Extracellular Matrix Proteome Remodeling in Human Glioblastoma and Medulloblastoma

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ABSTRACT: Medulloblastomas (MBs) and glioblastomas (GBMs) are high-incidence central nervous system tumors. Different origin sites and changes in the tissue microenvironment have been associated with the onset and progression. Here, we describe differences between the extracellular matrix (ECM) signatures of these tumors. We compared the proteomic profiles of MB and GBM decellularized tumor samples between each other and their normal decellularized brain site counterparts. Our analysis revealed that 19, 28, and 11 ECM proteins were differentially expressed in MBs, GBMs, and in both MBs and GBMs, respectively. Next, we validated key findings by using a protein tissue array with 53 MB and 55 GBM cases and evaluated the clinical relevance of the identified differentially expressed proteins through their analysis on publicly available datasets, 763 MB samples from the GSE50161 and GSE85217 studies, and 115 GBM samples from RNAseq-TCGA. We report a shift toward a denser fibrillary ECM as well as a clear alteration in the glycoprotein signature, which influences the tumor pathophysiology. MS data have been submitted to the PRIDE repository, project accession: PXD023350.

KEYWORDS: extracellular matrix, decellularization, glioblastoma, medulloblastoma, proteome, mass spectrometry

INTRODUCTION

Medulloblastomas (MBs) and glioblastomas (GBMs) stand out for their high incidence among central nervous system (CNS) tumors. They are prominently different in age-onset, origin site, and dissemination route—with MBs being the most common solid pediatric tumors, accounting for approximately 20% of the intracranial cases.1 MB mainly disseminates through cell shedding to the cerebrospinal fluid and subsequent re-implantation on distal leptomeninges.2 On the other hand, GBMs are the most common and aggressive adult CNS tumors with a characteristic high invasiveness of the normal surrounding tissue and an overall survival of 15 months.3 GBMs most commonly originate in the supratentorial region, with frontal tumors displaying higher overall survival and better response to treatment.4 Deep changes in the tissue microenvironment have been associated with the tumor onset and progression.

The CNS unique extracellular matrix (ECM) has been partially credited for its highly dynamic microenvironment. The ECM is a complex network of biomolecules, which not only acts as a physical scaffold for the tissue but also modulates cellular behavior, ion diffusion, synaptic connections, and neurotransmission.5 A normal adult brain ECM is highly hydrated and flexible, sparse in fibrillary components [such as collagen (CO), fibronectin (FINC), and vitronectin (VTNC)], and in basement membrane proteins (such as laminin). Also, the brain ECM is enriched in hyaluronic acid, lectin family of proteoglycans (such as versican and neurocan), thrombospondin, and tenascin C and R, among others. The use of the native brain ECM has been shown to accelerate the formation of electrophysiological active neuronal networks in neuron and glia co-cultures.6 Mainly, four types of ECM modifications have been reported during malignant transformation: alterations in the biosynthesis of ECM components, post-translational modifications, turnover, and modifications of cell–matrix interactions. Overall, a denser deposition and stiffer ECM has been reported in different tumor types when compared to the homeostatic tissue, favoring a more aggressive phenotype.7,8

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As the brain ECM comprises 15–20% of the volume of the tissue, conventional proteomic approaches dilute its components and might miss key alterations in its composition upon tumor progression. Aiming to identify differences between the ECM signature of MBs and GBMs that might contribute to the understanding of their distinct pathogenesis, we compared the proteomic profiles of human MB and GBM decellularized tumor samples between each other and their normal decellularized brain site counterparts. We validated key findings by using a protein tissue array with 68 MB, 55 GBM, and 5 normal cases. Next, we evaluated the clinical relevance of the identified differentially expressed ECM and ECM-associated proteins through their analysis available from the GEO microarray dataset of 763 MB cases (GSE85217) and 22 MB cases (GSE85217)10,11 and 115 GBM samples from The Cancer Genome Atlas (TCGA). We report not only a shift toward a denser fibrillar ECM but also a clear alteration in the glycoprotein signature that differs between these tumor types.

**EXPERIMENTAL SECTION**

Normal and Tumoral Brain Samples

Normal autopsy human brain samples were previously collected from individuals with ages ranging from 30 to 99yo (ethics committee approval #13041, 225/16) at the Sao Paulo Metropolitan Autopsy Service (Serviço de Verificação de Óbitos da Capital do Estado de Sao Paulo—SVOC-SP). Samples consisted of 4 cerebellums (CEs) and 10 isocortexes (ISO) (5 frontal and 5 parietal). Regarding the tumoral samples, written informed consent was obtained from all patients or their legally responsible person according to the ethical guidelines approved by the Ethics Committee of the School of Medicine, University of Sao Paulo (0600/10) and The Ethical Commission for Research Projects Analysis from the Clinical Board of The Clinical Hospital and School of Medicine, University of Sao Paulo (CAPPesq approval # 830/01, CONEP/MS approval # 373/02). A total of 5 MB (age ranging from 3 to 33yo, all male) and 8 GBM (age ranging from 29 to 60yo, three males: five females) samples were included in the study. Resected tumor and autopsy specimens were macrodissected, sectioned in 0.5–1 cm² portions, snapped-frozen in liquid nitrogen, and stored at −80 °C. The tumor specimens were categorized according to their World Health Organization classification and molecular subtypes by a neuropathologist as part of a routine diagnostic.

Preparation of Brain-Derived ECM

ECM enrichment through decellularization was achieved through a two-step process under mild agitation. The tissue fragments were incubated in 1% Triton-X (Sigma-Aldrich, St Louis, MO) for 15 min at room temperature following incubation in 2.0% deoxycholate (Sigma-Aldrich) until complete decellularization. All the steps were followed by three washes of 15 min with phosphate-buffered saline. The absence of remaining intact cells was certified by DNA quantification and microscopy analysis of decellularized tissues stained for H&E (data not shown).

Sample Preparation for Proteomic Analysis

Pellets containing ECM proteins were resuspended in 1% sodium deoxycholate containing 100 mM ammonium bicarbonate and sonicated using a probe tip sonicator three times on ice at 40% power for 30 s each. Extracted proteins (soluble fraction) and the pellet were reduced with 10 mM dithiothreitol for 30 min at 56 °C and subsequently alkylated in 40 mM iodoacetamide for 30 min in the dark. Following incubation, the samples were digested with trypsin 2% (w/w) overnight at 37 °C temperature. In order to determine the amount of trypsin for protein digestion, we concomitantly extracted a known weight of decellularized tissue using a buffer containing 8 M urea with protease inhibitors and quantified them using the Qubit fluorimetric detection assay (Thermo Fisher). Samples were acidified with 1% formic acid (FA) and centrifuged at 14,000g for 10 min to stop trypsin digestion and remove insoluble materials (e.g., lipids). The supernatant was collected and quantified using the Qubit fluorimetric assay (Thermo Fisher) before desalting. Samples were desalted using self-made microcolumns made with a C18 plug taken from a C18 disk (Sigma-Aldrich) and inserted in the constrict end of a P200 tip. The acidified samples were loaded onto the microcolumn by applying a gentle air pressure with the syringe and washed three times with 0.1% trifluoroacetic acid (TFA). Peptides were eluted with 50% acetonitrile (ACN), 0.1% TFA, followed by 70% ACN, 0.1% TFA12,13.

**Nanoflow Liquid Chromatography Coupled to Mass Spectrometry Analysis**

Tryptic peptide samples (1 µg) were resuspended in 0.1% FA and analyzed using an EASY-nLC system (Thermo Fisher Scientific, Waltham, MA) coupled to a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Peptides were loaded on a ReproSil-Pur C18-AQ (3 µm) column and separated in a gradient from 100% phase A (0.1% FA) to 45% phase B (0.1% FA, 95% ACN) during 100 min at a constant flow rate of 300 nL/min. LTQ-Orbitrap Velos was operated in the positive ion mode with data-dependent acquisition. Each mass spectrometry (MS) scan was acquired at a resolution of 60,000 full width at half-maximum, followed by 20 MS/MS scans of the most intense ions. Peptides were fragmented by collision-induced dissociation with a normalized collision energy of 35 and an activation time of 10 ms. Ions selected for MS/MS were dynamically excluded for a duration of 30 s. All raw data were accessed in Xcalibur software (Thermo Fisher Scientific).

**Data and Bioinformatics Analysis**

Raw files of liquid chromatography (LC)–MS/MS were processed into MaxQuant software v1.6.2.10 using the Andromeda search engine against the SwissProt Homo sapiens database (20,400 entries downloaded from Uniprot.org Jan/2019) with common contaminants for protein identification. Database searches were performed with the following parameters: precursor mass tolerance of 10 ppm, product ion mass tolerance of 0.6 Da, fully tryptic digestion and two missed cleavage were allowed, carbamidomethylation of cysteine (57.021 Da) was set as a fixed modification, and oxidation of methionine (15.994 Da) and protein N-terminal acetylation (57.021 Da) was set as a variable modification. Mass tolerance of 0.6 Da, fully tryptic cleavage were allowed, and carbamidomethylation of cysteine (57.021 Da) was set as a fixed modification, and oxidation of methionine (15.994 Da) and protein N-terminal acetylation (57.021 Da) were selected as variable modifications. A minimum peptide length of 7 was considered for the database search. All identifications were filtered to achieve a protein–peptide and PSM false discovery rate (FDR) of less than 1%, and a minimum of one unique peptide was required for protein identification. Protein quantification was based on the MaxQuant label-free algorithm using both unique and razor peptides for protein quantification. Protein abundance was assessed on label-free protein quantification (LFQ) based on the extracted ion chromatogram area of the precursor ions.
activating the matching between run features. Intensity-based absolute quantification (iBAQ) values were used to calculate the relative protein abundance within samples. MS data have been submitted to the PRIDE repository, project accession: PXD023350, username: reviewer_pxd023350@ebi.ac.uk, and password: UatLYU40. The total identified proteins were compared with MatrisomeDB (http://matrisomedb.pepchem.org/) containing 1027 ECM core and matrisome-associated proteins.14,15 The selection of statistically regulated proteins was performed using Perseus software (https://maxquant.net/perseus/). Quantitative analysis was carried out on the log2-values of LFQ. Pairwise comparisons were performed: (1) GBM versus cortex, (2) MB versus CER, and (3) GBM versus MB. Protein regulation was determined based on the Student $t$-test analysis with the Benjamini–Hochberg correction at a FDR ($q$-value) of less than 5%. Missing values were replaced in proteins identified in more than 50% of the samples using a width of 0.3 and a down shift of 1.8 in Perseus software. Gene

Figure 1. Workflow of the discovery and validation phases of the study. In the discovery phase, 14 normal and 13 tumor samples, 8 GBMs and 5 MBs, were decellularized, and the obtained ECM proteins were quantified through a high-resolution proteome-wide quantification LC–MS/MS. The differentially expressed proteins identified in the comparisons between the tumor and corresponding normal ECMs were validated at the protein level in an independent cohort of 68 MB and 55 GBM cases and MB microarray dataset from the GEO GSE85217 study ($n = 763$) and GSE50161 study ($n = 22$) and the GBM RNASeq dataset from TCGA ($n = 115$).
ontology and protein–protein interaction networks were identified using the STRING database (https://string-db.org/) and visualized using Cytoscape. The Human Protein Atlas was cross referenced to identify brain-enriched and specific proteins and proteins found in blood (https://www.proteinatlas.org/).

Public Database Analyses

The dataset from TCGA (http://cancergenome.nih.gov/) was downloaded from the Genomics Data Commons Data Portal, and the data were normalized by DE-seq. RPKM values were transformed to z-score to produce the heatmap. The TCGA cohort consisted of 115 GBM cases of proneural (29, PN), classical (38, CS), and mesenchymal (48, MS) subtypes. Gene expression in GBM cases (from TCGA) and GBM patients' overall survival were analyzed in the Gene Expression Profiling Interactive Analysis 2 (GEPIA2) online database. Microarray data of MB cases (763 cases) with a molecular subtype classification (144 of Group 3, 326 of Group 4, 223 of SHH, and 70 of WNT) of the GSE85217 study and 24 samples (2 normal CERs and 22 MBs) of the GSE50161 study from the Gene Expression Omnibus (GEO) database were analyzed by the online tool GEO2R.

Tissue Microarray and Immunohistochemistry

Three representative areas of 0.6 mm diameter from each tumor and normal brain tissue in FFPE were chosen by neuropathologists to build a tissue microarray (TMA) using an arraying machine (MTA-1, Beecher Instruments Inc., Sun Prairie, WI). The MB TMA consisted of 50 CS histopathologic cases, 6 desmoplastic (DSM)/extensive nodularity cases, 12 large cell (LC) cases, and 3 normal CER cases. GBM TMA consisted of 23 brain sites of five autopsy cases. GBM cases were classified based on the somatic mutation profile, as reported previously.

For immunohistochemical detection, TMA sections were deparaffinized, rehydrated, treated for endogenous peroxidase blocking, and subjected to antigen retrieval. Slides were immersed in 10 mM citrate buffer, pH 6.3 and ethylenediaminetetraacetic acid buffer, pH 9.3 for FINC and CO VI (CO6), respectively, and incubated at 122 °C for 3 min using an electric pressure cooker (Biocare Medical, Pacheco, CA). Specimens were then blocked and further incubated with anti-FINC (1:5,000 dilution, mouse monoclonal, ab6328, Abcam, Cambridge, United Kingdom) and with anti-CO VI (1:200 dilution, rabbit polyclonal, ab6588, Abcam) at 16–20 °C for 16 h. Development of the reaction was performed with the commercial kit NovoLink from Novocastra (Newcastle upon Tyne, United Kingdom) and with anti-CO VI (1:200 dilution, rabbit polyclonal, ab6588, Abcam) at 16–20 °C for 16 h. Development of the reaction was performed with the commercial kit NovoLink from Novocastra (Newcastle upon Tyne, United Kingdom). Development of the reaction was performed with the commercial kit NovoLink from Novocastra (Newcastle upon Tyne, United Kingdom) at room temperature using diaminobenzidine and Harris hematoxylin for nuclear staining. Optimization using positive controls suggested by the manufacturer of each antibody was performed in order to obtain an optimal dilution. Two observers (S.K.N.M. and I.F.M.) evaluated the staining intensity of tissue sections.
A semiquantitative scoring system considering both intensity of staining and the respective percentage of ECM stained area was applied as follows: no staining (score 0), 10–25% ECM area stained (score 1), 26–50% stained (score 2), 51–75% stained (score 3), and 76–100% stained (score 4).

Digital photomicrographs of representative fields were captured. Scores for the intensity were applied as follows: (1) slight, (2) moderate, and (3) strong reactivity. The labeling score index (LSI) was determined as intensity \( \times \) percentage of positive staining scores.

Statistical Analysis

The data distribution was tested using the Kolmogorov–Smirnov test. Expression levels were investigated by nonparametric Kruskal–Wallis and post-hoc Dunn tests among the groups. Correlation between relative protein expression values was assessed using the nonparametric Spearman-rho correlation test. Differences were considered statistically significant when \( p < 0.05 \). The analyses were performed in the SPSS for Windows, version 21.0 (IBM Corporation, Armonk, NY).

RESULTS

Quantitative Proteomics Applied to Human Brain-Derived ECM from Control and Tumor Cases

ECM from different human brain regions under normal and cancer conditions was obtained through an optimized protocol of decellularization. Cellular components were removed by an optimized sample treatment keeping intact the structure and morphology of the ECM. We applied a comprehensive workflow for the extracted ECM from 10 human frontal and parietal ISOs, 4 CERs, 5 MBs, and 8 GBMs using high-resolution and accuracy label-free quantitative LC–MS/MS-based proteomics combined with orthogonal validation by immunohistochemistry and in silico gene expression using the GEO and TCGA databases (Figure 1).

ECM samples were resuspended in the MS-compatible detergent sodium deoxycholate and subjected to probe-tip sonication to improve protein solubilization. The ECM samples were digested with trypsin, avoiding centrifugation and reducing sample loss. Proteomic analyses of the ECM from 27 samples were used to generate the first compendium.
of the human brain ECM. The total number of proteins identified analyzing simultaneously all the samples was 1495, and a total of 1124 proteins were identified with at least two peptides and one unique peptide (Figure 2A, Table S1). A total of 138 (12%) proteins were assigned as core matrisome (87) and matrisome-associate (51) proteins based on the human matrisome dataset. These proteins consisted on 22 COs, 55 ECM glycoproteins, 26 ECM regulators, 11 ECM-affiliated proteins, 10 proteoglycans, and 14 secreted factors (Figure 2A, Table S1). The iBAQ measurement normalized by the total number of identified proteins belonging to each class revealed a higher abundance of ECM compared to non-ECM (Figure 2B). Comparison of ECM protein categories between tumor and control samples evidenced a different composition, being tumors more enriched, especially with ECM glycoproteins, ECM regulators, and COs (Figure 2C). High abundances of COs and ECM glycoproteins can also be observed in tumor ECM (Figure 2D). The total number and percentage of human brain ECM compared to non-ECM proteins identified in this study are in accordance with previous studies on ECM isolated from other organs. However, the human brain ECM is composed of a higher number and abundance of glycoproteins (Figure 2C).

A clear separation of the control and tumor conditions was observed in PCA, indicating a specific ECM signature of tumor samples (Figure 3A). A higher abundance of ECM proteins was detected in tumors (MB and GBM) compared to the corresponding normal brain regions, indicating a role of ECM proteins in oncogenic processes.

Differential Quantitative Profile of ECM Proteins in MB and GBM Compared to Corresponding Normal Brain Sites

The protein composition of ECM in human MB and GBM brain tumors compared to ECM from the corresponding human brain regions under normal conditions was characterized. Additionally, ECM proteins of MB and GBM were also compared. Quantitative analysis of ECM proteins from MB versus CER, GBM versus ISO (frontal and parietal), and MB

Figure 4. Upregulated proteins in MB. (A) Proteins identified as upregulated in MB by proteomic analysis in comparison with those in CER and GBM. Benjamini–Hochberg test: *q ≤ 0.05, **q ≤ 0.005, ***q ≤ 0.0005, and ****q ≤ 0.00005. The dots represent the LFQ value of each sample and the horizontal line represents the mean LFQ value ± the standard deviation. (B) Immunohistochemistry for CO6. Representative images of normal CER, CS, DSM, and LC histological subtypes of MB. The bar graph represents the mean value of the LSI ± the standard deviation for normal control (CTL) and each of the MB histological subtypes. The dots represent the LSI of each analyzed case.
versus GBM showed differentially expressed proteins between groups, considering p-value < 0.05 with the Benjamini–Hochberg correction (Figure 3B).

A total of 19 ECM proteins out of 84 proteins were differentially expressed in MB compared with CER samples, being 15 upregulated and 4 downregulated (Figure 3B, Table S3): 7 COs, 10 ECM glycoproteins, and 2 proteoglycans. A total of 28 ECM proteins out of 163 proteins presented statistically different expressions between GBM and ISO samples, of which 21 and 7 were upregulated and downregulated, respectively (Figure 3B, Table S4): 4 COs, 16 ECM glycoproteins, 5 ECM regulators, 1 ECM-affiliated protein, and 2 secreted factors. Moreover, the comparison of expression of ECM proteins between MB and GBM resulted in 40 total proteins differentially expressed. Among them, 11 ECM core and associated proteins were upregulated in MB compared with those in GBM (Figure 3B, Table S5).

Four proteins were common to the comparison of both tumor and normal samples (GBM vs ISO and MB vs CER). In addition, seven proteins upregulated in MB were common to the analyses of MB versus CER and MB versus GBM. Therefore, a total of 46 ECM proteins were differentially expressed in all comparisons, as represented in the heatmap of Figure 3B, and a protein–protein interaction network is represented in Figure 3C.

**MB-ECM Composition**

The ECM of MB presented three chains of CO VI upregulated (CO6A1, CO6A2, and CO6A3), among other CO chains, such as CO1A1, CO5A1, and CO5A2. Among the ECM glycoproteins upregulated only in MB versus CER were fibulins (FBLN1 and FBLN5), fibrillins (FBN1 and FBN2), elastin (ELN), EMILINs (EMIL1 and EMIL3), and latent-transforming growth factor beta-binding protein 4 (LTBP4). Lumican (LUM), a proteoglycan, was also upregulated. Proteomic quantification data of these upregulated proteins in MB are presented in Figure 4A.

Orthogonal validation using immunohistochemistry confirmed an unequivocal expression of CO6 observed in MB samples, particularly high in the CS histopathological subtype, which contrasted with almost a complete absence of this protein in the control cerebellar tissue (Figure 4B).

Genes coding for ECM proteins and ECM-associated proteins were analyzed in molecular subtypes of MB from the in silico microarray studies of GEO85217 and GEO50161. Interestingly, 19 genes were differentially expressed in MB, and they were upregulated particularly in SHH and WNT molecular subtypes (Figure 5A). In comparison with a normal CER, nine of them were significantly upregulated, including four genes coding for CO chains (COL6A1, COL6A3, COL5A2, and COL1A1) (Figure 5B).
GBM-ECM Composition

Among the ECM glycoproteins upregulated only in the GBM were EGF-containing fibulin-like ECM protein 1 (FBLN3), fibrinogens (FIBA, FIBB, and FIBG), FINC, transforming growth factor-beta-induced protein (BGH3, TGFBI), von Willebrand factor (VWF), periostin (POSTN), tenasin A (TENA), and VTNC, while laminins (LAMA2, LAM5 and LAMB2) and tenasin R (TENR) were downregulated in the GBM. Other proteins classified as ECM regulators, such as histidine-rich glycoprotein (HRG), interalpha-trypsin inhibitor heavy chain H2 (ITIH2), plasminogen (PLMN), serpin H1 (SERPH), and prothrombin (THRB), as well as a related protein (ANXA2), and CO chains (COEA1 and CO5A3) and secreted factors (S10A9 and S10AB) were also upregulated in the ECM of GBM (Tables S3 and S4, Figure 6A).

Figure 6. Upregulated proteins in GBM. (A) Proteins identified as upregulated in GBM by proteomic analysis in comparison with the normal ISO. Benjamini–Hochberg test: *$q \leq 0.05$; **$q \leq 0.005$; ***$q \leq 0.0005$; and ****$q \leq 0.00005$. The dots represent the LFQ value of each sample and the horizontal line represents the mean LFQ value ± the standard deviation. (B) Immunohistochemistry for FINC. Representative images of normal ISO, PN, CS, and MS molecular subtypes of GBM stained for FINC. The bar graph represents the mean value of the LSI ± the standard deviation for normal control and each of the GBM molecular subtypes. The dots represent the LSI of each analyzed case.

In GBM, a differential expression of 21 genes coding for the proteins detected by proteomics was observed, with a preponderant upregulation in the MS molecular subtype through the RNAsSeq of TCGA cases of GBM (Figure 7A). A significant upregulation of the FINC coding gene (FN1) expression was observed in all three GBM molecular subtypes (PN, CS, and MS) compared to the normal brain (Figure 7B). The FN1 expression level affected the GBM patient outcome as a shorter overall survival time was associated with a higher FN1 expression (Figure 7C).

■ DISCUSSION

MBs and GBMs are CNS tumors of great clinical relevance that display very distinct features. Our study aims to help clarify the influence of the ECM on the intrinsic differences between these malignancies through the characterization of the matrisome and matrisome-associated proteins from their decellularized ECMs and their decellularized normal site counterparts.

Our results clearly indicate a shift in the matrisome signature of both tumor types toward the upregulation of CO when compared to normal samples. Altered CO expression signature and crosslinking patterns have been linked to tumor stiffening...
and aggressiveness. In turn, stiffer tumors have been associated with higher solid stress, interstitial fluid pressure, and angiogenesis and are known to display increased cellular migration and invasion. Although an increase in fibrillary components is observed in the matrisome signature of both tumor types, it is more significant in MBs. MBs originate in the CER, which normally presents a lower stiffness (around 2.38 kPa) compared to the ISO (frontal 3.15, occipital 3.21, parietal 2.87, and temporal lobe 3.17 kPa). Despite displaying high intratumoral heterogeneity, MBs have an average stiffness of 27 kPa. Our study demonstrated that MB-ECM presented a sharp shift toward fibrillary components compared to the homeostatic CER−ECM corroborating the change in its stiffness. We observed a significant increase of diverse types of CO with several fibril diameters: CO1 of 67 nm, CO5 of 9 nm, and CO6 of 5−10 nm. Moreover, we found an increase of FBNs (FBN1 and FBN2) and ELN. FBNs are cystein-rich glycoproteins incorporated into insoluble 10−12 nm microfibrils, which provide a scaffold for the deposition of ELN and confer strength and elasticity to the ECM. Mature microfibrils may contain both FBN1 and FBN2, where FBN2 forms the structural core of microfibrils and are masked by an outer layer of FBN1. FBN2 exposure in tumor endothelium has led to an increment in tumor angiogenesis through the local sequestration and activation of TGFβ. EMIL3 is another EMIL member upregulated in MB-ECM, lacking the C-terminal C1q domain that is present in all other members of the family, and it binds heparin with high affinity through its EMI domain. The availability and distribution of Wnt, Hedgehog, and bone morphogenic protein ligands are known to be regulated by heparan sulfate proteoglycans; therefore, EMIL3 may also participate in these processes. Additionally, proteins linked to CO processing were also found upregulated in MB, such as the case of LUM, a class II small leucine-rich proteoglycan, involved in the organization of CO fibrils, influencing their diameter and interfibrillar spacing. Enhanced cancer growth has been associated with LUM upregulation by integrin β1 activation mediated by the FAK signaling pathway, whereas the downregulation of LUM accelerated cancer cell invasion mediated by the Rho/LMK/colin pathway. In turn, integrin β1 present in stiff ECM was

Figure 7. Gene expression analysis in GBM molecular subtypes. (A) RNASeq analysis with the heatmap of genes coding for ECM proteins. Data are presented as the z-score of RPKM. (B) FN1 expression in GBM molecular subtypes across the TCGA dataset analyzed in the GEPIA 2 online database (http://gepia2.cancer-pku.cn). *p < 0.05. Y-axis is represented by log2 (TPM+1) for FN1 expression levels. (C) Kaplan−Meier curves for the overall survival rate in GBM cases separated by FN1 expression levels. Analysis was performed in GEPIA 2.
described to activate β-catenin in a focal adhesion kinase (FAK)-dependent manner and lead to the transcription of Wnt1 in MS stem cells,59 representing a positive feedback loop between ECM rigidity and the Wnt canonical pathway that might play a significant role in MBs, specially of the Wnt subtype. Furthermore, the Wnt subtype was previously described to display a higher expression of several TGFβ family members, including TGFβ1 (BGH3),40 found in our analysis. BGH3 is involved in the cellular interaction with CO fibers, inhibiting adhesion to the ECM and therefore stimulating cell detachment tumor migration.41

Equally important for the biological features of MB were the downregulated matrisome and matrisome-associated proteins. Four proteins were downregulated in MB-ECM: agrin (AGRIN), tubulointerstitial nephritis antigen-1 (TINAL), CO type 4 alpha 4 chain (CO4A4), and basement membrane-specific heparan sulfate proteoglycan (PGBM, HSPG2).

Considering our findings, the MB-ECM protein signature compared to normal CER displays a significant shift toward fibrillary components and to CO and elastic fiber-associated proteins. These alterations translate to a stiffer ECM and more aggressive phenotype and illustrate how the microenvironment enhances tumor hallmarks.

While MBs display a strong fibrillar signature, GBMs show substantial matrisome diversity, reflecting their high intratumoral heterogeneity. Nevertheless, an increase in ECM stiffness has also been reported to correlate with glioma progression, while the gliotic tissue presents a Young’s modulus (E) of 0.01–0.18 kPa, low-grade gliomas display the range of 0.05–1.4 Pa, and GBMs of 0.07–13.5 kPa.42 Herein, we describe that COSA3 was the most significantly overexpressed CO in GBM-ECM. ITIH2 was also increased and this protein stabilizes the ECM mesh by covalent binding to brain-enriched hyaluronic acid ECM.43 This indicates a favoring in HA interactions toward a more aggressive phenotype.

GBM major dissemination route is through the invasion of the surrounding normal tissue.5 GBMs’ highly invasive profile is supported by the upregulation of FINC (FN1) and BGH3 found in our analysis. Our results are in accordance with the previous knowledge that FINC is highly overexpressed in GBM and linked to the ECM ultrastructure.44 FINC leads to the activation of the PI3K/AKT pathway in an integrin-dependent manner, leading to proliferation, survival, invasion, and chemoresistance.45 Moreover, glioma stem-like cells exposed to FINC have a more differentiated phenotype, with decreased levels of Sox-2 and nestin and increased levels of glial fibrillary acidic protein (GFAP) and β-tubulin,56 highlighting the importance of FINC for glioma onset and progression. BGH3 binds to CO I, II, and IV and, therefore, mediates the cell–CO interaction. BGH3 is induced by TGFβ and inhibits cell adhesion and promotes cell migration.50 BGH3 was previously associated with the expression signature of MS GBMs, the subtype with poorer prognosis.47

We found an expressive modulation of members of the Tenascin family, large ECM glycoproteins that intermediate cellular response to ECM and are involved in basic cellular processes such as proliferation, migration, and differentiation.48 TENA was found to be significantly upregulated in GBM. ECM stiffness has been previously associated with TENA expression and a poorer prognosis in low-grade gliomas and GBMs. In the same study, the authors report that IDH1 mutation decreases TENA expression and ECM stiffness.52 Moreover, TENA interaction with FINC was also implicated in its interaction with aligned CO fibers, linking this glycoprotein to the modulation of the CO ultrastructure.53 Another member of the tenascin family, TENR, has the opposite profile, being significantly downregulated in GBM. TENR expression was inversely correlated to the glioma progression, and its expression might repress tumor invasion.54 These results suggest that an orchestrated modulation of tenasin proteins takes place during glioma progression, leading to a more aggressive phenotype.

As FINC, laminins are important components of the basement membrane, being crucial to cellular–ECM communication and modulating cell adhesion, differentiation, and migration. Here, we describe that laminin subunit α2 (LAM2A) and laminin subunit β2 (LAM2B) expressions were decreased in GBM-ECM. LAM2A promoter has been found to be hypermethylated in different carcinomas, with its suppression being linked to an increase in proliferation and invasion.51 Likewise, LAM2B was described to more efficiently activate integrin β1 when compared to Laminin subunit β1.52 Moreover, LAM2B has been predicted in silico to present a potential tumor suppressor activity.53 The importance of integrins in GBM progression was corroborated by POSTN increased expression in GBM-ECM. POSTN binds to integrins αvβ3 and αvβ5, leading to the activation of the Akt and PI3-kinase signaling pathways. Also, POSTN mediates TENA incorporation into the ECM meshwork, particularly TENA interaction with FINC,54 suggesting a direct link to ECM ultrastructure modulation, and its expression has been implicated in radioresistance in head and neck cancer cells.55 VTNC is another αvβ3 and αvβ5 integrin-associated protein that is increased in GBM-ECM. This multicmeric glycoprotein activates vascular endothelial growth factor receptor 2 via αvβ3.56 VTNC also binds to the plasminogen activator inhibitor-1 (PAI-1) and urokinase plasminogen activator receptor, mediating ECM degradation.57 Modulation of integrin signaling is also indicated by TINAL suppression. TINAL directly binds to integrins α5β1 and αvβ3, as to the epidermal growth factor receptor, leading to the inhibition of FAK and EGFR signaling pathways.58 Taken together, our results show that integrin signaling is a major determinant of the GBM microenvironment.

GBM’s high growth rate is associated with a characteristic impairment of the blood–brain barrier (BBB) and necrotic areas.59 Here, we detected a hyperexpression of fibrinogen chains A (FIBA), B (FIBB), and G (FIBG) in GBM-ECM. These three chains polymerize, forming an insoluble fibrin deposition as part of the coagulation pathway. Fibrinogens are only detected in the brain upon the disruption of the BBB.50 Therefore, it is not surprising that fibrinogen was detected at high levels in the GBM scaffold. Soluble fibrinogen inhibits binding of thrombospondin to FINC61 and therefore might play a role in ECM ultrastructure by influencing FINC biding partners.

Likewise, we observed a significant increase in PLMN protein. This serine protease is activated to plasmin by different agents, such as tPA, uPA, kallikrein, and factor XII.62 Plasmin is a proinflammatory agent and a key player in the ECM turnover by processing fibrin, FINC, and LAM.63 It is also implicated in the TGFβ receptor activation, which in turn will lead to increased uPA expression and PLMN processing.64 uPA is involved in TGFβ maturation by cleaving the N-
terminal of its latency-associated peptide (LAP) domain. The interactions between PLMN, uPA, and TGFβ constitute a positive feedback loop that results in the increase of metalloproteinase activity, FINC processing, and stimulates the epithelial to MS transition (EMT).

The high cellular motility and mechanical stress observed in cancer translate to a higher rate of damage to the plasma membrane. Here, we describe that the protein S100-A11 (S10AB) and annexin A2 (ANXA2) were increased in GBM-ECM. S10AB and ANXA2 are binding partners involved in plasma membrane repair in a Ca2+-dependent manner. These proteins are also involved in F-actin and β-tubulin reorganization, supporting pseudopodia and filopodia formation and thus cell motility. Another member of the S100 family, S10A9, is increased in GBM-ECM as well. S10A9 is part of the damage-associated molecular pattern, crucial secreted factors that foster a pro-tumorigenic and inflammatory microenvironment. S10A9 is mainly secreted by tumor-associated immune cells, mediating neutrophil adhesion to FINC in an integrin β2-dependent manner, and is associated with tumor aggressiveness.

Although of very different natures, we found common traits in MB and GBM. AGRIN is downregulated in both MB and GBM ECMs. AGRIN is a glycoprotein highly expressed in the synaptically active regions of the brain, such as the hippocampus and cortex. AGRIN is involved in synapsis formation and plasticity in cholinergic neurons, acting both in neuromuscular junctions and interneurons. Therefore, this result suggests that AGRIN downregulation might contribute to the destabilization of the neuronal networks at the vicinities of the tumor.

**CONCLUSIONS**

Our study represents the first comparative analysis of ECM-specific proteome mapping of site-specific alterations upon CNS malignant transformation, in which the proteome profiles of the CER and ISO were compared to MB and GBM, respectively. The matrisome and matrisome-associated sig-
natures of these two types of tumors reflect their distinct natures. MBs display a more fibrillary protein signature that supports their higher stiffness, ECM accumulation, and dissemination route dependent on impaired adhesion. Conversely, GBM protein signature not only supports an increased stiffness but also displays a higher proteoglycan and glycoprotein diversity, inducing a higher ECM turnover rate, migration, and invasion (Figure 8). Our results highlight the importance of the tissue microenvironment for tumor pathogenesis and to identify candidate targets for therapeutic interventions. The characterization of ECM composition in brain tumors will allow to analyze the crosstalk between the ECM and the cellular components and to investigate related signaling pathways for the search of novel therapeutic strategies in organoid models.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.1c00251.

- Total proteins identified and quantified in ECM-enriched human brain samples (XLSX)
- Regulated proteins identified in ECM-enriched human brain samples comparing GBM + MB versus ISO + CER (XLSX)
- Regulated proteins identified in ECM-enriched human brain samples comparing MB versus CER (XLSX)
- Regulated proteins identified in ECM-enriched human brain samples comparing GBM versus cortex (XLSX)
- Regulated proteins identified in ECM-enriched human brain samples comparing GBM versus MB (XLSX)
- Total proteins identified and quantified in ECM-enriched human brain samples (Table S1); regulated proteins identified in ECM-enriched human brain samples comparing GBM+MB vs isocortex-cerebellum (Table S2); regulated proteins identified in ECM-enriched human brain samples comparing MB vs Cerebellum (Table S3); regulated proteins identified in ECM-enriched human brain samples comparing MB vs Cortex (Table S4); regulated proteins identified in ECM-enriched human brain samples comparing GBM vs MB (Table S5) (PDF)

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Notes

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**ABBREVIATIONS**

ACN, acetonitrile; AGRIN, agrin; ANXA2, annexin A2; BBB, blood–brain barrier; BGH3TGFβI, TGFβI transforming growth factor-beta-induced protein ig-h3; CER, cerebellum; CID, collision-induced dissociation; CNS, central nervous system; CO, collagen; CO4A4, collagen type 4 alpha 4 chain; CS, classical; CTL, normal control; DAMP, damage-associated molecular pattern; DSM, desmoplasic; ECM, extracellular matrix; ELN, elastin; EMIL1, EMILIN1; ETM, epithelial to mesenchymal transition; FA, formic acid; FAK, focal adhesion kinase; FBN1, fibulin; FBN, fibrillin; FDR, false discovery rate; FIBA, hyperexpression of fibrinogen chain A; FIBB, hyperexpression of fibrinogen chain B; FIBG, hyperexpression of fibrinogen chain G; FENCFN1, FN1fbronectin; GBMs, glioblastomas; GEO, gene expression omnibus; GEPIA2, ling interactive analysis 2; iBAQ, intensity-based absolute quantification; ISO, isocortex; ITIH2, interalfa-trypsin inhibitor heavy chain 2; LAMA2, laminin subunit α2; LAMB2, laminin subunit β2; LAP, latency-associated peptide; LC, large cell; LSI, labeling score index; LUM, lumican; MBs, medulloblastomas; MS, mesenchymal; NCE, normalized collision energy; PAI-1, plasminogen activator receptor; VTNC, vitronectin.

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