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Laminin alpha 2 enables glioblastoma stem cell growth

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Abstract

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Objective—Glioblastomas (GBMs) are lethal cancers that display cellular hierarchies parallel to normal brain. At the apex are GBM stem cells (GSCs), which are relatively resistant to conventional therapy. An important driver of malignancy and self-renewal in GSCs are interactions with the adjacent perivascular niche. Extracellular matrix (ECM) cues instruct neural stem/progenitor cell-niche interactions and the objective of our study was to elucidate its composition and contribution to GSC maintenance in the perivascular niche.

Methods—We interrogated human tumor tissue for immunofluorescence analysis and derived GSC from tumor tissues for functional studies. Bioinformatics analyses were conducted by mining publicly available databases.

Results—We find that laminin ECM proteins are localized to the perivascular GBM niche and inform negative patient prognosis. To identify the source of laminins, we characterized cellular elements within the niche and found that laminin α chains were expressed by non-stem tumor cells and tumor associated endothelial cells (ECs). RNA interference targeting laminin $\alpha 2$ inhibited GSC growth and self-renewal. In co-culture studies of GSCs and ECs, laminin $\alpha 2$ knockdown in ECs resulted in decreased tumor growth.

Interpretation—Our studies highlight the contribution of non-stem tumor cell-derived laminin juxtacrine signaling. As laminin $\alpha 2$ has recently been identified as a molecular marker of aggressive ependymoma, we propose that the brain vascular ECM promotes tumor malignancy through maintenance of the GSC compartment providing not only a molecular fingerprint but also a possible therapeutic target.

Introduction

Among primary intrinsic brain tumors, GBMs [World Health Organization Grade IV] are the most prevalent and most lethal. Despite aggressive therapies including surgical resection, radiation, and chemotherapy, GBM patients have a median survival of 15 months [1]. GBMs are angiogenic, refractory to many therapies, and characterized by a high degree of cellular heterogeneity and a propensity to invade surrounding tissue [2]. Recent work has demonstrated that GBM has a defined hierarchy with a self-renewing cancer stem cell (CSC) at the apex [3]. Although the CSC hypothesis remains controversial due to unresolved issues in identifying the CSC and the cell-of-origin, numerous studies have shown that GBM contain cells functionally validated as CSCs through secondary transplantation assays and are key contributors to therapeutic resistance, angiogenesis, and invasion into surrounding tissues [4–11]. It is notable that many of these characteristics are shared with neural stem cells. Hence, a better understanding of the biology of GSCs may impact the development of more effective GBM therapies with possible extension to many other nervous system diseases in which neural stem and progenitor cells may be involved.

Within a tumor, the stem cell-like tumor cells are preferentially located in defined anatomical locations and are regulated by extrinsic contributions of their microenvironment or niche [12]. For brain tumors, the most well defined niche is adjacent to blood vessels, known as the perivascular niche [13], an area enriched in GSCs and distinct from the blood brain barrier and neurovascular unit. A variety of cell types, including GSCs, non-stem tumor cells, and vascular components (ECs, pericytes) are present in this niche. Within this microenvironment, GSC maintenance is facilitated through a variety of mechanisms including growth factor signaling, cell-to-cell communication, and cell-to-ECM interactions [14]. Recent work from our research group and others has elucidated the contributions of growth factors (including vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF- β)) and cell-to-cell communication (e.g. Notch signaling) to niche maintenance [9, 15, 16]. Because of its importance, substantial efforts have been made to target the perivascular niche with the anti-VEGF antibody, bevacizumab [17]. However,

clinical efficacy of bevacizumab has been mixed, underscoring the complexity of niche targeting and demonstrating the need to better define the biology of the microenvironment [18].

The contributions of ECM interactions to GSC maintenance still remain poorly understood. It has recently become appreciated that the ECM is more than a structural component and, for stem cells in particular, is pivotal in instructing cell fate choices and concentrating growth factors to defined niches. In the developing and adult brains, neural progenitor cells (NPCs) rely on ECM components for growth and survival [19–21]. Many of these interactions are guided by the laminin family of proteins, each consisting of an α , β , and γ subunit, and their receptors that include specific integrins, dystroglycan, and syndecans. While there are 15 known isoforms, the key distinguishing features between laminins are their structure, binding partners, and tissue specific expression. The α chain, in particular, mediates most of the receptor binding and hence is responsible for generating different cellular responses [22]. The use of laminin α chain mutant mice to examine nervous system disorders highlights the diversity of phenotypes and underscores the specific role of each laminin isoform [23]. Further highlighting the differences between laminin isoforms is the specific expression in NPC niches. These niches are enriched in several members of the laminin family (including $\alpha 2$, $\alpha 4$, and $\alpha 5$ chain) and the laminin-specific integrin (namely $\alpha 6$ and $\beta 1$) are highly expressed on NPCs [24–27]. Mice with targeted disruption of either integrins $\alpha 6$ or $\beta 1$ or laminin $\alpha 2$ have profound NPC defects underscoring their pivotal role in neural development [24, 27–29]. Building on this developmental relationship, we previously evaluated integrin expression on GSCs and found elevated levels of integrin $\alpha 6$ [30]. Importantly, integrin $\alpha 6$ enriches for GSCs and targeting integrin $\alpha 6$ attenuates self-renewal and tumor formation. The laminin $\alpha 5$ and laminin-411 (composed of $\alpha 4$, $\beta 1$, and $\gamma 1$ chains) expression informs glioma grade and poor patient survival [31, 32]. The importance of this relationship is further highlighted by adherent GSC culturing methods that rely on laminin [33, 34].

Understanding the repertoire of signals present in the perivascular niche which orchestrate GSC maintenance represents an immediate priority as it has direct relevance to therapeutic approaches for GBM and will provide insights into GSC biology. Here we have utilized human patient specimens to evaluate the laminins present on GSCs within the perivascular niche and to show that laminin $\alpha 2$, which is provided by non-GSCs and ECs, is critical for GSC maintenance. Our results also demonstrate that ECM components protect GSCs from radiation-induced damage, which highlights the contribution of the niche to therapeutic resistance, and must be considered in the design of next generation GBM therapies.

Methods

Genomic data and processing

Custom Agilent 244,000 feature Gene Expression Microarray and Affymetrix-HT-HG-U133A GeneChip gene expression data for 354 and 246 glioblastomas, respectively, and associated clinical data were obtained from the Open-Access and Controlled-Access Data Tiers Portal (<http://tcga-data.nci.nih.gov/tcga/findArchives.htm>) of The Cancer Genome Atlas (TCGA) Pilot Project (<http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp>) upon National Human Genome Research Institute (NHGRI) approval. Gene expression data were background corrected and normalized through the Robust Multigene Average (RMA) algorithm [35] as described by TCGA [36]. Furthermore, a unified and integrated gene expression data set created by combining individual platform (Affymetrix-HT-HG-U133A GeneChip, Affymetrix HuEx GeneChip, and Custom Agilent 244,000 feature Gene Expression Microarray) gene level expression measurements for 202 TCGA glioblastoma samples and associated molecular subtype [37] and clinical data were retrieved from <http://>

tcga-data.nci.nih.gov/docs/publications/gbm_exp/. The assumption of proportional hazards was tested using interactions of the predictor variable (laminin $\alpha 2$ expression) with time based on the Cox proportional-hazards regression model. Cumulative hazard functions were estimated at the mean of laminin $\alpha 2$ expression. P-values for the association of laminin $\alpha 2$, integrin $\alpha 6$, and integrin $\beta 1$ expression with molecular subtypes of GBM were computed with the use of the Wilcoxon-rank sum test. To determine the correlation of laminin $\alpha 2$ chain with brain tumor expression a microarray database (Oncomine, Compendia Bioscience, Ann Arbor, MI <http://www.oncomine.org>) was queried. To determine the correlation between laminin chains and patient survival, the NCI REpository for Molecular BRAin Neoplasia DaTa bioinformatics database (REMBRANDT, <https://caintegrator.nci.nih.gov/rembrandt/>) was queried. To determine imaging differences between laminin $\alpha 2$ high and low patients, two expression probes were used to assess LAMA2 mRNA expression from a previously published dataset of 53 glioblastomas which included both imaging and gene expression analysis [38]. Values were expressed as ratios of red-to-green fluorescence dye intensity (log2R/G) estimated by the robust multigene average preprocessing algorithm. Linear regression confirmed a significant correlation of expression values between both probes ($p < 0.000001$). Locally weighted least squares (LOWESS) smooth fit confirmed the appropriateness of a linear regression analysis. Patients in the top and bottom quartile were then evaluated for edema based on MRI evaluation (scored 0 – 2).

Glioblastoma stem cell derivation, culture, and analysis

Human GBM cells were derived under written informed consent and approved IRB protocols from Cleveland Clinic, University Hospitals-Seidman Cancer Center at Case Western Reserve University, and Duke University to establish xenografts in BalbC nu/nu (athymic nude) mice for maintenance of the tumor hierarchy as previously published [30, 39, 40]. GBM xenografts for 3 tumors (556, 1966, and 387) were used for experimental studies. Only low (<5) passage cells were used for analysis to minimize cellular drift [41] and the majority of cells were used immediately after dissociation. Unenriched glioma cells were derived from tumor xenografts using a papain dissociation kit (Worthington) and cultured overnight in neurobasal media supplemented with B-27 (Life Technologies, 1x), EGF (R&D Systems, 20ng/ml), and FGF-2 (R&D Systems 20ng/ml). For RNA studies, GSCs were enriched based on previously validated cell surface marker expression by flow cytometry (CD133/2-APC, Miltenyi, or integrin $\alpha 6$ -FITC, BD). For functional studies, GSCs were enriched using CD133/2 magnetic beads and cultured in supplemental neurobasal media as described above. GSCs were functionally assayed for self renewal, multi-lineage differentiation, stem cell marker expression and tumor propagation as previously described. Non-stem tumor cells were processed in parallel and cultured as we previously described [30, 39, 40].

Brain tumor endothelial cell derivation, culture, and analysis

For isolation of brain tumor endothelial cells (BTECs), established protocols were used as previously described [42] for 3 separate primary GBM specimens. Purity was confirmed by immunofluorescence staining with anti-CD31 (Sigma, 1:200) and anti-von Willibrand factor (Abcam, 1:200) antibodies. Normal brain endothelial cells were purchased Cell Systems and cultured in conditions similar to BTECs prior to in vivo injection.

Immunostaining analysis

Immunostaining analysis on human GBM specimens or tumorspheres was done as previously described using 10 μ m frozen sections obtained from the Duke University Brain Tumor Center Tissue Bank and histologically confirmed by a neuropathologist (R.E.M.) [30] using antibodies against integrin $\alpha 6$ (Millipore, 1:100), CD31 (Dako (mouse) or Abcam (rabbit), 1:200), laminin $\alpha 2$ (Millipore, 1:100), laminin $\alpha 3$ (Millipore, 1:100), laminin $\alpha 5$

(Dako, 1:50), pan-laminin (laminin $\alpha 1$, $\beta 1$, $\gamma 1$, Sigma, 1:100), fibronectin (Sigma, 1:100), collagen IV (Abcam, 1:100) and species appropriate secondary antibodies (Alexa 488, 568, or 633, Invitrogen, 1:200). Nuclei were counterstained with Hoechst 33342 (1:1000 dilution from a 5 mg/ml stock solution, Invitrogen). All imaging was done using a Leica SP-5 confocal microscope as described previously [30] and images were processed and assembled in Photoshop (Adobe). Tissue microarray analysis was performed as previously described [43] using the laminin $\alpha 2$ antibody (Millipore).

Quantitative real time PCR (qPCR)

qPCR analysis was done as previously described using an ABI 7900HT system with using SYBR-Green Mastermix (SA Biosciences). For analysis, fractions of marker positive and negative tumor cells (i.e. integrin $\alpha 6$ or CD133) cells were sorted directly into lysis buffer and RNA was extracted using the RNeasy kit (Qiagen) and cDNA was synthesized using the Superscript III kit (Invitrogen). BTECs were expanded in vitro as described above and processed using the same methodology as the marker positive and negative tumor cells. For qPCR analysis, the threshold cycle (C_T) values for each gene were normalized to expression levels of β -Actin. Dissociation curves were evaluated for primer fidelity and only threshold cycles below 35 cycles were reported.

The following primers were used:

β -actin	Forward	5'-AGAAATCTGGCACCACACC-3'
	Reverse	5'-AGAGGCGTACAGGGATAGCA-3'
Laminin $\alpha 1$	Forward	5'-AGGTCAGTGGCCATGTAAGG-3'
	Reverse	5'-ACCCACAGGAGACACAGGTC-3'
Laminin $\alpha 2$	Forward	5'-GGTGACTCTGAAGGCTGAG-3'
	Reverse	5'-GGGCAACAATCTCTGGATGT-3'
Laminin $\alpha 3$	Forward	5'-CATTTCTACGCCTGCTTTCC-3'
	Reverse	5'-GCACAAGCTTCCAGTCTTCC-3'
Laminin $\alpha 4$	Forward	5'-CCTCCTCAATCAAGCCAGAG-3'
	Reverse	5'-TGCTTAACGGCATCACTGAG-3'
Laminin $\alpha 5$	Forward	5'-GAGAGCCCTTTGTGCTGAAC-3'
	Reverse	5'-GCCTCGTAGTATGCGCTAGG-3'

Laminin $\alpha 2$ lentiviral short hairpin RNA (shRNA) construct production

Lentiviral shRNA constructs were prepared as we previously reported [30]. In short, using lipofectamine 2000 (Invitrogen), 293FT cells were co-transfected with packaging vectors psPAX2 and pCI-VSVG (Addgene) and lentiviral vectors directing expression of shRNA (Sigma) specific to laminin $\alpha 2$ (TRCN0000083857 (KD1) and TRCN0000083853 (KD2)) or a non-targeting control (NT) shRNA (SHC002) to produce virus. Media on the 293FT cell cultures were changed 18 hours after transfection and viral supernatants were collected 24 and 48 hours later, filtered, and concentrated for immediate use or stored at -80°C for future use.

Cell proliferation and tumorsphere formation assays

For cell proliferation assessments, cell populations of interest were plated at a density of 1000 cells/well in a 96-well plate in quadruplicate as previously described [30] using the CellTiter-Glo assay kit (Promega). Cell number was measured every other day and normalized to the initial reading. For Caspase 3/7 analysis, parallel wells were evaluated using the Caspase 3/7 substrate (Promega) and normalized to cell growth values. For

tumorsphere formation assessments, 96-well plates were seeded at a density of 1 or 10 cells per well per condition as previously described [30]. Reported numbers represent a minimum of 16 wells per condition and were calculated ten days after plating. Tumorspheres containing more than 20 cells were scored.

Immunoblotting analysis

Protein analysis was done by immunoblotting as previously described [40]. Briefly, 10 μ g of total protein was loaded per condition and probed using an anti-laminin α 2 antibody (Millipore, 1:500). Immunoblotting with an α -Tubulin antibody (Sigma, 1:500) was used as a loading control and 250 ng of purified merosin (containing laminin α 2, Millipore) was used as a positive control. Species specific horseradish peroxidase (HRP) conjugated secondary antibodies were used for detection (Invitrogen, 1:2000).

Cell mixing studies

For co-culture studies, ECs were infected with either a non-targeting control or a laminin α 2 knock down construct (KD1) as described above in non-GSCs. In vivo mixing studies were done by labeling GSCs with a yellow fluorescent protein reporter virus (Sigma) for identification and 1,000 GSCs were mixed with 100,000 ECs were transplanted into the cortex of immune compromised mice as previously described with 5 mice per group [44].

DNA damage assessments

DNA damage was induced by exposing the cells to the indicated dose of ionizing radiation (IR; J.L. Shepherd Mark 1 Model 30, 137 Cesium Irradiator; J.L. Shepherd and Associates). Relative survival was measured using CellTiter-Glo (Promega) at 48 or 72 hours following irradiation. GSCs (specimen 387) were grown either on purified laminin α 2 (Sigma) or with the equivalent protein concentration (10 μ g/ml) of bovine serum albumin (BSA) as a control. Resolution of DNA damage was evaluated through staining for phospho-H2AX (S139) (γ H2AX; Millipore, 1:1000) 24 hours after irradiation using methods described above. A minimum of 50 cells per condition were quantified from four independent trials with cells bearing greater than ten γ H2AX foci and graphed as a percentage of total cells counted.

Statistical analysis

Reported values are mean values $\pm$ standard error from studies done in at least triplicate. Unless otherwise stated, one-way ANOVA was used to calculate statistical significance with p-values are detailed in the text and figure legends.

Results

Laminin chain expression inversely correlates to patient prognosis in malignant gliomas

As laminins may promote GSC maintenance in culture [33, 34], we evaluated their expression with regards to tumor malignancy in human patient populations. We queried a microarray database (Oncomine) to compare the relative expression levels of laminin α 2 between NPCs and GBM, and found significantly elevated levels of laminin α 2 in GBM tissue compared to human NPCs (Fig. 1A). Laminin α 2 was also significantly higher in GBM compared to other less malignant tumors (Astrocytoma, Oligodendroglioma, Mixed Glioma) suggesting a correlation to tumor grade (Fig. 1B–E). We assessed the association of laminin α 2 expression in 354 glioblastoma patients of the TCGA (University of North Carolina dataset) and Cox proportional hazards regression modeling revealed a significant association between LAMA2 expression and poor patient survival (Fig. 2A, $p = 0.03$ by Cox model, hazard ratio for death: 1.11; 95% CI: 1.01–1.23). This association was confirmed in a second data set of 246 TCGA glioblastoma patients profiled independently on a different

genomics platform (Broad Institute; $p = 0.04$ by Cox model, hazard ratio for death: 1.16; 95% CI: 1.00–1.34). The genomic pre-processing algorithms for the microarray data yielded estimates of laminin $\alpha 2$ gene expression whose units of measurement complicate interpretation of Cox model hazard ratios for continuous versions of these predictors. For easier interpretation of hazard ratios, we have estimated cumulative hazard curves for the minimum, median, and maximum expression of laminin $\alpha 2$ in each of the two data sets, which demonstrated similar cumulative hazard functions (Fig 2A). We found a strong negative correlation between laminin $\alpha 2$ RNA levels and patient survival (Figure 2B). A statistically significant negative correlation was also seen with key laminin receptors (integrin $\alpha 6$ and integrin $\beta 1$), laminin $\alpha 4$, and laminin $\alpha 5$ but not with laminins $\alpha 1$ or $\alpha 3$ (Table 2). Additionally, only laminin $\alpha 2$ among the alpha-laminins was informative of extended survival in patients with low expression (Figure 2B, Table 2). To confirm the difference in laminin $\alpha 2$ expression between tumor types at the protein level, we evaluated laminin $\alpha 2$ immunoreactivity on a tissue microarray containing non-tumor neural tissue and varying grades of gliomas (Grade II, Grade III, GBM). The specificity of the laminin $\alpha 2$ chain specific antibody was validated by probing for purified laminin $\alpha 2$, which was only detected by the laminin $\alpha 2$ but not laminin $\alpha 3$ and $\alpha 5$ antibodies (Supplementary Fig. 1). The tissue microarray analysis confirmed that laminin $\alpha 2$ was increased in GBM specimens as compared to Grades II and III (Supplementary Fig. 2). Statistical analysis revealed that there was significantly higher expression of laminin $\alpha 2$ in GBM versus lower grade tumors and a significant difference in survival between GBM and lower grade tumors ($p < 0.05$, data not shown).

The recent identification by Philips [45] and the TCGA [45] of GBM subgroups distinguished by specific gene expression profiles and genetic alterations suggests that intertumoral heterogeneity of GBMs may be phenotypically important. To evaluate if laminin $\alpha 2$ was associated with a glioma molecular subtype, we interrogated the TCGA dataset and found that laminin $\alpha 2$ was significantly higher in the Mesenchymal and Classical subtypes as compared with the Neural and Proneural subtypes (Fig. 2C). To evaluate if laminin receptor expression had a similar trend, we evaluated integrins $\alpha 6$ and $\beta 1$ expression in TCGA and found a similar increase in the Mesenchymal and Classical subtypes as compared with the Neural and Proneural subtypes (Supplementary Fig. 3). The laminin $\alpha 2$ molecular subclass results and survival differences were also confirmed in a recently published dataset [38] (data not shown). Collectively, these results emphasize the correlation between laminin chain expression and patient prognosis and are suggestive of a role for laminins in tumor progression.

Laminins are expressed in the perivascular glioblastoma stem cell niche

As the perivascular niche is a reservoir of GSCs and previous studies of normal brain have identified laminins as resident ECM on blood vessels [31, 32], we explored the expression of laminins in the GSC microenvironment. Using sections from human GBM tumors, we confirmed the presence of laminins in the perivascular niche using a pan-laminin antibody (recognizing the $\alpha 1$, $\beta 1$, and $\gamma 1$ subunits, Figure 3A), in conjunction with integrin $\alpha 6$ and CD31 to mark GSCs and blood vessels, respectively. As we previously reported, integrin $\alpha 6$ was present on both vascular and non-vascular cells in the perivascular niche with high selectivity (Fig. 3) [30]. To evaluate laminin chain specific expression patterns, we utilized chain specific antibodies against laminins $\alpha 2$, $\alpha 3$, and $\alpha 5$. Laminin $\alpha 2$ (Fig. 3B), $\alpha 3$ (Fig. 3C), and $\alpha 5$ (Fig. 3D) co-labeled with integrin $\alpha 6$ on both vascular and non-vascular cells in the area adjacent to the vasculature. Previous work in NPCs [28, 46] and our results on the expression pattern of laminin $\alpha 2$ in the perivascular niche suggest that GSCs may bind laminin $\alpha 2$. To test this hypothesis, we evaluated GSCs cultured from xenografted tumors grown as tumorspheres, which are derived from GSCs and contain a heterogeneous mixture

of tumor cells including GSCs and non-GSCs. In tumorsphere cultures, we found elevated levels of laminin $\alpha 2$ in conjunction with integrin $\alpha 6$ expression, suggesting that laminin $\alpha 2$ is important for GSC growth in vitro (Fig. 3E). Evaluation of additional ECM ligands determined fibronectin and collagen IV were expressed on the vasculature (Fig. 4). While fibronectin expression was limited to the vascular compartment (Fig. 4A), collagen IV was detected on non-vascular cells adjacent to the blood vessels (Fig. 4B), suggesting the presence of basement membrane structures containing both laminins and collagen IV. We also evaluated tenascin-C, another ECM protein expressed in GSCs [6], upregulated in GBM tumors [47, 48], and linked with NPC migration [49]. Expression of tenascin-C was detected in the perivascular compartment and throughout the tumor (Supplementary Fig. 4A). Additionally, while elevated in glioma patients, tenascin-C itself was not informative for patient survival as analyzed by bioinformatics (Supplementary Fig. 4B), suggesting that the specific laminin chain expression and link to patient survival is unique to laminin chains and not a general ECM characteristic.

Non-stem cell tumor cells and endothelial cells provide niche laminins

Having identified laminins as highly expressed in the perivascular niche, we interrogated the cellular source for the laminins to determine paracrine, juxtacrine, or autocrine communication. By comparing GSC marker positive (integrin $\alpha 6$) and negative cells from three xenografted tumors (propagated in nude mice), we found that non-GSCs had elevated RNA levels of laminin $\alpha 2$, $\alpha 3$, and $\alpha 5$ as compared to GSCs (Fig. 5). In particular, laminin $\alpha 2$, which had a negative correlation with patient survival, was preferentially expressed in the non-GSC population (Fig. 5A). Laminin $\alpha 3$ also had an elevated expression in non-GSCs (Fig. 5B). There were limited differences in laminin $\alpha 4$ expression between GSCs and non-GSCs (Fig. 5C). However there was significant enrichment of laminin $\alpha 5$ in non-GSCs (Fig. 5A). Analysis of GSCs enriched on CD133 expression showed similar results (Supplementary Fig. 5).

To evaluate laminin expression levels of endothelial cells, we compared the qPCR results from the GSCs in the above-mentioned analysis to brain tumor associated endothelial cells (BTECs). We found limited expression of laminin $\alpha 1$. Laminin $\alpha 2$ was greatly elevated in BTECs as compared to GSCs (Fig. 6A), however laminin $\alpha 3$ expression was not as elevated in BTEC as compared with GSCs (Fig. 6B). Both laminins $\alpha 4$ (Fig. 6C) and $\alpha 5$ (Fig. 6A) were also elevated in BTECs as compared with GSCs. These results demonstrate that GSCs express the least amount of several laminin chains ($\alpha 2$, $\alpha 3$, $\alpha 5$) and suggest a juxtacrine relationship, where GSCs receive ECM ligands from their microenvironment.

Laminin $\alpha 2$ is essential for glioblastoma stem cell maintenance

To disrupt laminin $\alpha 2$ function, we utilized two non-overlapping short hairpin RNA (shRNA) sequences against laminin $\alpha 2$ compared to a control non-targeting shRNA sequence not found in the human genome transduced by lentivirus (validated in Fig. 7A). As non-GSCs contained higher levels of laminin $\alpha 2$, validation of knockdown for laminin $\alpha 2$ was performed on non-GSCs derived from a xenografted tumor and laminin $\alpha 2$ knockdown did not result in substantial changes in laminins $\alpha 3$ and $\alpha 5$, confirming specificity for laminin $\alpha 2$ (Supplementary Fig. 6). A similar reduction in laminin $\alpha 2$ levels was observed in GSCs by immunoblotting (data not shown). The impact of disrupting laminin $\alpha 2$ function on GSCs was evaluated with several validated in vitro assays to measure cell proliferation, survival, and self-renewal, key aspects of the GSC phenotype. GSCs with attenuated laminin $\alpha 2$ had significantly decreased growth (Fig. 7B) and increased cell death (Fig. 7C) when compared to a non-targeting control. Additionally, tumorsphere formation was reduced in GSCs with attenuated laminin $\alpha 2$ (Fig. 7D). To determine if addition of exogenous laminin $\alpha 2$ could rescue the growth phenotype, we added purified laminin $\alpha 2$ (10 $\mu\text{g/ml}$) to GSC

cultures with attenuated laminin $\alpha 2$ and observed a partial rescue in Caspase 3/7 activity (Fig. 7E). The introduction of shRNAs into GSCs was also controlled for by comparing uninfected GSCs. While non-targeting controls showed reduced growth and increased cell death as compared with uninfected GSCs, laminin $\alpha 2$ attenuation greatly decreased growth and increased cell death substantially as compared with both controls (Supplementary Fig. 7). Taken together, these results demonstrate that laminin $\alpha 2$ is pivotal for GSC maintenance (which includes survival, proliferation, and self-renewal).

To determine the in vivo significance of EC laminin $\alpha 2$ contribution to tumor growth, we utilized a co-culture system in which GSCs were labeled and cultured with an equal number of control or shRNA laminin $\alpha 2$ targeted ECs. GSC only controls were used to verify strong Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling (data not shown). Intracranial transplantation was done with 1000 GSCs (labeled with a constitutively active yellow fluorescent protein reporter) and 100,000 ECs with control or laminin $\alpha 2$ shRNA. Attenuation of laminin $\alpha 2$ resulted in significantly increased tumor latency (Fig. 7F), confirming that laminin $\alpha 2$ is critical for the tumorigenic process. Taken together, these results demonstrate that laminin $\alpha 2$ is important for GSC maintenance and that laminin $\alpha 2$ from other cells (ECs, non-GSCs) can impact GSC growth.

Laminin $\alpha 2$ promotes glioblastoma stem cell survival and DNA repair in response to radiation

From a clinical standpoint, radiation is the most effective non-surgical therapy for GBM patients but remains palliative [1]. We previously demonstrated that GSCs were preferentially resistant to radiation-induced damage and contributed to recurrence after radiation treatment in laboratory models [10]. In a medulloblastoma mouse model, perivascular cells expressing a stem cell marker (Nestin) have an increased incidence of survival [50]. Based on these findings, we hypothesized that ECM interactions may increase GSC survival after radiation. To test this directly, GSCs were cultured with purified laminin $\alpha 2$ or a protein control (bovine serum albumin, BSA) and we observed a significant increase in survival 48 and 72 hours after single doses (1, 3, or 6 Gy) of ionizing radiation (Fig. 8A, B). To evaluate if exposure to laminin $\alpha 2$ facilitated increased DNA repair, γ H2AX foci was assessed and found to be significantly reduced 24 hours after exposure to 3 Gy of ionizing radiation when GSCs were cultured in the presence of laminin $\alpha 2$ (Fig. 8C, D). No significant difference was detected between the groups with no ionizing radiation (Fig. 8C, D). These results confirm the importance of laminin $\alpha 2$ mediated cell survival after radiation and provide a mechanism by which the perivascular niche promotes GSC survival after irradiation.

Discussion

Despite advances in the diagnosis and treatment of GBMs, these cancers remain universally fatal. Most systemic cancers are lethal due to metastases but GBMs cause death due to invasion into normal brain preventing surgical resection and striking therapeutic resistance [2] supporting the brain as a unique environment for cancer. The laminins are likely to be a biomarker of more aggressive molecular subgroups (in the case of GBM, the Classical and Mesenchymal subtypes). It is notable that another group using gene expression studies of ependymomas found that laminin $\alpha 2$ levels informed a poor prognosis [51], suggesting that this specific laminin may be particularly influential in tumor growth. From a clinical perspective, the role of laminins in brain tumors is central to the difficulty of effective treatment, namely therapeutic resistance. Previous work has demonstrated a protective role for the perivascular niche from ionizing radiation in a mouse model [50] and cell culture studies demonstrated the increased expression of integrin $\beta 1$ after radiation [52]. Our study demonstrated that the ECM, a key component of the vasculature, increased survival and

resulted in a more rapid resolution of DNA damage after radiation. Extending these findings, GBM patients with the highest quartile of laminin $\alpha 2$ expression had a higher incidence of edema as assessed by imaging as compared with the lowest quartile of laminin $\alpha 2$ patients (Supplementary Fig. 8). Taken together, our findings suggest that interaction with the ECM in the perivascular niche, involving such proteins as laminins, is likely to be an extrinsic mechanism by which cells survive after radiation. Given these findings, exploring approaches to disrupt the interaction with the ECM to serve as a radiation sensitizer should be an immediate priority. Such an approach has recently been demonstrated with Notch signaling, a key component in GSC maintenance [16, 53], where Notch inhibition alone had a modest effect on GSC survival but when combined with radiation, had a major impact on GSC survival. Reports have suggested a cross-talk between Notch signaling, integrins, and laminins which indicates that the integrin-laminin relationship may be amenable to targeting for radiation sensitization [54–57]. Targeting integrins alone is also being actively explored in the context of malignant gliomas [58]. Our results highlight the role of laminins in the perivascular niche and suggest that their contribution is greater than that of a structural protein but rather are key in GSC maintenance.

The α chains may serve as a good starting point for functional analysis as they mediate most receptor binding events and the expression of the 5 α chains is responsible for generating laminin diversity throughout the body [22]. Laminins $\alpha 1$, $\alpha 2$, and $\alpha 5$ are structurally similar and contain a short arm consisting of globular domains while laminins $\alpha 3$ and $\alpha 4$ have a highly truncated arm [22]. Mutant mice of laminin alpha chains have further demonstrated the tissue specific expression and function of laminins. Within the nervous system, laminin $\alpha 2$ deficient mice display a radial glia detachment phenotype [28] and laminin $\alpha 2$ and $\alpha 4$ mice have myelination defects [23]. Expression and functional studies in the neoplastic brain will likely contribute key information with regards to the differing roles of the diverse laminin chains in tumorigenic processes.

In the context of brain tumors, GSCs reside within a perivascular niche [13] and interact with non-stem tumor cells and host tissue (including endothelial cells, pericytes, immune cells, and reactive astrocytes). Our results indicate that laminin is generated from the endothelial cells and non-GSCs (non-stem tumor cells) and suggest a model in which the GSCs have a receptor (i.e. integrin $\alpha 6$) and the ligand is being provided by the niche cells. The concept of stem cells requiring signals from the niche for their maintenance is well-established and is supported by several examples in brain tumors. For example, GSCs have elevated levels of interleukin (IL)-6 receptor and non-GSCs secrete IL-6, which via STAT3 signaling is required for GSC maintenance [59]. There are also several examples involving the vasculature. In a mouse glioma model, endothelial cells secrete endothelial nitric oxide synthase (eNOS) which activates Notch signaling in GSCs [60]. In human glioma models, endothelial cells influence GSC growth and self-renewal via Notch signaling by presenting appropriate ligands [16, 53]. Additionally, a secreted factor analysis of human brain vasculature identified the mammalian target of rapamycin (mTOR) pathway as being critical for GSC growth [61]. These examples highlight the importance of cell communication within the niche and future studies integrating the role of the niche cells in promoting GSC maintenance are likely to be informative.

To date, there is limited direct evidence for the function of laminins in GSCs and the perivascular niche. Previous studies examining the perivascular niche may have, in part, addressed the contribution of laminins as the exterior of the vasculature is enriched in laminins. These contributions encompass a variety of diverse biological processes including survival, migration/invasion, adhesion, and stem-cell maintenance. Our work highlights the role of laminin $\alpha 2$ in GSC survival, which has also been reported when integrin $\alpha 6$ is attenuated. This is likely to reflect an inherent role of the ECM signaling in the perivascular

niche. Laminins also have a major role in glioma cell migration and invasion, which has been demonstrated by previous studies and while yet to be fully elucidated, is likely to involve laminin interactions on blood vessels [31]. An additional role laminin signaling may have is that of stem-cell maintenance. Additionally, laminins may have an instructive role in the recently characterized phenomenon of GSCs assuming EC phenotypes [62–65]. Future studies evaluating a role for perivascular ECM in this process will be critical in determining mechanism by which this plasticity occurs in brain tumors. To fully appreciate stem cell maintenance is a difficult task as many assays measure self-renewal as a surrogate in combination with survival and proliferation (i.e. sphere formation assay). Due to their location in the niche and their association with many key signaling pathways (EGFR, mitogen-activated protein kinase), it is likely that laminins promote stem cell maintenance and subsequent signaling focused studies will be required to determine the exact mechanisms [22, 66]. Laminins may also play a role in adhesion to the niche [20]. This is a relatively unexplored area and many questions remain unresolved as to how the niche instructs the GSC phenotype. It is likely that laminins play a central role in this process and may involve adhesion receptors such as integrin $\alpha 6$, but live imaging studies may be required to fully appropriate interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Laminin $\alpha 2$ chain is elevated in GBM

Box and whisker plot (A) of expression level for laminin $\alpha 2$ using Oncomine (<https://www.oncomine.org/>) demonstrates significantly higher expression ($p = 2.08 \times 10^{-5}$ as assessed by one-way ANOVA) of laminin $\alpha 2$ in GBM tissue as compared with neural progenitor cells (NPCs). Box and whisker plot (B–E) of expression level for laminin $\alpha 2$ using Oncomine (<https://www.oncomine.org/>) demonstrates significantly higher expression ($p = 5.92 \times 10^{-6}$ (B), $p = 0.011$ (C), $p = 7.87 \times 10^{-14}$ (D), $p = 6.89 \times 10^{-6}$ (E) as assessed by one-way ANOVA) of laminin $\alpha 2$ in GBM tissue as compared with tissue from Oligodendroglioma (Oligo) tumors, Astrocytoma, or mixed glioma (Oligodendroglioma and Astrocytoma).

Laminin $\alpha 2$

A University of North Carolina dataset

Broad Institute dataset

Figure 2. Laminin $\alpha 2$ chain expression correlates with glioma patient survival, and is differentially expressed in GBM molecular subtypes

Cumulative hazard curves (A) plotted to estimate the risk of death according to time at the minimum (Low, green), median (blue), and maximum (High, red) of laminin $\alpha 2$ expression in two independent data sets of the TCGA using two different genomic platforms (University of North Carolina, left; Broad Institute, right), demonstrates similar cumulative hazard functions. 354 patient samples profiled by the University of North Carolina; $p = 0.03$ by Cox model for laminin $\alpha 2$ expression as a continuous variable, hazard ratio for death: 1.11 (95% CI, 1.01–1.23). 246 patient samples profiled by the Broad Institute; $p = 0.04$ by continuous Cox model, hazard ratio for death: 1.16 (95% CI, 1.00–1.34). Kaplan-Meier survival plot (B) for glioma patients with differential tumor laminin $\alpha 2$ expression calculated by NCI REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT)

bioinformatics database (<https://caintegrator.nci.nih.gov/rembrandt/>) at a level of two-fold upregulation indicates that laminin $\alpha 2$ levels inversely correlates with patient survival (red line indicates two-fold upregulation, green line indicates two-fold downregulation, and yellow line represents intermediate expression with number of patients per group indicated in legend). The log-rank p -value for significance of difference of survival between up-regulated group and intermediate group is 0.0007, difference of survival between up-regulated group and down-regulated group is 0.004, and difference of survival between down-regulated group and intermediate group is 0.04. Box plots (C) of association of laminin $\alpha 2$ with molecular subtype in 188 GBMs. Values for gene dosage are presented as log2R/G ratios as estimated by the robust multigene average preprocessing (RMA) algorithm. The box plots show the smallest and largest observations (upper and lower whiskers, respectively), the interquartile range (box), and the median (black line). Data points that are more than 1.5 times the interquartile range lower than the first quartile or 1.5 times the interquartile range higher than the third quartile were considered to be outliers. The p value was calculated with the use of the Wilcoxon rank-sum test and displayed in Table 1.

Figure 3. Unique laminin chain expression in the perivascular niche

Confocal micrographs of immunostaining analysis from GBM patient specimens demonstrate that laminins (detected using a pan-laminin antibody reacting with $\alpha1$, $\beta1$, and $\gamma1$ chains, red, **A**) are co-expressed with a laminin receptor, integrin $\alpha6$ (Int $\alpha6$, green), in the perivascular niche (marked by an antibody against CD31, purple). Chain specific antibodies demonstrate that laminin $\alpha2$ (Ln $\alpha2$, **B**), $\alpha3$ (Ln $\alpha3$, **C**), $\alpha5$ (Ln $\alpha5$, **D**) are co-expressed with integrin $\alpha6$ (green) in the perivascular niche. Confocal micrographs of immunostaining analysis from GSCs derived from a xenografted tumor (tumor 387) grown as spheres (**E**) show expression of laminin $\alpha2$ (red) in conjunction with integrin $\alpha6$ (green).

Yellow arrow indicates area of interest, inset in bottom right. Nuclei counterstained with Hoechst 33342, scale bar = 25 μm

Figure 4. Differential non-laminin ECM expression in the perivascular niche
 Confocal micrographs of immunostaining analysis from GBM patient specimens demonstrate that fibronectin (Fn, red, **A**) expression was limited to the vascular compartment while Collagen IV (Col IV, red, **B**) showed perivascular co-expression with a laminin receptor, integrin $\alpha 6$. White arrow indicates area of interest, yellow arrow indicates area shown in inset. Nuclei counterstained with Hoechst 33342. Scale bar = 25 μ m

Figure 5. Laminins produced by non-GSCs in vivo

Summary of quantitative real time PCR results from xenografted GBM tumors (556, 1966, 387) enriched based on integrin α 6 expression and analyzed without cell culture expansion demonstrates that GSC marker negative cells (non-GSCs, black bars) have elevated levels of laminin chain transcripts when compared to GSC marker positive cells (GSCs, red bars). Bar graph of quantitative real time PCR results for laminin α 2 (**A**), laminin α 3 (**B**), laminin α 4 (**C**), and laminin α 5 (**D**) demonstrates that non-GSCs consistently contain significantly higher levels of laminin α 2, α 2, and α 5 mRNA. *, $p < 0.001$ as assessed by one-way

ANOVA for all groups except laminin $\alpha 3$ specimen 387 and laminin $\alpha 5$ specimen 556. *, $p < 0.01$.

Figure 6. Laminins produced by ECs

Summary of quantitative real time PCR results from primary brain tumor endothelial cells (BTEC, red bars) indicate consistently elevated laminin chains $\alpha 2$ (A), $\alpha 4$ (C), $\alpha 5$ (D) and decreased laminin $\alpha 3$ (B) expression when compared to GSCs (black bars). Prior to calculation of ratios between GSCs and BTECs, all data were first normalized to actin cycle number.

T387

Figure 7. Laminin $\alpha 2$ is required for GSC growth

For functional studies, laminin $\alpha 2$ shRNA were generated and validated by immunoblotting in non-GSCs, with purified laminin $\alpha 2$ used as a positive control (A). Growth and self-renewal of GSCs (derived from tumor 387) was compromised upon laminin $\alpha 2$ shRNA mediated knockdown (KD) as shown in tumorsphere formation assay (B) and ATP based growth assay (C) graphs. Laminin $\alpha 2$ knockdown also resulted in increased cell death as shown by Caspase 3/7 activity assay graphs (D). Addition of exogenous purified laminin $\alpha 2$ provided a partial rescue as shown by Caspase 3/7 activity assay graphs (E). In vivo studies where 1000 GSCs were co-transplanted with 100,000 ECs with NT or laminin $\alpha 2$ knock

down (KD1) demonstrate that EC derived laminin $\alpha 2$ contributes to tumor growth as shown in Kaplan-Meier survival graph (**F**). *, $p < 0.001$ as assessed by one-way ANOVA for sphere formation assays; *, $p < 0.001$ for NT vs both KD1 and KD2. †, $p < 0.001$ for KD1 vs KD2 as assessed by one-way ANOVA for growth assays; *, $p < 0.01$ and **, $p < 0.001$ by one-way ANOVA for rescue studies; $p < 0.01$ as assessed by long-rank analysis for in vivo studies.

Figure 8. Laminin α 2 exposure attenuates radiation induced GSC cell death and increases DNA repair

GSC survival is increased after exposure to purified laminin α 2 as compared to protein control (0.5% bovine serum albumin, BSA) as shown with ATP-based cell survival assays 48 (A) and 72 (B) hours after single dose ionizing radiation (IR) of 0, 1, 3, or 6 Gy. Analysis of γ H2AX 24 hours after exposure to 0 or 3 Gy IR shows increase foci (>10 foci) in BSA cultures as compared with laminin α 2 at 3 Gy IR as shown in representative micrographs (C) and summary bar graph (D). Scale bar represents 10 μ m. **, $p < 0.01$, ***, $p < 0.001$ as assessed by one-way ANOVA.

Table 1
Summary of association of laminin $\alpha 2$, integrin $\alpha 6$, and integrin $\beta 1$ with GBM molecular subtypes

Table summarizing number of samples evaluated using TCGA for each GBM molecular subtype (Classical, Mesenchymal, Neural, and Proneural) and p values calculated with the use of the Wilcoxon rank-sum test for laminin $\alpha 2$ between each subtype.

Association of Laminin α2, Integrin α6, and Integrin β1 with GBM molecular subtype			
Subtype		Number of Samples	
Classical		51	
Mesenchymal		56	
Neural		27	
Proneural		54	
Statistics – p-values based on Wilcoxon rank-sum test			
Laminin α2			
Mesenchymal	Neural	Proneural	
0.436	1.4×10 ⁻⁵	4.5×10 ⁻¹⁴	Classical
	0.001	1.8×10 ⁻¹²	Mesenchymal
		0.001	Neural

Table 2
Correlation between integrin and laminin chain expression and glioma patient survival

Table summarizing survival results for glioma patients with differential tumor integrin $\alpha 6$, integrin $\beta 1$, or laminin α chain expression calculated by NCI REMBRANDT bioinformatics database at a level of two-fold upregulation or downregulation. Integrins $\alpha 6$, $\beta 1$, and laminin $\alpha 2$ expression levels inversely correlate with patient survival. The log-rank p -value for significance of difference of survival between up-regulated group and all other groups is given in p-value column and the numbers of patients per given condition is indicated in the n column. ND indicates not determined.

Integrin/ Laminin	2 fold upregulated		2 fold downregulated	
	n	p-value	n	p-value
$\alpha 6$	62	0.023	7	0.29
$\beta 1$	158	0.000003	ND	-
Ln $\alpha 1$	130	0.06	29	0.84
Ln $\alpha 2$	77	0.00007	7	0.047
Ln $\alpha 3$	2	0.98	4	0.36
Ln $\alpha 4$	208	0.0014	1	-
Ln $\alpha 5$	215	0.00006	3	0.72