabolic activity of EGFR⁺ cells in GelMA-HA matrices, suggesting a protective effect of the HA-environment to erlotinib is mediated via CD44 activation. Comparatively, cell metabolic activity significantly increased in the presence of matrix-bound HA for EGFR^{vIII} (GBM6) xenografts, and remains elevated (not significantly due to an increase in metabolic activity of cells in GelMA-only gels) in the presence of CD44 inhibition. Confirming results seen across a range of erlotinib doses, the effect of erlotinib on the metabolic activity of EGFR^{vIII} GBM6 cells was significantly reduced in the presence of matrix bound-HA. However, this effect is not observed when an equivalent amount of soluble HA is added within the GelMA matrix (Figure S2, Supporting Information). Similar discrepancies of erlotinib effect between soluble and HA-bound environments are observed for the rest of GBM xenografts. This suggests that matrix-bound HA provides an extrinsic signal that may alter GBM response to EGFR molecular subtype therapies.

2.5. Matrix-Immobilized HA Impacts PI3K Activity in Response to Erlotinib

We subsequently examined whether erlotinib treatment alters EGFR associated pathways differently as a result of the presence of matrix-bound HA, quantifying the effect of erlotinib and CD44 inhibitory treatment on total/phosphorylated PI3K expression for the series of GBM xenografts. Here, we focus on identifying combinations of therapeutic interventions that lead to a reduction of p/t-PI3K signal relative to the control condition for each PDX variant (culture in the absence of erlotinib and anti-CD44).

PI3K phosphorylation results are largely consistent with the metabolic activity observed in GBM10 and GBM6 after erlotinib and anti-CD44 treatments in GelMA and GelMA-HA hydrogels (Figure 5). Erlotinib treatment significantly decreases PI3K phosphorylation in PTEN^{wt} EGFR^{vIII} PDX cells (GBM6) in the absence of HA, but treatment with anti-CD44 leads to enhanced p/t-PI3K expression in GelMA hydrogels. The combinatorial treatment of erlotinib and anti-CD44 only leads to significant reduction in p/t-PI3K in PTEN⁻ GBM10 within HA-functionalized GelMA, while dual erlotinib/anti-CD44 treatment activates PI3K phosphorylation in EGFR⁺ PTEN^{wt} GBM12.

2.6. Matrix-Bound HA Alters Secretion of MMP-2 and Soluble HA in a Manner Influenced by EGFR^{vIII} Expression

Based on findings suggesting matrix-bound HA may alter the response of three GBM PDX specimen variants to the EGFR inhibitor erlotinib, we subsequently explored activation of matrix remodeling and HA synthetic processes as a function of matrix HA content (Figure

6a). HA secretion within GelMA hydrogels was relatively similar for all groups in the presence of erlotinib (3 d, 10×10^{-6} M). While exposure to erlotinib in HA-decorated GelMA hydrogels did not alter the secretion of HA in GBM10 and GBM12, HA secretion was significantly enhanced in EGFR^{VI} GBM6 in response to erlotinib in the presence of matrix-bound HA (Figure 6a). Interestingly, MMP-2 secretion was also enhanced in all xenograft lines (GBM10, 12, 6) after exposure to 10×10^{-6} M erlotinib for 3 d. While highest for EGFR⁺ GBM12, MMP-2 secretion was enhanced in the presence of matrix-bound HA for all xenograft variants (Figure 6b).

3. Discussion

Glioblastoma tumors are often marked by the amplification, overexpression, and mutation of EGFR, with a constitutively active EGFR^{VI} mutant associated with poorest clinical outcomes.^[11,32] However, current TKIs such as erlotinib fail in the clinic. The failure to predict efficacy in such overexpressed targets as EGFR suggests compensatory signaling capable of bypassing these pathways. Such compensatory signaling mechanisms, despite the obvious significance, are poorly understood in GBM. Previous work has focused largely on intracellular processes and alterations of expressed proteins in response to kinase inhibitors.^[33] However, these may account for a portion of the total mechanisms associated with RTK-mediated resistance. Our project focuses on the use of tissue engineering tools capable of exploring compensatory pathways mediated by the extracellular environment. Here, we explicitly consider the potential for the CD44-HA complex, a relevant compensatory pathway associated with chemoresistance in other cancers,^[34] to alter GBM cell response to a model TKI erlotinib.

We employ a GelMA-based hydrogel to explore this question, leveraging our previously documented ability to modify hydrogel composition (e.g., HA content) and biophysical properties (e.g., stiffness, swelling)^[13a,14,30b] to isolate cell-matrix interactions in response to erlotinib treatment. Together our results suggest that the activation of EGFR-CD44 complex in response to erlotinib is dependent on the local matrix environment, and further, such interactions may initiate a feedback loop capable of modulating GBM resistance to erlotinib. While hydrogel elastic properties were adjusted to remain consistent across the tested range of matrix-bound HA, increasing HA content is balanced by a reduction in gelatin concentration, and likely slightly reduces the number of available integrin binding domains in the cell microenvironment, potentially influencing tumor cell behavior. While small given the amount of HA added to the system, these findings highlight opportunities to further optimize hydrogel chemistries to orthogonally tune matrix biophysical properties and inclusion of biomolecular stimuli.^[35] Embracing the complexity and heterogeneity of patient tumors, we examined the behavior of three patient-derived xenograft specimens that maintain patient morphologic and molecular characteristics^[36] within these HA-functionalized gelatin microenvironments.

Our finding of a lack of cell death in response to erlotinib exposure in GelMA hydrogels is consistent with previous findings that sensitivity to EGFR inhibition in cellular growth assays is correlated with arrest in G1 phase (Figure 3).^[37] In addition, our finding that GBM cell aggregations are reduced with erlotinib exposure, especially in GBM6 specimens, is also

consistent with previous results suggesting that EGFR inhibition leads to actin cytoskeleton reorganization, preventing multicellular tumor spheroid formation (Figure S1, Supporting Information).^[38] Importantly, these results suggest the erlotinib dosage tested in these experiments is not cytotoxic, but are of sufficient dosage to explore GBM cell response to combinations of matrix biophysical environments and erlotinib. Further, our observation that the metabolic activity of EGFR^{vIII} cells (GBM6) is increased in HA-containing matrices with erlotinib exposure supports the conception that tumor ECM may provide a protective environment against EGFR inhibitors in select tumor subtypes. This finding is further enhanced by our hypothesis that activation of the EGFR-CD44 complex in response to erlotinib is dependent on local matrix environment, specifically the presence of matrix-immobilized HA, and further may initiate a feedback loop capable of modulating GBM resistance to erlotinib. This EGFR-CD44 activation may explain the increased phosphorylation of PI3K downstream. The amplified effect of erlotinib in the presence of matrix-bound HA and CD44 blocking on GBM12 cells metabolic activity (Figure 4), suggests an interaction between EGFR and hyaluronan main receptor CD44. The unaltered metabolic activity for EGFR^{vIII} GBM6 cells with CD44 blocking suggests that there may be other pathways activated in EGFR^{vIII} within HA matrices besides Akt/ERK. Since HA concentration varies depending on the tumor type and location (Figure S4, Supporting Information).

Around 40% of GBM samples display mutations or homozygous deletions of PTEN, a tumor suppressor protein that can alter PI3K signaling.^[3] Since EGFR⁺, PTEN^{wt} cells seem to be the most responsive to these inhibitors, some effects of EGFR inhibition may be found in downstream molecules of PI3K network (e.g., Akt), where PTEN plays a more important role. However, data report that inhibition of Akt is not as efficient as inhibition of PI3K.^[28,29] Immunoblotting assays in our PDX laden constructs show the important influence of matrix-bound hyaluronan in defining the effect of erlotinib exposure. Quantification of PI3K signaling via densitometry (Figure 5) suggests EGFR GBM10 (PTEN⁻) are more susceptible to EGFR inhibition if CD44 is also blocked in HA-containing matrices. Here, CD44 blockade is more effective than erlotinib and both erlotinib and anti-CD44 show a further decrease in p-PI3K concentration. However, CD44 blockade is ineffective on PTEN^{wt} tumors GBM 12 and 6, and can rather promote PI3K phosphorylation. The increase in HA secretion by GBM6 stimulated by erlotinib in HA-matrices may lead to the observed lack of inhibition of PI3K pathway in these matrices. Further, our findings that EGFR^{vIII}, PTEN^{wt} GBM 6 responds to erlotinib through enhanced soluble HA production while GBM10, 12, and 6 show enhanced MMP-2 production in response to erlotinib (Figure 6), factors known to increase cell malignancy and to remodel the matrix environment populated by the cells. These findings are consistent with the production of MMP2 in response to HA-CD44^[39] and EGFR-CD44^[24] interactions, where low molecular-weight HA saccharides are able to stimulate cell proliferation, CD44 cleavage, and tumor cell invasion. Moreover, the promotion of CD44 cleavage has also been reported in several solid tumors, including glioma.^[40]

In summary, our results indicate that a combination of both matrix-bound and soluble HA influence GBM sensitivity and resistance to EGFR tyrosine kinase inhibitors. The use of these 3D in vitro systems helps us understand that the reason why potentially susceptible

tumors to erlotinib (EGFR^{vIII}, PTEN^{wt} GBM 6) have failed in the clinic may be due to the presence of extracellular HA, that prevents inhibition of PI3K pathway (Figures 3–5). Interestingly, the inhibition of a membrane receptor compels glioma cells to activate other kinase receptors depending on the matrix composition: e.g., PI3K phosphorylation rate increases in GBM10 cells when using erlotinib in gelatin matrices and in GBM12 when using anti-CD44 in HA-containing matrices. These findings suggest it is of critical importance to define the spatiotemporal heterogeneity of PDX tumor microenvironment as a function of molecular signature and tumor progression, and then employ biomimetic tissue engineering tools to define the regulatory nature of the extracellular matrix on GBM cell activity and therapeutic resistance as a complementary assay to predict efficacy of EGFR inhibitors in GBM.

4. Conclusions

We demonstrate here that PDX GBM tumors can be cultured and maintained in vitro within GelMA and hyaluronan-modified GelMA hydrogels. The resulting GBM cell laden hydrogels allow us to investigate the method of action of a particular TKI, erlotinib, across three patient-derived xenograft specimens with disparate EGFR status. Our results suggest an important effect of matrix-immobilized HA signaling in erlotinib efficacy and highlights the importance of this extracellular component in molecular subtype therapy design. This system opens up the possibility of culturing tumor tissue directly from the patient biopsies in order to achieve fast drug screening of available treatment options. Our results suggest matrix-bound HA (alone or in combination with soluble HA) may be a key factor in the negative response of vIII tumors to EGFR inhibitors such as erlotinib. Further, traditionally non-responsive GBM tumors to EGFR inhibitors (PTEN⁻, GBM10) may become susceptible in combination with anti-CD44 therapies or other types of HA-receptor blockades. Taken together, while it remains difficult to predict erlotinib activity of EGFR inhibitors in clinical trials, the benefits of EGFR inhibition in GBM may be found in combination to other inhibitors of ECM signaling, and that biomimetic culture models provide a platform to explore the intersection of molecular subtype inhibitors with the matrix-associated signaling pathways to reveal novel therapeutic strategies.

5. Experimental Section

Hydrogel Fabrication and Cell Encapsulation

GelMA and hyaluronic acid methacrylate (HAMA) were prepared as described previously.^[13a] GelMA scaffolds were produced with concentrations of 0, 0.3, 0.5, 0.7, 1 and 2 wt% HAMA in phosphate-buffered saline (PBS, Invitrogen) by UV photopolymerization in the presence of 0.1 wt% Irg2959 as a photoinitiator. Prepolymer solution was photopolymerized (10 mW cm⁻², 365 nm) between glass slides (1 mm thick). After polymerization, 5 mm disks were generated using a Miltex biopsy punch. Patient-derived GBM cells (Mayo Clinic) were cultured within these hydrogels at a concentration of 4 million cells mL⁻¹. Different mice per xenograft were used in the data collection and used at passage one. Dispersed GBM tumor tissue was received from Mayo Clinic. Upon arrival,

cells were dissociated enzymatically (Gibco TrypLE, ThermoFisher Scientific), counted, and mixed with the prepolymer solution, followed by UV curing to form the cell laden hydrogel.

Patient Tissues and Xenograft Cells

The xenografts used in this study were established with tumor tissue from patients undergoing surgical treatment at the Mayo Clinic, Rochester, Minnesota. These studies were approved by the respective Institutional Review Boards and only samples from patients who had provided prior consent for use of their tissues in research were included. All xenograft cells used in this study were obtained using an orthotopic tumor model for glioblastoma on a protocol^[41] approved by the Mayo Institutional Animal Care and Use Committee. The molecular characterization of PDX was performed by Mayo Clinic as previously described.^[36b,c,42] Dispersed xenograft tumor tissue was provided by Mayo Clinic for hydrogel culture. The invasive potential of GBM xenograft cells (GBM6 highly invasive and GBM10/GBM12 poorly invasive) was provided by Mayo Clinic. A qualitative invasion rate was provided by the surgeon after observation of the GBM tumor during resection of the original mass. The invasion rate varied from 0 to 7, where GBM10, GBM12, and GBM6 were 0, 2 and 6, respectively.

Cell Culture

PDX lines GBM10 (EGFR⁻/PTEN⁻; poorly invasive in vivo), GBM12 (EGFR⁺/PTEN⁺), and the constitutively activated vIII mutant GBM6 (EGFR^{vIII}/PTEN⁺; highly invasive in vivo) were maintained at Mayo Clinic at 37 °C in a 5% CO₂ environment in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U mL⁻¹ and 100 µg mL⁻¹), shipped in supplemented DMEM media, and were used upon receipt. For hydrogel cultures, patient-derived xenograft cells encapsulated in the GelMA hydrogel were incubated on an orbital shaker in low adhesion well plates containing standard culture media for 48 h. Subsequently, erlotinib (2, 10, or 100 µM in 0.2 × 10⁻³ M DMSO) was added to the culture media, with analysis of cell populations performed after 3 d in culture versus a DMSO only control.

Analysis of Cell Viability and Proliferation

Cell proliferation was analyzed as change in total number of cells within the hydrogel via DNA quantification (Pico Green, ThermoFisher Scientific) as previously described.^[14] Results were normalized against the initial number of encapsulated cells (at day 0). Metabolic activity analyzed viability, assessed by an MTT assay (Vybrant, ThermoFisher Scientific) as previously described.^[43] Briefly, growth medium was replaced by a 12 × 10⁻³ M 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt solution in DMEM. MTT is reduced by viable cells to a colored, water-insoluble formazan. The precipitated salt was dissolved in DMSO followed by measure of absorbance at 540 nm, with results normalized against the recorded MTT assay for cells initially encapsulated in the hydrogel (at day 0). Cell viability was further evaluated by confocal imaging as described previously (Zeiss LSM710).^[13a] Prior to imaging, gels were incubated in PBS containing 4 × 10⁻³ M calcein AM ($\lambda_{em} \approx 515$ nm, Invitrogen) to stain viable cells and 1.5 × 10⁻³ M propidium iodide ($\lambda_{em} \approx 617$ nm, Invitrogen) to stain dead cells.

Extracellular Matrix Secretion and Gene Expression Analysis^[13a]

Media samples were collected during culture, pooled, then stored at -80°C until analysis. Production of HA and MMP-2 was determined via ELISA assay following the manufacture's protocol (R&D Systems, Minneapolis, MN). Protein synthesis was calculated relative to cell number at each time point. Alternatively, RNA was extracted from cell-laden hydrogels at day 7 via an RNeasy Plant Mini kit (Qiagen, Valencia, CA).^[44] RNA isolated from the lysates and reverse transcribed to cDNA as previously described. Primers sequences are listed in Table S1 of the Supporting Information (Integrated DNA Technologies, Coralville, IA). Expression levels of HIF-1, VEGF, MMP-2, MMP-9, and fibronectin (Fn) were examined relative to the housekeeping gene GAPDH. RT-PCR data was analyzed using the delta-delta Ct ($\Delta\Delta\text{Ct}$) method,^[45] with all results presented as a relative fold induction, calculated as $2^{-\Delta\Delta\text{Ct}}$, normalized to expression levels of cells in GelMA hydrogels (in the absence of HAMA) as the control.

Protein Immunoblotting

At each experimental time point, cells were lysed using RIPA buffer on ice for 30 min. After lysis and determination of protein concentration, samples were separated on a 10% polyacrylamide precast electrophoresis gel (Biorad # 456-1096) and transferred onto a nitrocellulose membrane (Amersham Protan, GE Healthcare, #10600012). Proteins were visualized by ECL western blotting detection reagents (Pierce, ThermoFisher Scientific), according to manufacturer's instruction, and following antibodies were used (all from Cell Signaling): rabbit anti-PI3 Kinase p85, rabbit anti-phospho-PI3 Kinase Class III (Ser249), and rabbit anti- β -actin; followed by goat-anti-rabbit IgG conjugated to horseradish peroxidase.

Tissue Samples Hematoxylin and Eosin (H&E) and Hyaluronan Staining

Intracranial tumor specimens were created from the PDX lines as part of a separate study. Brains from mice with intracranial tumors were removed, fixed in 10% neutral buffered formalin, breadloafed, and then embedded in paraffin. Five micrometer sections were used for H&E staining that was performed according to a standard protocol. Hyaluronan staining was performed following a previously described protocol.^[46] Briefly, sections were deparaffinized with Histoclear (Electron Microscopy Sciences) and rehydrated with ethanol/1× PBS (100%, 70%, 50%, 25%, and 3 × 0%), and hyaluronan was detected by incubating sections with $5\text{ }\mu\text{g mL}^{-1}$ bHABP (Seikagaku, Tokyo, Japan) overnight at room temperature. Sections were mounted in ProLong Gold (ThermoFisher).

Statistical Analysis

All analyses were performed using a one-way analysis of variance followed by Tukey's HSD post hoc test. Significance level was set at $p < 0.05$. At least $n = 3$ samples were examined for cell viability assays, at least $n = 5$ samples for biosynthesis assays, and at least $n = 3$ samples were examined for PCR analysis. Error was reported in figures as the standard deviation unless otherwise noted.

Supplementary Material

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Figure 1.

Amplification, overexpression, and mutation of the wild-type (wt) EGFR tyrosine kinase has been identified in 50% of GBM patients. Often, the genetic aberration of wtEGFR (EGFR+) comes with the expression of the mutant EGFR variant III (EGFRvIII) which is expressed in 30% of GBM tumors. Overexpression of EGFR leads to the activation of an additional constitutive pathway. GBM is also associated with aberrant HA secretion, tissue stiffening, and overexpression of the HA receptor CD44. CD44 constitutively colocalizes and coimmunoprecipitates with EGFR in GBM. HA mediates the formation of a complex including CD44 and EGFR.

Figure 2.

a) Gene expression profiles of GBM xenograft cells in 3D culture of gelatin with increasing concentrations of HA. b) Metabolic activity measured by MTT, c) cell number, and d) PI3K expression of encapsulated xenograft cells in GelMA gels with 0 versus 1 wt% HA. RT-PCR values are relative to GelMA-only gels and normalized to GAPDH. Total gelatin/HA concentration is 7 wt% and results are shown at day 7. Dash line indicates control. $*p < 0.05$ as compared to 0 wt% HA hydrogels.

Figure 3.

a) Live/dead (green/red) images of encapsulated tumor cells after addition of 2 and 10 μ M erlotinib to media. Images on the right column show cells in HA/gelatin hydrogels versus gelatin-only gels. Metabolic activity of tumor cells exposed to different concentrations of erlotinib within gelatin b) and Gelatin + HAMA c) hydrogels. Cells were exposed to erlotinib for 3 d, with data normalized to cultures in DMSO alone (dashed line). Scale bar is 200 μ m. * $p < 0.05$ as compared to GBM10.

Figure 4.

Metabolic activity, measured by MTT, of GBM tumor cells within gelatin and gelatin/hyaluronan hydrogels. The effect of erlotinib is influenced by the addition of the anti-CD44 antibody, especially in HA-containing matrices. Concentration of erlotinib is 10 μ M and anti-CD44 is 3 μ g mL⁻¹. * $p < 0.05$, between erlotinib and erlotinib + anti-CD44 groups.

Figure 5. Western blot analysis of the effect of erlotinib and anti-CD44 treatments on the inhibition of PI3K phosphorylation in encapsulated PDX cells from GBM10, GBM12, and GBM6. β -actin protein was used as the internal control. Quantification of protein intensity is represented on the left for cells within GelMA and GelMA + HAMA matrices. Concentration of erlotinib is 10 μM and anti-CD44 is 3 $\mu\text{g mL}^{-1}$. DMSO is used as control. * $p < 0.05$, as compared to control (—/— erlotinib/anti-CD44 for each PDX specimen).

Figure 6.

a) HA secretion in media after a dose of erlotinib (day 3). HA from gelatin-only gels is compared to gelatin/1 wt% HA gels. b) Corresponding MMP-2 secretion amounts after a dose of erlotinib. HA secretion is compared between gelatin gel containing 0 and 1 wt% HA. Data are normalized to control in DMSO and relative to cell number. Erlotinib concentration is 10 μ M. * $p < 0.05$, between gelatin and gelatin + hyaluronan hydrogels.