NEURAL STEM CELLS OF THE SUBVENTRICULAR ZONE: FROM NEUROGENESIS TO GLIOBLASTOMA ORIGIN

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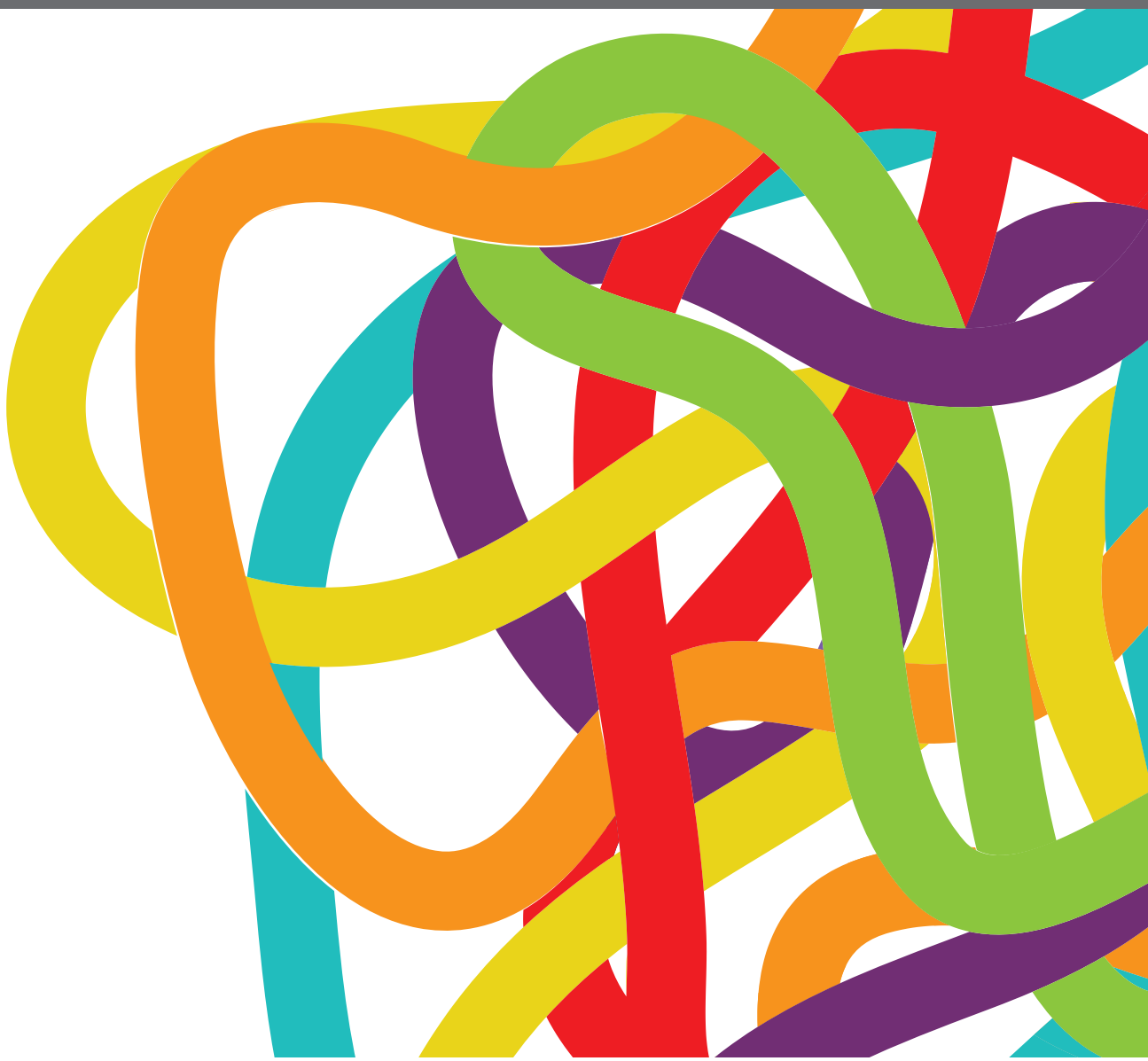

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NEURAL STEM CELLS OF THE SUBVENTRICULAR ZONE: FROM NEUROGENESIS TO GLIOBLASTOMA ORIGIN

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EDITORIAL
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Editorial: Neural Stem Cells of the Subventricular Zone: From Neurogenesis to Glioblastoma Origin

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Keywords: glioblastoma, subventricular zone, neurogenesis, cerebrospinal fluid, glioma stem cells

Editorial on the Research Topic

Neural Stem Cells of the Subventricular Zone: From Neurogenesis to Glioblastoma Origin

Glioblastoma (GBM) is the most common and aggressive primary tumour of the adult central nervous system. Patients with a GBM diagnosis present a poor prognosis and median survival of less than 2 years. Despite the use of standard of care, which includes surgery, chemotherapy and radiation, recurrence is almost inevitable. Understanding the cellular and molecular cues that underlie the origin and development of this aggressive tumour, may lead to the identification of new therapeutic targets to fight GBM progression and recurrence. The Research Topic "Neural stem cells of the subventricular zone: from neurogenesis to glioblastoma origin" includes 13 articles which provide a general view on the influence of the subventricular zone (SVZ), a region that contains neural stem cells (NSCs) in the adult brain, in the origin and progression of GBM (**Figure 1**).

Extremely invasive, GBMs cannot be completely resected by surgery, and are resistant to radiation and chemotherapy. A subpopulation of cells called glioma stem cells (GSCs), which exhibit NSC properties, are responsible for drug resistance and tumour recurrence. A couple of articles in this Topic analyse similarities and differences between NSCs of the SVZ and GSCs. Lombard et al. describe some features shared by GSCs and NSCs and show that the SVZ is a preferred destination site for GSCs, probably due to chemoattractive cues released within the SVZ. The authors summarize some studies showing the role of the chemokine CXCL12 and the protein pleiotrophin as key players in the migration of GSCs from the tumour mass towards the SVZ. In addition, they discuss possible reasons by which GSCs nested in the SVZ benefit from a supportive environment and present an increased resistance to radiation and chemotherapy. In line with this work, Bakhshinyan et al. review different mechanisms involved in the intrinsic and extrinsic regulation of SVZ-derived NSCs that are shared by GSCs. Their analysis shows that the dysregulation of these mechanisms may induce aberrant growth signals, increased invasiveness, sustained angiogenesis or evasion of apoptotic death. The analysis of the differences between NSC and GSC regulatory mechanisms may offer new avenues for the generation of novel targeted therapies for GBM. Noteworthy, NSCs residing in the other neurogenic niche of the adult mammalian brain, the hippocampus, have not been involved in gliomagenesis. Fontán-Lozano et al. provide possible explanations for this fact by analysing cellular and molecular differences between the SVZ and the hippocampal niches, as well as genotypic and phenotypic characteristics of the NSCs present in both niches that might confer SVZ NSCs more opportunities to become

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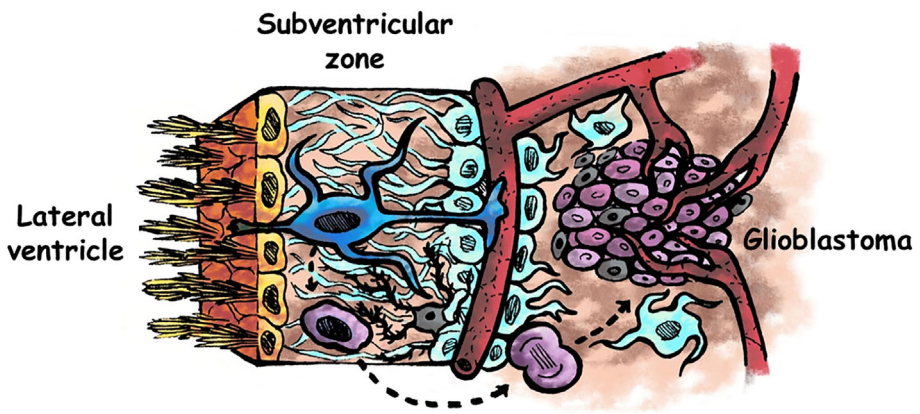

FIGURE 1 | Schematic representation of the human adult subventricular zone and an adjacent glioblastoma. Ependymal cells are represented in yellow, neural stem cells in blue, astrocytes in pale blue, microglia in grey, glioma stem cells in purple, and other type of glioma cells in pale purple. Neural stem cells of the subventricular zone may acquire driver mutations that generate glioma stem cells, which divide to form the tumour mass. Factors present in the cerebrospinal fluid flowing the lateral ventricles may intervene in the genesis and/or in the progression of glioblastomas. Modified from (1).

tumorigenic than hippocampal NSCs. One of the main differences between these two neurogenic niches is the direct contact with the cerebrospinal fluid (CSF), existing in the SVZ but not in the hippocampus. Thus, factors present in the CSF might intervene in NSC biology and in GBM development (**Figure 1**). Indeed, an interesting research article published in this Topic by Carrano et al. investigates the effect of human CSF on primary-cultured GSCs. They show that CSF derived from GBM patients induces an increase in proliferation and migration of GSCs *in vitro*. The transcriptome analysis of GBM cells exposed to CSF reveals alterations in gene expression in pathways promoting cell malignancy. In addition, the authors test these effects *in vivo*, by injecting GBM cells encapsulated in a hydrogel containing human CSF and demonstrate that animals receiving this combination generate larger and more proliferative tumours than controls. These reported effects of CSF on GBM malignancy could be partially responsible for the observed increased aggressiveness of those tumours close to the SVZ. The relevance of the proximity to SVZ in GBM malignancy has also been analysed by some other authors in this Topic. Ripari et al., in an interesting experimental approach, show that animals that receive GBM engraftments close to the lateral ventricles (and therefore to the SVZ) develop larger and more proliferative tumours than animals with GBM grafts distal to the lateral ventricles. GBM proximity to lateral ventricles also leads to decreased median survival of the animals. A noticeable aspect of this study is that the authors also analyse the influence of GBM proximity on the SVZ population of NSCs and progenitors. They demonstrate that tumour proximity to the lateral ventricles induces a decrease in the proliferation of SVZ NSCs and their progeny. These results emphasize the importance of deciphering bidirectional molecular signalling between GBM and the SVZ to identify pathways contributing to tumour progression in patients with GBM located proximal to the SVZ. In relation to this, the article by Mistry et al. analyses possible correlations between patient survival and GBM distance to the SVZ and demonstrates a significantly decreased survival when the tumour contacted the SVZ. However, they do not report a survival correlation with the GBM-SVZ distance. Their results have clinical relevance to test differential effectiveness of SVZ radiation in patients with SVZ-contacting or non-contacting GBMs. Other predictive factors for GBM progression that may help to make more personalized and precise treatments have been studied by Jiang et al. They show that SVZ involvement is correlated with higher risk for non-local progression in patients with IDH-wildtype GBM. This correlation is also described for male gender and MGMT promoter methylation.

Experimental models of GBM are crucial to understand the mechanisms involved in the progression of this devastating disease and to find more efficient treatments. Besides, as IDH-wildtype and IDH-mutant GBMs differ in their cell of origin and in their genetic alterations, different animal models need to be designed for both GBM types. Gomez-Oliva et al. have carefully evaluated the different experimental models used in the study of GBM. They first describe advantages and disadvantages of classical approaches such as cell cultures from GBM cell lines or patient-derived cells and xenografts to continue with more novel approaches such as genetically engineered mouse models, organotypic cultures, brain organoids or 3D-bioprinted mini-brains. Kim et al. give an overview of genetic alterations and cell-of-origin in IDH-wildtype and IDH-mutant GBMs and discuss recent genetically engineered mouse models in which NSCs or progenitor cells are transformed by specific genetic alterations to model either IDH-wildtype or IDH-mutant GBM.

There is consensus that IDH-wild type GBM may arise from accumulation of somatic mutations in SVZ NSCs and/or in glial precursor cells that confer growth advantages resulting in uncontrolled proliferation. These driver mutations could originate

from genetic alterations as well as by epigenetic modifications. Two articles of this Topic analyse this possibility. Lozano-Ureña et al., based on previous reports describing a dysregulation of the imprinting pattern in different tumours, show that there is an extensive alteration in the expression of imprinted genes in GBMs. Furthermore, they demonstrate that adult NSCs from the human SVZ cannot be distinguished from GBM cells based on imprinted gene expression data, which supports the hypothesis that NSCs are the cells-of-origin of IDH-wild type GBMs. Valor and Hervás-Corpión review multiple epigenetic activities that are involved in glioma malignancy and some therapeutic approaches proposed to overcome these epigenetic changes. For instance, they describe in detail the role of Polycomb repressive complexes or the histone variant H3.3 in the maintenance of the GSC phenotype.

This Research Topic also includes some articles providing potential therapeutic strategies for GBM. Rackov et al. analyse the effect of Nilo1, a monoclonal antibody that marks NSCs and early progenitors, on patient-derived GSCs. They show that Nilo1 recognizes GSCs and reduces cell viability and self-renewal in a subset of GSCs. Their results open the possibility of studying the effect of this antibody-based therapy in preclinical studies alone or in combination with other drugs. Another therapeutic option for GBM has been discussed by Geribaldi-Doldán et al. focusing on one important component of both SVZ and GBM niches: the microglia. Microglial cells within the GBM microenvironment acquire a tumour-supportive phenotype, which is analysed by the authors providing details on some relevant molecules and epigenetic mechanisms involved in its acquisition. Accordingly, they discuss possible therapies based on microglia as a target to complement the currently used treatments for this disease. Also, the already mentioned articles by Lombard et al. and Bakhshinyan et al., include interesting analyses on the SVZ as a potential therapeutic target in GBM. Additionally, research discussed in the articles by Carrano et al. and Ripari et al. provide evidence of the existence of factors present in the CSF and/or in the SVZ that promote GBM malignancy, which encourages the study of the identification of molecules responsible for these effects with the goal of their use as biomarkers and/or targets for this disease.

Overall, the collection of articles contained in this Research Topic contributes to the understanding of GBM from different perspectives. Firstly, it provides an analysis of the similarities and differences between GSCs and NSCs of two adult neurogenic niches: the SVZ and the hippocampus. Secondly, it highlights the role of the SVZ in GBM tumour progression and patient survival, identifying molecules in the CSF as responsible for tumour progression, aggressiveness and malignancy. Finally, it discusses the most adequate models to study GBM, and to end with, it suggests new therapeutic targets providing potential strategies for the treatment of GBM.

AUTHOR CONTRIBUTIONS

EM, NZ, CC, and HG-C contributed equally to this Research Topic by acting as Guest Editors and writing the editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Potential Therapeutic Effects of the Neural Stem Cell-Targeting Antibody Nilo1 in Patient-Derived Glioblastoma Stem Cells

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Glioblastoma (GBM) is the most devastating and least treatable brain tumor with median survival <15 months and extremely high recurrence rates. Promising results of immune checkpoint blockade obtained from pre-clinical studies in mice did not translate to clinic, and new strategies are urgently needed, particularly those targeting GBM stem cells (GSCs) that are held responsible for drug resistance and tumor recurrence. Patient-derived GSC cultures are critical for finding effective brain tumor therapies. Here, we investigated the ability of the recently described monoclonal antibody Nilo1 to specifically recognize GSCs isolated from GBM surgical samples. We employed five patient-derived GSC cultures with different stemness marker expression and differentiation potential, able to recapitulate original tumors when xenotransplanted *in vivo*. To answer whether Nilo1 has any functional effects in patient-derived GSCs lines, we treated the cells with Nilo1 *in vitro* and analyzed cell proliferation, cell cycle, apoptosis, sphere formation, as well as the expression of stem *vs.* differentiation markers. All tested GSCs stained positively for Nilo1, and the ability of Nilo1 to recognize GSCs strongly relied on their stem-like phenotype. Our results showed that a subset of patient-derived GSCs were sensitive to Nilo1 treatment. In three GSC lines Nilo1 triggered differentiation accompanied by the induction of p21. Most strikingly, in one GSC line Nilo1 completely abrogated self-renewal and led to Bax-associated apoptosis. Our data suggest that Nilo1 targets a molecule functionally relevant for stemness maintenance and pinpoint Nilo1 as a novel antibody-based therapeutical strategy to be used either alone or in combination with cytotoxic drugs for GSC targeting. Further pre-clinical studies are needed to validate the effectiveness of GSC-specific Nilo1 targeting *in vivo*.

Keywords: Nilo1, antibody, glioblastoma, glioma stem cells, neural stem cells, immunotherapy

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BACKGROUND

Glioblastoma (GBM, World Health Organization grade IV glioma) is the most aggressive and least treatable brain tumor. Current therapy for newly diagnosed GBM includes maximal surgical resection followed by concurrent radiation therapy with temozolomide (TMZ) and subsequent adjuvant TMZ therapy. Despite this standard of care treatment, median overall survival has only been extended to 14.6 months and 5-year survival rates are less than 10% (1). In addition, there is no effective treatment at the time of recurrence, which occurs in most of the patients. Bevacizumab – a humanized monoclonal antibody against VEGF (vascular endothelial growth factor) – was the most promising therapeutic agent for recurrent GBM. However, clinical trials have shown that, while it prolongs progression-free survival for 3 months, this does not translate to increased overall survival (2, 3). In fact, anti-VEGF therapy results in increased tumor invasiveness at the time of progression, which challenges surgical resection of recurrent GBM (4) and possibly even worsens the quality of life (3).

Significant progress in immuno-oncology has led to new treatments, such as immune checkpoint blockade, CAR (chimeric antigen receptor) T cell therapy, cytokine therapy, oncolytic viruses and dendritic cell and peptide vaccines. Currently, immune checkpoint blockade utilizing monoclonal antibodies against PD-1 (programmed cell death-1) or its ligand (PD-L1) is being extensively studied in GBM clinical trials (e.g., NCT02336165, NCT02617589, NCT02550249, NCT02017717); however, their efficacy so far has been very limited. Only a small subset of patients (8%) showed objective responses in a trial of anti-PD-1 in recurrent GBM (5), and these responses were transient due to acquired resistance mechanisms (6). New candidate immunotherapeutics are thus needed to be used in combination with immune checkpoint blockade and overcome GBM resistance mechanisms.

The model of cancer initiating cells proposes that tumor growth depends on a small population of undifferentiated cells, termed cancer stem cells (CSCs) because of their self-renewal ability and multilineage differentiation potential (7, 8). Due to their slow cell cycle and overexpression of efflux pumps, CSCs are held responsible for driving tumor progression and recurrence after treatment with irradiation and cytotoxic drugs. In fact, such treatment might lead to CSC enrichment after eliminating other cancer cells. Identifying CSCs and specifically targeting signaling pathways responsible for maintenance of their tumor-initiating and stem cell properties are thus of high clinical relevance. Nonetheless, specific targeting of glioblastoma stem-like cells (GSCs) is still challenging, given that truly specific GSC markers have not been described thus far. GSCs express markers associated with neural stem cells (NSCs), such as CD133, Nestin, CD44 and CD90. Substantial evidence suggests that GSCs originate from NSCs that undergo malignant transformation and migrate from subventricular zone (SVZ) to distant regions of the brain (9–11). In accordance with this hypothesis, GSCs share many features with NSCs of the SVZ, like high proliferative and migration potential, association with vasculature and reciprocal communication with perivascular niche (12). Interestingly, key signaling pathways responsible for NSC maintenance, proliferation, differentiation and migration, like EGFR, PDGFR, p53 or PTEN, are frequently altered in GBM. Novel therapeutic strategies directed to target not only GSCs, but also their putative cells of origin – NSCs of the SVZ – are thus worth considering for clinical implementation (12).

Nilo1 (neural identification lineage from olfactory bulb) is a monoclonal antibody generated after immunization of hamsters with olfactory-bulb-derived mouse neurospheres (13). Nilo1 specifically marks NSCs and early progenitors in the mouse brain (13), however, it is also able to recognize a homologous antigen in human neurospheres derived from GBM patients (14). Nilo1 treatment arrests mouse neurosphere proliferation (13), suggesting that it might recognize functionally relevant molecule involved in NSC stem cell maintenance. Nonetheless, whether Nilo1 affects human GSC functions remained unknown.

The aim of this study was to analyze the effects of Nilo1 treatment in patient-derived GSC cultures, which represent indispensable *in vitro* model for GBM basic studies and drug development (15, 16). We previously characterized these cells and showed that they express stem cell markers, grow as 3D neurospheres in serum-free conditions, and form tumors when xenotransplanted to immunodeficient mice brain, recapitulating the phenotype and gene expression of the original tumor (17). Our previous study revealed that Nilo1 indeed recognizes human GSCs (14), however, in the present work we observed that the effects of Nilo1 varied between GSC lines derived from different patients. Namely, one GSC line was completely resistant to Nilo1 treatment, while four other lines were sensitive. In three of those lines, Nilo1 led to slowing down the cell cycle and triggered differentiation, which was accompanied by the induction of cell cycle inhibitor p21. Most strikingly, in one GSC line Nilo1 completely abrogated self-renewal and led to apoptosis, associated with the induction of Bax. Overall, our data show that Nilo1 targets a functionally relevant molecule for GSC maintenance and suggest that patient-derived GSCs can be stratified according to their differential Nilo1 sensitivity. This establishes Nilo1 as a potential therapeutic agent to be used in combination with existing immunotherapy to improve GBM clinical outcome.

Abbreviations: 7-AAD, 7-aminoactinomycin D; ANOVA, Analysis of variance; CAR, chimeric antigen receptor; CDK2, cyclin-dependent kinase 2; CSCs, cancer stem-like cells; DAMPs, danger-associated molecular patterns; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; GBM, glioblastoma multiforme; GSCs, GBM stem-like cells; MRI, magnetic resonance imaging; Nilo1, neural identification lineage from olfactory bulb 1; NSC, neural stem cells; OPC, oligodendrocyte precursor cells; PBS, phosphate buffered saline; PD-1, programmed death-1; PD-L1, programmed death ligand-1; RT-PCR, real time polymerase chain reaction; SD, standard deviation; SVZ, subventricular zone; Thy-1, thymocyte differentiation antigen 1; TMZ, temozolomide; VEGF, vascular endothelial growth factor.

METHODS

Isolation of GSCs, Cell Culture, and Differentiation

Glioblastoma stem-like cells were isolated from five freshly obtained GBM samples. All patients gave informed consent and the use of tumor samples was approved by Hospital La Fe (Spain) Ethics Committee. All patient-derived GSCs used in this study have been previously characterized and have generated tumors when xenotransplanted into nude mice [Ref. (17), and unpublished data]. GSCs cell expansion was carried out in serum-free DMEM/F-12 supplemented with N2, 300 ng/ml hydrocortisone, 2 μg/ml heparin, 30 ng/ml triiodothyronine, 10 ng/ml EGF and 20 ng/ml FGF-2. GSCs were routinely allowed to form spheres during 10 days in culture, dissociated using Accutase and then split 1:10. Medium was replaced every 3–5 days. For differentiation, the GSCs were allowed to form spheres during 6 days and then the medium was replaced with differentiation medium, containing the same basal media supplemented with 10% FBS and lacking EGF and FGF-2. All experiments were performed in mycoplasma-free conditions.

Mesenchymal Stem Cell Culture

Human adipose tissue samples were obtained at private plastic surgery clinic (Clinica Dra. Isabel Moreno) from lipoaspiration procedures from 8 healthy patients under surgery by aesthetic reasons, aged between 18 and 35, following written informed consent and ethical research project approval by both Clinica Dra Isabel Moreno and Hospital General Foundation in Valencia ethical boards under the research project of Dr. Escobedo-Lucea. All the patients were previously screened for human immunodeficiency virus (HIV), hepatitis C and other infectious diseases. Cells were obtained following the protocol established from Planat-Benard (18), with a few modifications. Briefly, samples were digested in a solution of 1 mg/ml collagenase type I from Clostridium Histolyticum (Gibco, Grand Island, NY, United States) for 90 min at 37°C. The cells were then washed with 0.5% of HSA in Hank's balanced salt solution (Gibco, Grand Island, NY, United States) and after discarding mature adipocytes, seeded in culture flasks with growth medium, Dulbecco's modified Eagle's medium (Invitrogen) supplemented with human or bovine serum mesenchymal stem cell qualified (Gibco, Grand Island, NY, United States), in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The medium was replaced every 3 days. When primary culture became subconfluent, cells were detached using Tryple (Invitrogen) and subcultured in growth medium.

Fluorescence Confocal Microscopy

Glioblastoma stem-like cell tumorspheres or dissociated single cells were plated on Matrigel-coated coverslips, fixed in 4% paraformaldehyde for 10 min and blocked with 10% BSA/0.05% Tween for 1 h at room temperature. Primary Nilo1 monoclonal antibody was generated by the fusion of hamster B cells and the mouse myeloma X63Ag8 (13) and purified in CNB-CSIC (Madrid, Spain). Cells were incubated with Nilo1 1:100 overnight at 4°C, followed by 1:200 FITC-conjugated anti-hamster secondary antibody from BD. F-actin was stained with Phalloidin-iFluor 647 (1:40, 1 h at room temperature, Abcam) and nuclei with DAPI (1:5000, 10 min at room temperature, Sigma). For stem-like and differentiation markers we used anti-GFAP (1:500, Dako) and anti-OLIG2 (1:500, Millipore), followed by Alexa fluor 555 goat anti-rabbit antibody (1:1000) from Invitrogen. To estimate cell viability within the tumorspheres, 5 μg/ml 7-AAD (BioLegend) was used. Coverslips were mounted with Fluorsave (Calbiochem). The images were taken using the glycerol ACS APO 20x NA0.60 immersion objective of a confocal fluorescence microscope (SPE, Leica-Microsystems) and analyzed using FIJI software.

Nilo1 Treatment and Cell Viability Assay

Glioblastoma stem-like cell tumorspheres or dissociated single cells were plated in 96-well plate at a density of 3000 cells per well and treated with Nilo1 monoclonal antibody or *InVivo*MAb hamster anti-mouse CD3ε (BioXCell) as irrelevant control, at a concentration of 0.5 mg/ml for indicated time points. For 7-day treatment, the media was replenished once on Day 3. Cell viability was assessed using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay from Promega, according to manufacturer's instructions.

Neurosphere Formation Assay

After tumorsphere dissociation, GSCs were plated in 96-well plate at a density of 50 cells per well and treated as above, with 6 replicates per condition. Neurospheres were counted and photographed after 21 days.

RNA Extraction and RT-PCR Analysis

Total RNA was extracted with TRIzol (Sigma) according to the manufacturer's instructions and 1 μg RNA was retro-transcribed using cDNA kit from Applied Biosciences. Real-time PCR was performed using TB Green Premix Ex Taq RR420 (Takara) and detected by ABI PRISM 7900HT (Applied Biosystems). Data were analyzed using SDS 2.4 software (Applied Biosystems), normalized to the expression of β-actin and represented as the fold-change with respect to controls. All primers were synthesized, desalted and purified by Sigma, and the sequences were as follows: *NES*, 5′-GAGGTGGCCACGTACAGG-3′ (forward) and 5′-AAGCTGAGGGAAGTCTTGGA-3′ (reverse); *PROM1*, 5′-GGAAACTAAGAAGTATGGGAGAACA-3′ (forward) and 5′-CGATGCCACTTTCTCACTGAT-3′ (reverse); *OLIG2*, 5′-AGCTCCTCAAATCGCATCC-3′ (forward) and 5′-ATAGTCGTCGCAGCTTTCG-3′ (reverse); *PDGFRA*, 5′-CCACCTGAGTGAGATTGTGG-3′ (forward) and 5′-TCTTCAGGAAGTCCAGGTGAA-3′ (reverse); *S100B*, 5′-GGAAGGGGTGAGACAAGGA-3′ (forward) and 5′-GGTGGAAAACGTCGATGAG-3′ (reverse); *GFAP*, 5′-GTGGTGAAGACCGTGGAGAT-3′ (forward) and 5′-GTCCTGCCTCACATCACATC-3′ (reverse); *MAP-2*, 5′-CCTGTGTTAAGCGGAAAACC-3′ (forward) and 5′-AGAGACTTTGTCCTTTGCCTGT-3′ (reverse).

Flow Cytometry

Glioblastoma stem-like cell tumorspheres were dissociated using Accutase (5 min, 37°C) and cells were stained with anti-PDGFRA-PE (BD), -CD133-APC (Miltenyi Biotec), -CD24-FITC (BD) and -CD90-FITC (Beckman Coulter) at the dilution of 1:100. The cells were analyzed on Attune Acoustic Focusing Cytometer from Applied Biosystems. The data were analyzed using FlowJo software (Tristar).

Apoptosis Analysis

Following Nilo1- or irrelevant control-treatment, the tumorspheres were dissociated with Accutase (5 min, 37°C), stained with Pacific Blue Annexin V Apoptosis detection kit (BioLegend) according to the manufacturer's protocol, and analyzed by flow cytometry.

Cell Cycle Analysis

To analyze cell cycle, dissociated tumorspheres were washed with PBS, permeabilized with detergent, stained with PI for 30 min at 37°C according to the manufacturer's instructions (DNA-Prep Reagent Kit, Beckman Coulter) and analyzed by flow cytometry.

Statistical Analysis

Statistical significance was determined by unpaired two-tailed Student's *t*-test for comparisons between two groups, or by 1- or 2-way ANOVA for multiple comparisons, followed by Bonferroni *post hoc* test. Differences were considered significant when $p < 0.05$. All statistical analyses were conducted using Prism 8 software (GraphPad).

RESULTS

Human GBM Neurospheres Stain Positively for Nilo1

Nilo1 has been shown to recognize NSCs in mice, as well as human GBM cells derived from patients. While Nilo1 arrests proliferation in mouse NSCs, whether Nilo1 has such an effect on human GSCs remained unknown. In this study, we used GSC-enriched cultures isolated from five GBM patients (GBM18, GBM27, GBM38, GBM123, and GBM128B) and grew them as neurospheres in growth-factor-enriched stem cell medium (19). Nilo1 gave positive staining in all five tested lines (**Figure 1A**, left panels), confirming our previous findings that Nilo1 recognizes human GBM neurospheres (14). To visualize cell morphology, neurospheres were dissociated into single cells and F-actin (filamentous actin) was stained with phalloidin. Although the relative abundance of Nilo1-positive cells varied between different GSC lines, only a small proportion of cells were positive for Nilo1 within each line (**Figure 1A**, right panels).

Nilo1 Treatment Reduces Cell Viability and Self-Renewal Properties in a Subset of GSCs

To investigate the effect of Nilo1 stimulation on patient-derived GSCs, we allowed neurospheres to form during 10 days in stem cell culture, and then treated them for 7 days with 0.5 mg/ml Nilo1, the highest concentration that efficiently inhibited proliferation in mouse neurospheres (13). The numbers of viable cells were similar in Nilo1- and irrelevant antibody control-treated cells, except in GBM38, where Nilo1 treatment slightly reduced the numbers of viable cells compared with control (**Figure 1B**). Cell cycle analysis showed that during neurosphere formation G1 phase lengthens overtime and that the division cycles become longer resulting in decreased proportions of actively proliferating cells at Day 10 compared with Day 3 of cell culture (**Supplementary Figure S1**). We thus hypothesized that Nilo1 treatment might have an effect on the growth of single cells during neurosphere formation. To investigate this, we dissociated the neurospheres and treated them with Nilo1 or an irrelevant control antibody for 3 or 7 days. As before, Nilo1 didn't show any effect on the viability of GBM18 and GBM123 cells compared with control (**Figure 1C**). However, in this setting Nilo1 treatment significantly reduced viable cell numbers in GBM27 and GBM128B cells (**Figure 1C**). Most strikingly, Nilo1 completely abrogated cell growth in GBM38 (**Figure 1C**). To exclude the possibility that Nilo1 treatment would be toxic to normal stem cells, we performed a 7-day-Nilo1 treatment on adipose tissue-derived mesenchymal stem cells. Our results confirmed that the viability of normal stem cells was not altered, suggesting that Nilo1 effects are restricted to GSCs (**Supplementary Figure S2**).

Since Nilo1 treatment affected the viability of replicating single cells and not the fully formed spheres, we considered that Nilo1 might interfere with GSC self-renewal properties. We thus performed sphere-formation assay over the course of 3 weeks to investigate whether Nilo1 treatment would affect the ability of GSCs to form neurospheres. As in viability assay, Nilo1 didn't show any effect on sphere formation in GBM18 and GBM123 cells compared with control treatment (**Figure 2** and **Supplementary Figure S3**). However, Nilo1 effectively reduced GSC stemness in GBM27, GBM38 and GBM128B, as indicated by decreased number of spheres at 21 days after plating (**Figure 2** and **Supplementary Figure S3**). Increased proportions of adherent cells were observed in Nilo1-treated GBM18 and GBM128B (**Figure 2** and **Supplementary Figure S3**), suggesting that Nilo1 might interfere with sphere formation by inducing cell differentiation. Again, most striking effect was detected in GBM38, where not a single sphere could be observed after the Nilo1 treatment (**Figure 2**). To discard the possibility that some GSC lines were particularly sensitive to Nilo1 treatment due to suboptimal growing conditions and/or cell death, we performed 7-AAD staining of GBM27, GBM38, and GBM128B control neurospheres to check for the presence of non-viable cells at the outermost neurosphere layers as a sign of non-optimal growth (20). Nonetheless, we didn't detect a significant number of 7-AAD-positive cells in external layers of these neurospheres, suggesting they were healthy and viable (**Supplementary Figure S4**). Collectively, our data showed that Nilo1 stimulation

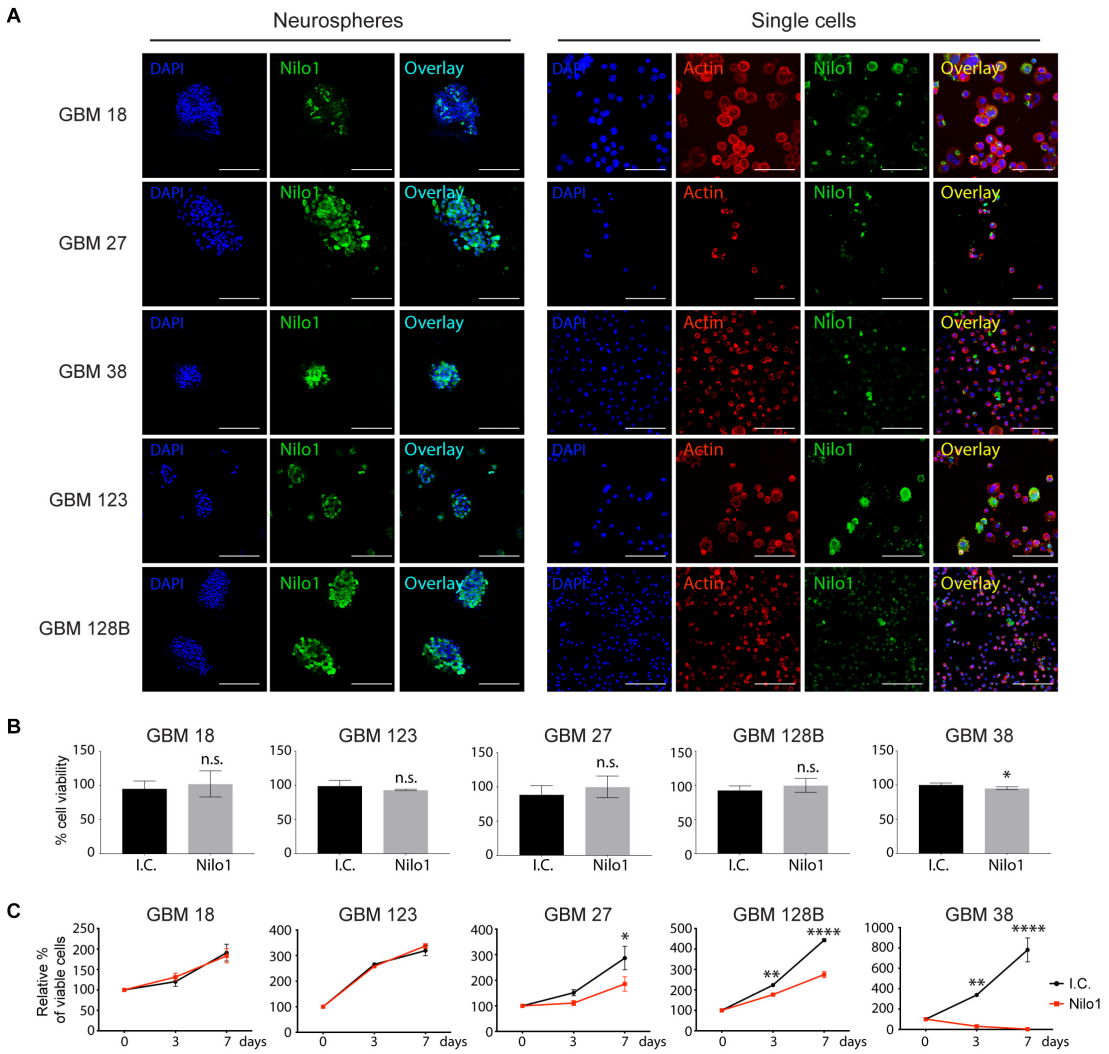

FIGURE 1 | Nilo1 treatment reduces the viability of GSCs. **(A)** Confocal microscopy showing Nilo1-positive staining in five lines of patient-derived GSCs grown as neurospheres (left) or after dissociation to single cells (right). DAPI is shown in blue. Scale bar shows 100 μm. **(B)** MTS assay showing the effect of Nilo1 (0.5 mg/ml) 7-day-treatment on GSC tumorsphere viability compared with the i.c. (irrelevant control, hamster anti-mouse CD3ε). Data were normalized to i.c. treatment and show mean ± SD ($n = 3$), $^{*}p < 0.05$, Student's *t*-test. **(C)** GSCs were dissociated to single cells and treated with Nilo1 or i.c. (0.5 mg/ml) for 3 or 7 days. MTS assay showed a reduction in GSC viability after Nilo1 treatment. Data were normalized to day 0 and show mean ± SD ($n = 3$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, two-way ANOVA (with Bonferroni correction).

significantly reduces stemness in three out of five tested patient-derived GSC lines.

Differentiation Reduces the Ability of Nilo1 to Recognize GSCs

Our data showed that Nilo1 affects GSC self-renewal properties, which suggested that Nilo1 specifically targets cells in stem-like state. To investigate this, we next asked whether Nilo1 capacity to recognize GSCs would decrease upon their differentiation. Like NSCs, GSCs have the ability to differentiate into three downstream cell lineages: neuron, astrocyte and oligodendrocyte (9). However, differentiation efficiency and lineage choice vary significantly between each GSC line (15). To induce GSC differentiation, we first allowed tumorspheres to form during 6 days in presence of EGF and FGF-2, and then removed growth factors from culture medium and added 10% FBS. We noted that GSCs did not undergo cell death upon growth factor withdrawal, but instead tumorspheres attached to the plate and the cells started to migrate away from the sphere and change their morphology. To assess the efficacy of GSC differentiation, we analyzed stem *vs.* differentiation markers both at the levels of mRNA and protein expression after 4 days of differentiation. Of note, similar results were obtained using a 10-day differentiation protocol (data not shown). Following differentiation, GBM18 significantly downregulated *NES* (Nestin), a cytoskeletal protein typically expressed by NSCs and progenitor cells in developing brain (21), and *PROM1* (Prominin1, i.e., CD133), a cell

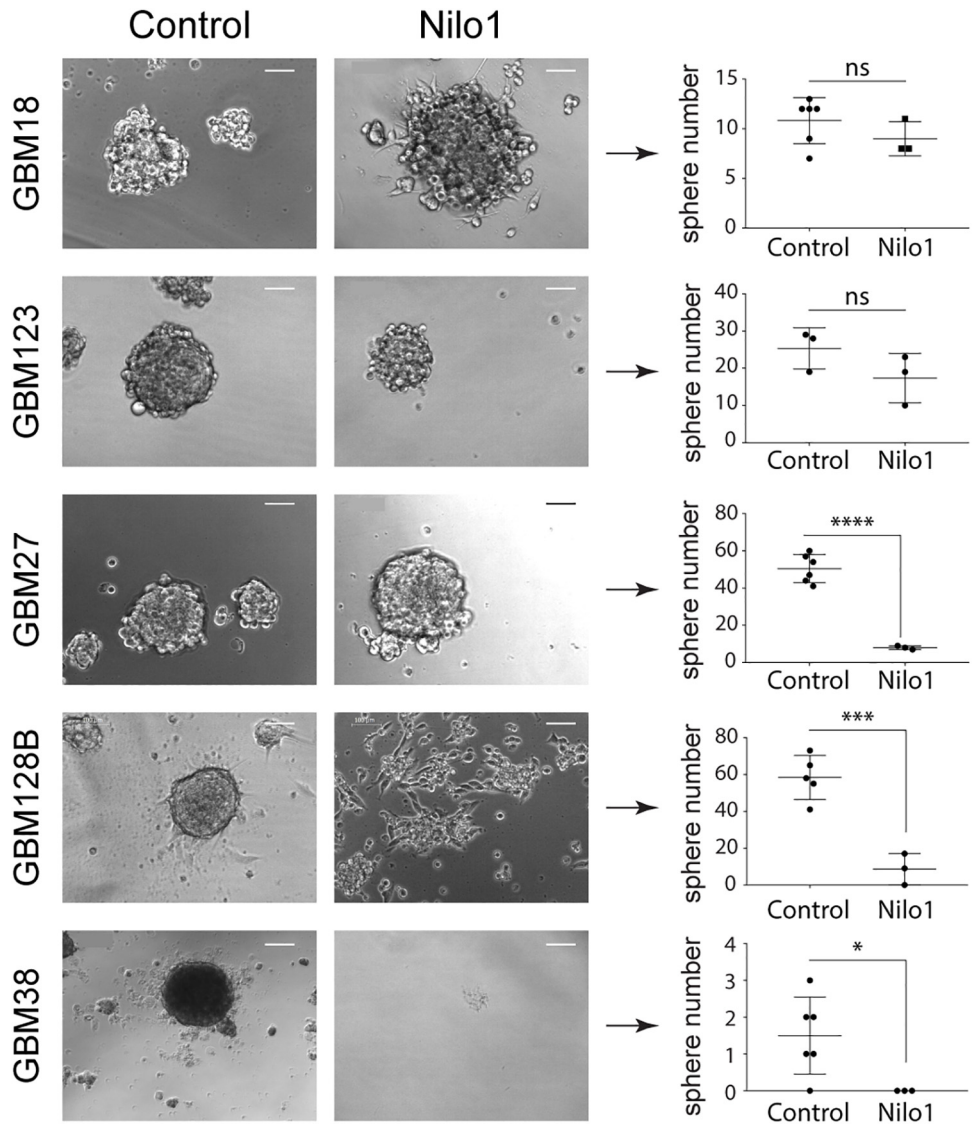

FIGURE 2 | Nilo1 treatment reduces GSC self-renewal properties. For sphere-formation assay, GSC neurospheres were dissociated and 50 cells were plated per well in a 96-well plate. GSCs were treated with Nilo1 or irrelevant control (i.c.) at 0.5 mg/ml and spheres were counted after 3 weeks. Scale bar shows 100μm. Data show mean ± SD ($n = 6$), $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$, Student's *t*-test. NS, not significant.

surface marker of NCSs (22) (**Figure 3A**). In addition, we detected decreased expression of oligodendrocyte lineage markers (*OLIG2* and *PDGFRA*) in differentiated GBM18 cells, which was confirmed by flow cytometry (**Figure 3A**) and immunofluorescence analysis (**Figure 3B**). Compared with their stem-like counterparts, differentiated GBM18 cells showed an increase in CD24 levels, which has been correlated with neuronal differentiation and neuronal maturation (23) (**Figure 3A**). In GBM27 line, differentiation induced significant decrease of *NES* and *OLIG2* expression, paralleled by an increase in astrocyte markers *S100B* and *GFAP* (**Figure 3C**). Increased levels of GFAP after differentiation were confirmed by immunofluorescence analysis (**Figure 3D**), showing that GBM27 adopted an astrocyte phenotype. In addition, flow cytometry analysis revealed that differentiated GBM27 cells upregulated CD24 (**Figure 3E**), which can be found in neurons undergoing differentiation as well as astrocytes (24). In GBM38, differentiation induced an increase in CD24 levels, suggesting their neuronal differentiation (**Figure 3F**). Stem-like GBM38 cells showed relatively high levels of GFAP which was decreased after differentiation (**Figure 3G**). Conversely, the levels of OLIG2 increased, suggesting the presence of oligodendrocyte differentiation in GBM38 (**Figure 3G**). As evidenced by increased GFAP levels and decrease in *NES* and *OLIG2* expression, GBM123 differentiated into astrocytes (**Figures 3H,I**). Finally, GBM128B line evidently differentiated into neurons, as we detected decreased levels of *PDGFRA* and *OLIG2* together with increased expression of CD24 and *MAP2* (**Figures 3J–L**).

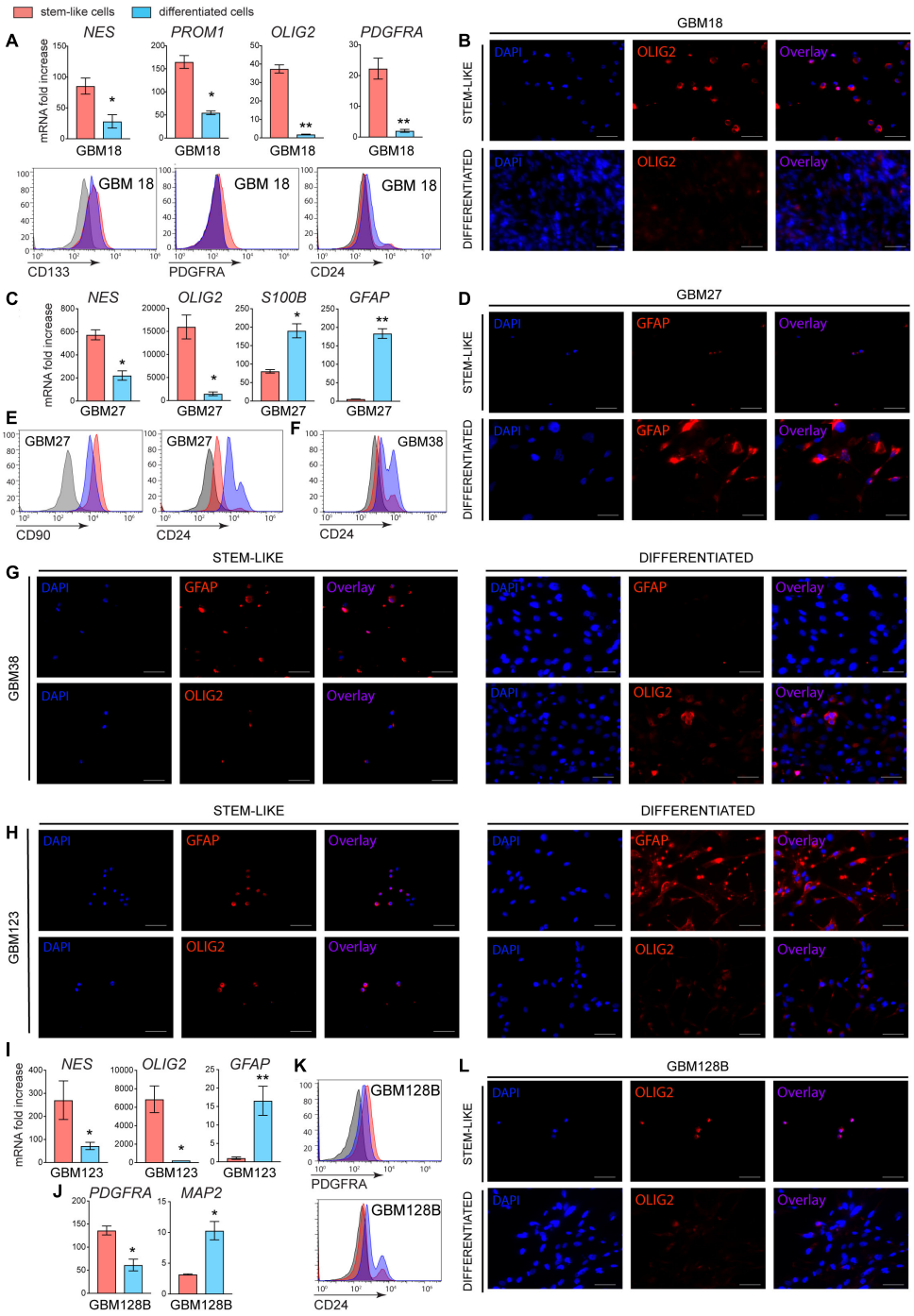

FIGURE 3 | Stemness and differentiation markers in stem-like and differentiated GSCs. GSCs were grown in FBS-free media supplemented with growth factors for 10 days (stem-like cells). To induce differentiation, GSCs were grown in the same media for 6 days to form tumorspheres and then changed to growth-factor-free media supplemented with 10% FBS for additional 4 days (differentiated cells). **(A)** RT-PCR and flow cytometry analyses of mRNA and surface protein expression for GBM18. **(B)** Immunofluorescence analysis showing OLIG2 levels in GBM18. **(C)** RT-PCR analysis and **(D)** immunofluorescence showing the induction of astrocyte markers in GBM27. Flow cytometry showing increased CD24 surface levels in GBM27 **(E)** and GBM38 **(F)** after differentiation. **(G)** Immunofluorescence showing a decrease in GFAP and an increase in OLIG2 levels in GBM38 after differentiation. **(H)** Immunofluorescence analysis of GBM123, showing an increase in GFAP and decrease in OLIG2 levels after differentiation. **(I)** RT-PCR showing increased GFAP expression in GBM123. **(J)** RT-PCR and **(K)** flow cytometry showing decreased PDGFRA and increased neuronal markers MAP2 and CD24 in GBM128B following differentiation. **(L)** Immunofluorescence showing a decrease in OLIG2 levels in differentiated GBM128B cells. Gene expression analysis was done for all GSC lines at once and the data were normalized to GBM38, so the relative fold change can be appreciated between different GSC lines. Data show mean ± SD ($n = 3$), $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, Student's *t*-test. For flow cytometry, negative staining control is in gray. Shown are representative histogram plots of two experiments performed. For immunofluorescence, white bar shows 50 μm. Shown are representative images of two independent experiments performed.

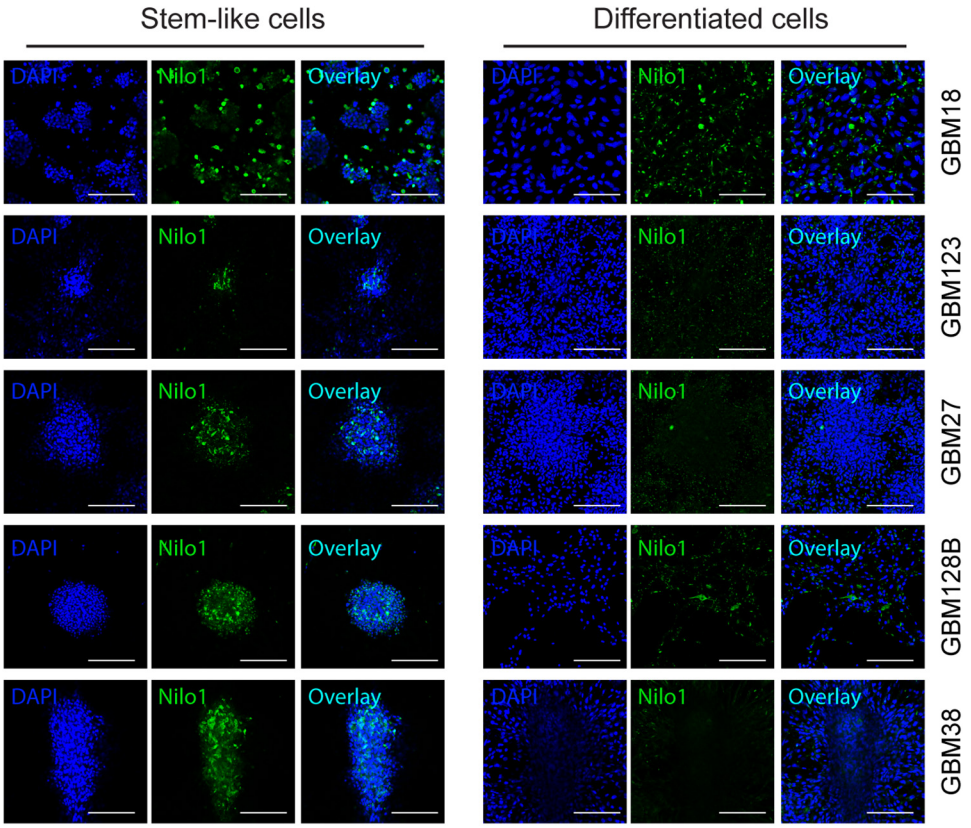

FIGURE 4 | Nilo1 recognizes GSCs in stem-like state. GSCs were grown as in **Figure 3**. Confocal microscopy showing reduced proportions of Nilo-1 positive cells in differentiated GBM123, GBM27, GBM128B and GBM38 cells **(right)** compared with their stem-like equivalents **(left)**. Scale bar shows 100 μm.

We next analyzed the ability of Nilo1 to recognize GSCs cultured in stem cell conditions *vs.* those cultured in differentiation medium. Confocal microscopy (**Figure 4**) showed that, compared with tumorspheres grown in stem cell medium (left panels), the proportions of Nilo1-positive cells dramatically decreased along with the loss of stem cells upon GSC differentiation (right panels). Overall, these data showed that the decrease of Nilo1 ability to recognize GSCs correlates with the degree of their differentiation, which is in line with the hypothesis that Nilo1 identifies stem-like GSCs.

Nilo1 Induces Apoptosis in GBM38

The effect of Nilo1 treatment was the most evident in GBM38 cells, since it sharply reduced cell viability and completely abrogated sphere formation in these cells (**Figures 1C**, 2). To get an insight of the mechanism by which Nilo1 kills GBM38 cells, we tested the ability of Nilo1 to induce apoptosis. After 7 days of Nilo1 treatment, flow cytometry analysis revealed significantly increased AnnexinV$^+$/7-AAD$^+$ dead cell percentage (33 *vs.* 47%) in Nilo1-treated cells compared with controls (**Figure 5A**, upper panels). In addition, the percentage of apoptotic AnnexinV$^+$/7-AAD$^-$ cells was increased (4 vs. 13%), which suggested that Nilo1 acts through induction of apoptosis. To verify this, we assessed the ratio between apoptosis promoter *BAX* and apoptosis inhibitor *BCL2* expression, which is known to determine the cell's susceptibility to die in response to apoptotic stimulus (25). Indeed, *BCL2* tended to decrease already at 3 days after Nilo1 treatment, and it was significantly decreased at 7 days, while the expression levels of *BAX* were significantly increased at this time point in Nilo1-treated cells compared with controls (**Figure 5A**, lower panels). These results demonstrated that Nilo1 inhibits GBM38 growth by inducing apoptosis.

Nilo1 Arrests Cell Cycle in GBM18, GBM27, and GBM128B

We also tested the capacity of Nilo1 to induce cell death in other GSCs, however flow cytometry showed similar proportions of apoptotic and dead cells in GBM18, GBM27, GBM123 and GBM128B after 3 (data not shown) and 7 days of Nilo1 compared with control treatment (**Figure 5B**). As shown in **Figure 1C**, Nilo1 treatment reduced the numbers of viable cells in GBM27 and GBM128B, although these cells continued to grow during the course of the experiment. This finding indicated that Nilo1 might affect cell cycle progression in these cells, without having a pro-apoptotic effect. We therefore analyzed cell cycle profiles in GSCs, and found that Nilo1 treatment increased the percentage of cells in G0/G1 phase (69.7 vs. 74.6% in GBM27 and 63.0 vs. 69.2% in GBM128B, **Figure 5C**) compared to

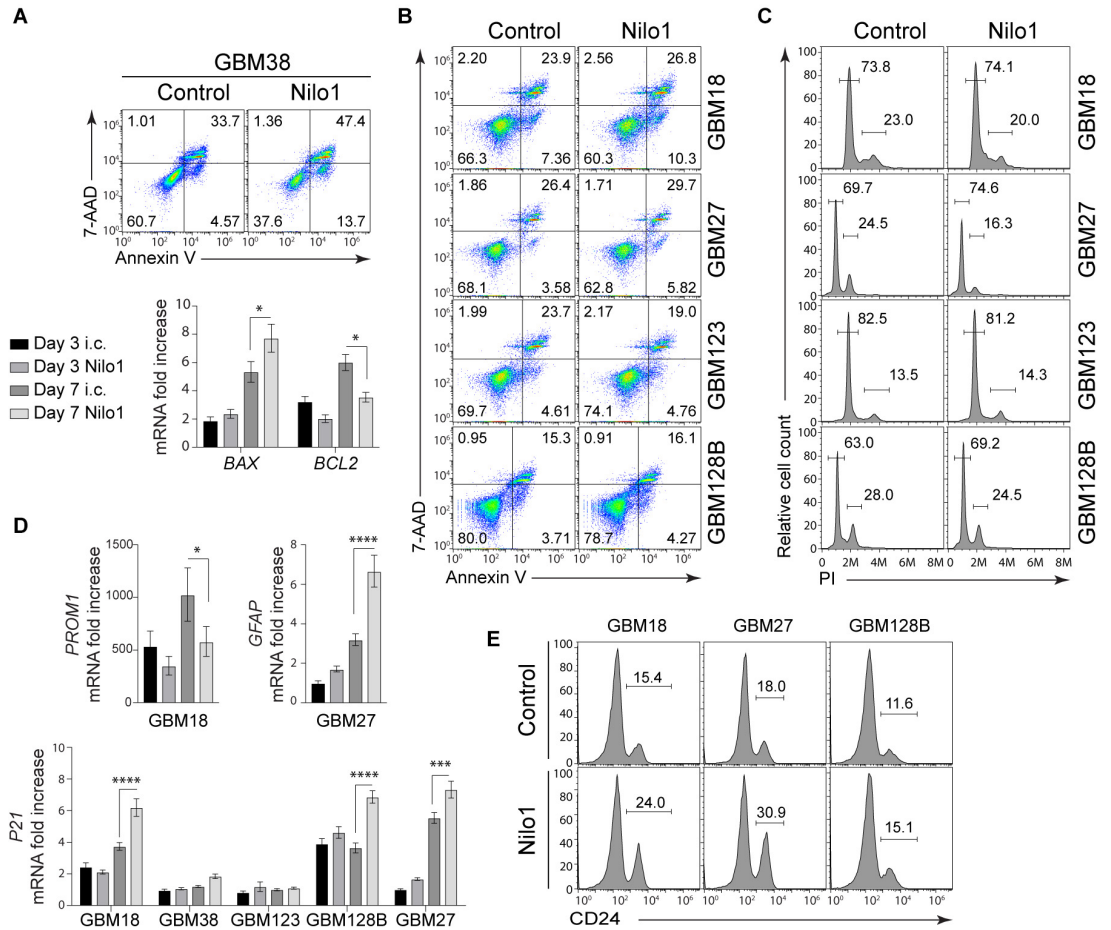

FIGURE 5 | Nilo1 induces apoptosis and cell cycle arrest in GSCs. Neurospheres were dissociated and treated with Nilo1 (0.5 mg/ml) or with hamster anti-mouse CD3ε) as an irrelevant control (i.c.) for 7 days. **(A)** Flow cytometry analysis shows increased proportions of apoptotic (Annexin V^+) cells and dead (PI^+/Annexin V^+) cells in GBM38 after Nilo1 treatment (upper panel). RT-PCR analysis shows increased *BAX* and decreased *BCL2* expression in GBM38, indicative of apoptosis (lower panel). **(B)** Flow cytometry analysis showing no significant change in apoptotic (Annexin V^+) cells and dead (PI^+/Annexin V^+) cells in GBM18, GBM27, GBM123 and GBM128B after Nilo1 treatment. **(C)** Cell cycle analysis shows decreased proportions of proliferating (G2/M) and increased proportions of G1-arrested (G1/G0) GBM27 and GBM128B cells after Nilo1 treatment. **(D)** Nilo1 treatment reduces the expression of stemness marker *PROM1* in GBM18 and induces the expression of astrocyte marker *GFAP* in GBM27. The expression of *P21* is significantly induced in GBM18, GBM128B and GBM27 after 7-day Nilo1 treatment. **(E)** Flow cytometry analysis showing and increase of CD24, a neuronal differentiation marker, in GBM18, GBM27 and GBM128B after Nilo1 treatment. Panels **(A–C,E)** shown are representative histograms or dot plots of two experiments performed. Panels **(A)** and **(D)** data were normalized to GBM38 day 3 i.c. and show mean ± SD ($n = 3$), $^*p < 0.05$, $^{****}p < 0.0001$, 1- or 2-way ANOVA (with Bonferroni correction).

controls. In line with this, the proportions of proliferating cells in G2/M phase were reduced (24.5 vs. 16.3% in GBM27 and 28.0 vs. 24.5% in GBM128B, **Figure 5C**), suggesting that in presence of Nilo1 cells continue growing but at lower speed. This phenomenon of slowing down the cell cycle is known to occur during differentiation (26) and has been associated with neurogenic differentiation of neural stem-like progenitor cells during brain development (27). Therefore, these results, together with the fact that adherent cells were observed in Nilo1-treated GBM18 and GBM128B cells (**Figure 2**), led us to hypothesize that Nilo1 treatment might be inducing GSC differentiation. We thus examined the expression of stemness and differentiation markers, and found that Nilo1 treatment led to a reduction of Prominin1 (CD133) in GBM18, and induction of *GFAP* in GBM27 (**Figure 5D**). The induction of astrocyte-specific gene expression in GBM27 clearly pointed to a differentiation-inducing effect of Nilo1. Furthermore, RT-PCR analysis revealed that Nilo1 significantly induced the expression of p21 – a cyclin-dependent kinase 2 (CDK2) inhibitor – in GBM18, GBM27 and GBM128B (**Figure 5D**). Overexpression of cell cycle inhibitors is a key event ultimately leading to cell cycle arrest and induction of cell type-specific gene expression in cancer cells (28). In support of this, flow cytometry analysis showed that Nilo1 treatment significantly induced the expression of CD24, a marker of neuronal differentiation, in GBM18, GBM27, and GBM128B. Collectively, our findings thus point to a p21-dependent mechanism by which Nilo1 acts to reduce proliferation and induce differentiation in GSCs.

DISCUSSION

Nilo1 monoclonal antibody was generated against mouse neurospheres and it specifically recognizes NSCs in the mouse brain (13, 14). This antibody can be coupled with magnetic nanoparticles to identify mouse NSCs in their niche *in vivo*, and track their migration by MRI (magnetic resonance imaging) in response to brain damage (14). *In vitro*, Nilo1 treatment arrests mouse neurosphere proliferation, which suggested that Nilo1 targets a molecule functionally relevant for stem cell maintenance (13). In addition to mouse NSCs, Nilo1 was previously shown to recognize a homologous antigen within GBM patient-derived neurospheres (14). This raised the possibility that Nilo1 might be the first therapeutic drug targeting GSCs (29); however this remained to be investigated. In this work, we establish that, (1) Nilo1-specific targeting of GBM neurospheres depends on their stem-like phenotype, (2) Nilo1 affects the viability of GSCs but not normal stem cells, (3) in a portion of patient-derived GSCs Nilo1 treatment affects cell cycling and triggers differentiation in parallel with p21 induction, and (4) Nilo1 treatment kills a subset of patient-derived GSCs through a Bax-associated apoptotic mechanism.

Glioblastoma is characterized by extreme inter- and intra-tumoral heterogeneity, resulting in substantial differences in clinical characteristics and response to treatment. This heterogeneity is also reflected in GSC populations, showing different molecular and functional phenotypes that could explain why the effects of Nilo1 treatment varied between different GSC lines. For their growth and stemness maintenance, GSCs rely on a very complex network of signaling pathways, including Notch, Hedgehog and Wnt. Whether due to chromosomal instability, or different signaling events, yet our GSCs showed a remarkable difference in the expression of Notch, Hedgehog and Wnt downstream effectors (**Supplementary Figure S5**), which regulate key events in cell fate determination and proliferation. As there is considerable redundancy among these pathways, some GSCs lines might bypass the effects of Nilo1 and continue to grow despite blocking one route.

It would certainly be of relevance to find a marker defining Nilo1-sensitive GSC subtype. Currently, there is no universal marker for isolating GSCs or distinguishing them from NSCs. Our data showed that only GBM18 expressed CD133 (Prominin1), confirming that CD133 is not a universal marker for GSCs (30). GBM18, GBM27, and GBM123 expressed Nestin, while GBM128B showed the expression of PDGFRA and OLIG2. GBM38 lacked all putative stem cell markers except for GFAP, which is highly expressed in astrocytoma (31). Yet, GBM38 exhibits the most aggressive tumor growth *in vivo* (32) and was shown to be more resistant than other lines to a panel of drugs currently used in clinical practice (17). The fact that Nilo1 most effectively killed GBM38 cells therefore has twofold implications. First, our findings suggest that Nilo1 might be used in combination with current drugs to kill the most resistant GSCs. Second, in combination with therapeutic strategies that specifically target GSCS by their markers – such as CART (chimeric antigen receptor T cell) therapy – Nilo1 might aid to eliminate GSCs that lack most of the widespread CSC markers. We consider these possibilities worth investigating in future studies.

Glioblastoma stem-like cells, like normal stem cells, are resistant to conventional therapy that affects more differentiated cells of the bulk tumor. Therapies designed to specifically kill CSCs or target the pathways that maintain their stem-cell state and induce differentiation, might thus prove useful in the clinic. Because CSCs express specific markers, antibody-based therapies are considered as an effective approach to induce CSC cell death either directly, or to be used as antibody-drug conjugates. Indeed, anti-CD44 antibody can induce differentiation and apoptosis in leukemia and bladder cancer cells (33, 34), while $CD133^{+}$ cancer cells can be targeted using an anti-CD133 antibody conjugated with cytotoxic drug (35). Nonetheless, there are currently no specific markers that tell apart CSCs from NSCs, and on-target/off-tumor toxicity represents one of the major challenges of CSC-targeted therapy. Nilo1 was first described as a monoclonal antibody that labels mouse NSCs (13), which raised the possibility that Nilo1 might also recognize NSCs in humans. This will certainly need to be explored in preclinical studies; nonetheless, our results showed that Nilo1 treatment did not have any toxic effects on normal human mesenchymal stem cells. To avoid its interaction with NSCs, using Nilo1 conjugated with gold nanoparticles was proposed for location-restricted photo-ablation therapy. In that case, any Nilo1 + NSCs would be protected by their location (in SVZ), which is distinct from the location of the tumor (36). Alternatively, recent studies revealed that glioblastomas may originate from NSCs of the SVZ that undergo malignant transformation and give rise to GSCs (12). Having this in mind, therapeutic strategies directed toward common molecular targets in NSCs and GSCs might be critical for achieving better prognosis for GBM patients.

An important factor for developing antibody-based therapies is the presence of blood–brain barrier (BBB). Intact BBB efficiently prevents crossing of systemically administered drugs and monoclonal antibodies larger than 400 Da from the bloodstream into the brain parenchyma (37). GBM is often characterized by a disruption of BBB and open endothelial tight junctions, which allow limited amounts of drugs to reach the tumor site. Nonetheless, the extent of BBB disruption is difficult to determine and varies from patient to patient. To bypass BBB, locoregional delivery using intracerebral placement of catheters can be used as a strategy for enhancing intraparenchymal drug delivery (37). Pharmacodynamics and pharmacokinetics studies will need to be performed to determine the *in vivo* efficacy of Nilo1 antibody.

Our results establish Nilo1 as a potential therapeutic drug targeting GSCs, with several possible applications. First, our data show that Nilo1 labels GSCs, hence conjugating Nilo1 with a dye might allow for better visualization of highly infiltrative GBM tumors. Second, Nilo1 treatment directly induced cell death in one patient-derived GSC line. In addition, the fact that Nilo1 treatment disabled sphere formation and triggered differentiation in a subset of patient-derived GSCs, suggested that these cells, by losing self-renewal and stemness properties, might become susceptible to standard chemotherapy and radiotherapy. Finally, the fact that all tested GSC lines were Nilo1-positive raises the

possibility that conjugating Nilo1 with a cytotoxic drug might encompass the full spectrum of GSC molecular subtypes. We believe that future studies exploring this wide range of possible applications might set the stage for Nilo1 humanized antibody development and clinical trials.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

All patients gave informed consent and the use of tumor samples was approved by the Hospital La Fe (Spain) Ethics Committee.

AUTHOR CONTRIBUTIONS

ÁA-S and VG-R conceived the study. ÁA-S, VG-R, and GR designed most of the experiments, analyzed the data, and wrote the manuscript. GR performed most of the experiments, with the help of GI, DU, PP, and CQ. CE-L provided the mesenchymal stem cells. All authors read, discussed, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.01665/full#supplementary-material

FIGURE S1 | Cell cycle analysis for GBM38 line at different time points during neurosphere formation.

FIGURE S2 | MTS assay showing that Nilo1 treatment does not affect the viability of mesenchymal stem cells. Data were normalized to day 0 and show mean ± SD (n = 3).

FIGURE S3 | Sphere formation assay at day 14, showing that Nilo1 treatment impedes sphere formation in GBM18, GBM128B, and GBM38. Scale bar shows 100 μm.

FIGURE S4 | Immunofluorescence analysis of GBM27, GBM38 and GBM128B tumorspheres showing the proportions of non-viable cells stained with 7-AAD. Scale bar shows 50 μm.

FIGURE S5 | RT-PCR showing different expression levels of Hedgehog (*GLI1*), Notch (*HEY2*), and Wnt (*BMP4*) downstream effectors in GSC lines.

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Classification of Progression Patterns in Glioblastoma: Analysis of Predictive Factors and Clinical Implications

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Background: This study was designed to explore the progression patterns of IDH-wildtype glioblastoma (GBM) at first recurrence after chemoradiotherapy.

Methods: Records from 247 patients who underwent progression after diagnosis of IDH-wildtype GBM was retrospectively reviewed. Progression patterns were classified as either local, distant, subependymal or leptomeningeal dissemination based on the preoperative and serial postoperative radiographic images. The clinical and molecular characteristics of different progression patterns were analyzed.

Results: A total of 186 (75.3%) patients had local progression, 15 (6.1%) patients had distant progression, 33 (13.3%) patients had subependymal dissemination, and 13 (5.3%) patients had leptomeningeal dissemination. The most favorable survival occurred in patients with local progression, while no significant difference of survival was found among patients with distant progression, subependymal or leptomeningeal dissemination who were thereby reclassified into non-local group. Multivariable analysis showed that chemotherapy was a protective factor for non-local progression, while gender of male, subventricular zone (SVZ) involvement and O^6-methylguanine-DNA-methyltransferase (MGMT) promoter methylation were confirmed as risk factors for non-local progression ($P < 0.05$). Based on the factors screened by multivariable analysis, a nomogram was constructed which conferred high accuracy in predicting non-local progression. Patients in non-local group could be divided into long- and short-term survivors who differed in the rates of SVZ involvement, MGMT promoter methylation and reirradiation ($P < 0.05$), and a nomogram integrating these factors showed high accuracy in predicting long-term survivors.

Conclusion: Patients harboring different progression patterns conferred distinct clinical and molecular characteristics. Our nomograms could provide theoretical references for physicians to make more personalized and precise treatment decisions.

Keywords: glioblastoma, progression, subventricular zone, prognosis, nomogram

INTRODUCTION

Glioblastoma (GBM) is the most common primary central nervous system malignancy in adults which confers a gloomy prognosis even after receiving maximal safe resection and chemoradiotherapy (1–3). More than half of patients will undergo progression at the time of six months post operation and the majority will die in 2 years (3, 4). According to the previous studies, about 80% GBM cases would suffer progression within primary treatment field (5, 6). However, patients with different progression patterns, including distant metastasis and leptomeningeal spread, have been increasingly reported in recent years (7, 8). It's noteworthy that there is no broad consensus on the classification of patients' progression patterns (9). Furthermore, controversy still remains regarding the clinical implication of different progression patterns (6, 7, 10).

To better address the clinical practice of patients with GBM, we should have a comprehensive understanding of predictive factors and prognostic potential of different progression patterns, especially for non-local failure. There is increasing evidence that the subventricular zone (SVZ) may be involved in the progression of GBM, because the dysregulated neural stem cells of SVZ can leave the niche and migrate over long distances, and finally contribute to the oncogenesis (11, 12). Numerous studies have shown that patients whose lesions involved the SVZ have worse clinical outcomes and more aggressive patterns of recurrence (13, 14). Moreover, those with SVZ involvement have been demonstrated to show a higher propensity to chemotherapy and radiation resistance (15). These findings have manifested the significance of the SVZ as a critical factor for GBM progression and treatment resistance.

Therefore, in this study, we retrospectively reviewed the clinical and molecular data of 247 patients who underwent the first progression after diagnosis of isocitrate dehydrogenase (IDH) wildtype GBM. To our knowledge, it presents the largest sample committed to clarifying the progression patterns of GBM after 2010 when Response Assessment in Neuro-Oncology (RANO) criteria was established (16). The primary objective of our study was to explore the role of SVZ in GBM progression and establish the prognostic significance and features of different progression patterns.

MATERIALS AND METHODS

Patients Cohort

Records from a consecutive series of 247 patients who were diagnosed with primary GBM and experienced progression in Beijing Tiantan Hospital were retrospectively reviewed. The inclusion criteria were: pathologically diagnosed as GBM, molecular analysis showed a wildtype IDH, tumor located in supratentorial region based on the preoperative magnetic resonance (MR) images, tumor underwent progression after operation, and hospitalization from September 2011 to December 2019. The exclusion criteria were: patients received adjuvant radiotherapy or chemotherapy before resection, loss of follow-up, concurrent with other malignancies or death from other lethal diseases.

Pathological Evaluation

For histopathological evaluation, the resected tumor tissues were fixed in 10% formalin and embedded in paraffin wax. Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) were performed on the slices of 5 μm thick. All slices were reviewed by experienced neuropathologists according to the WHO classification system (17, 18). IHC staining was performed on a Ventana BenchMark XT autostainer (Ventana Medical System Inc, Tucson, Arizona) with antibodies against epidermal growth factor receptor (EGFR, Invitrogen), P53 (Invitrogen), and Ki-67 (Invitrogen). The specific experiment protocol and interpretation principle have been elaborated in a prior study (19). Ki-67 index was defined as either high level (≥30%) or low level (<30%) based on the percentage of IHC-positive cells (10).

In addition, telomerase reverse transcriptase (TERT) and IDH mutations were detected by Sanger sequencing using a HITACHI 3500xL Dx Genetic Analyzer (Applied Biosystems Inc, USA). The promoter region of TERT was amplified with the following primers: TERT-F, 5'-GTCCTGCCCCTTCACCTT-3' and TERT-R, 5'-CAGCGCTGCCTGAAACTC-3'. The primers of IDH were as follows: IDH1-F, 5'-ACCAAATGGCACC ATACG-3' and IDH1-R, 5'-TTCATACCTTGCTTAATGGGG-3'; IDH2-F, 5'-GCTGCAGTGGGACCACTATT-3' and IDH2-R, 5'-TGTGGCCTTGTACTGCAGAG-3'. Abnormality of chromosome 1p and 19q and O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation were analyzed with fluorescence *in situ* hybridization (FISH) and pyrosequencing, respectively, according to a prior study (20) (**Figure S1**).

Evaluation of Progression Pattern and SVZ Involvement

All progression patterns were analyzed by an independent review team consisted of a neuroradiologist and a neurosurgeon who were blinded to the outcome of patients. Tumor progression patterns were defined according to the following definition criteria: (1) local progression: recurrence contiguous with the resection cavity or original tumor site (**Figure 1A**). (2) distant progression: focal recurrence that was not contiguous with the resection cavity or original tumor site (**Figure 1B**). (3) subependymal dissemination: lesions disseminated along with the subependymal zone (**Figure 1C**). (4) leptomeningeal dissemination: leptomeningeal contrast enhancement around the contours of the gyri and sulci or multiple nodular deposited in the subarachnoid space. (**Figures 1D, E**). The SVZ was considered involved if tumors with enhanced lesion touching the lining of the lateral ventricle (21).

Treatment

Once patient was radiologically diagnosed with GBM, maximal safe resection was attempted. In order to evaluate the extent of resection (EOR) of each patient, a MR was routinely performed within 48-72 hours after operation. EOR was determined according to the following equation: (preoperative tumor volume – postoperative tumor volume)/preoperative tumor volume, based on the contrast-enhanced T1 weighted imaging.

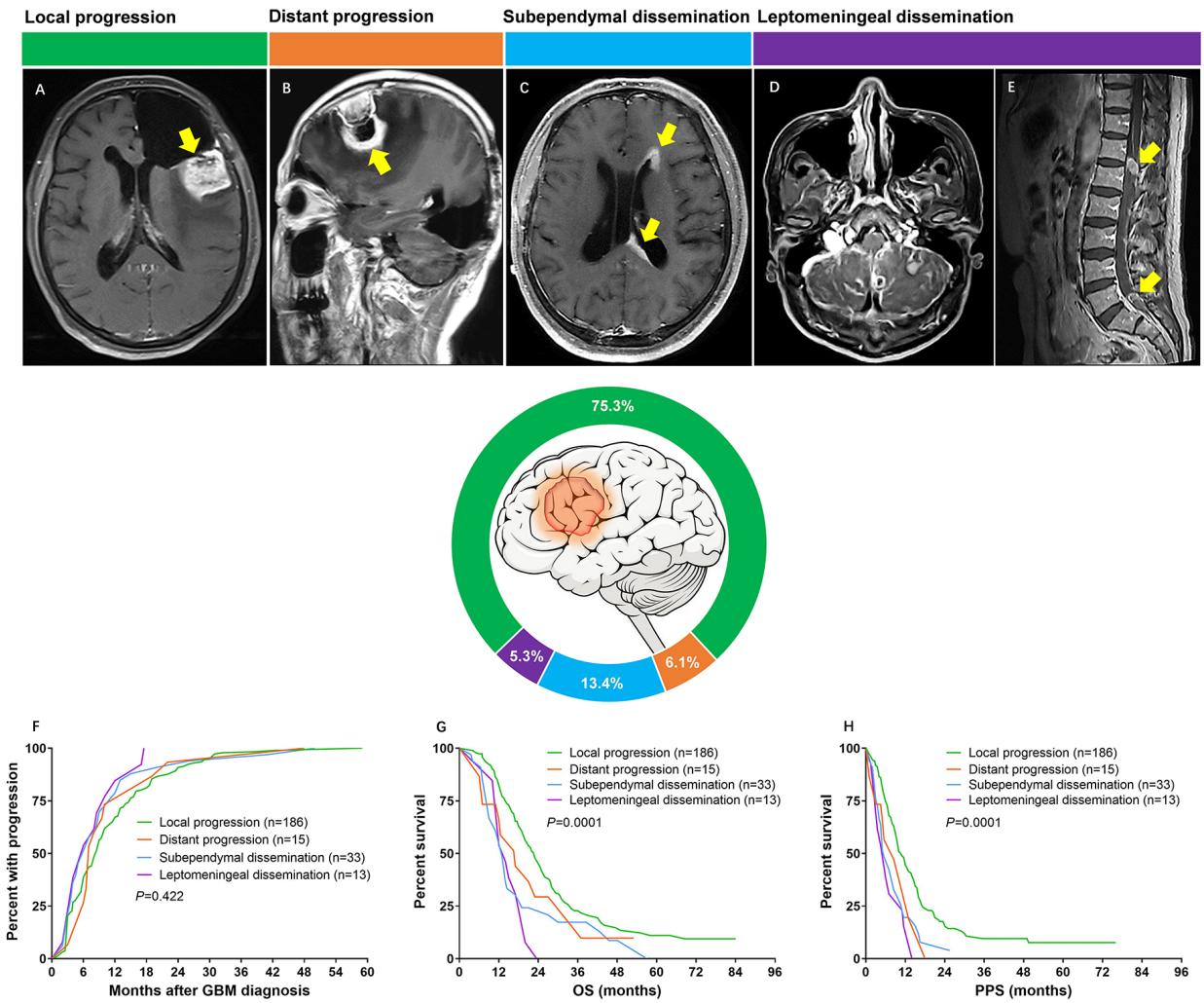

FIGURE 1 | **(A)** Axial T1 image showing tumor recurred at the resection cavity (yellow arrow). **(B)** Sagittal T1 image showing a new lesion far from the original resection cavity was found (yellow arrow). **(C)** Axial T1 image showing subependymal dissemination (yellow arrows); **(D)** Axial T1 image showing superficial leptomeningeal dissemination; **(E)** Sagittal T1 image of spine demonstrating enhanced lesions (yellow arrows). **(F)** Median time from diagnosis to development of progression was 8.5 months for local progression, 7.0 months for distant progression, 6.0 months for subependymal dissemination and 6.0 months for leptomeningeal dissemination (P = 0.422). **(G)** Median OS was 23.0 months for patients with local progression, 17.0 months for patients with distant progression, 13.0 months for subependymal dissemination and 14.0 months for leptomeningeal dissemination (P = 0.0001). **(H)** Median PPS was 11.0 months for patients with local progression, 8.5 months for patients with distant progression, 5.5 months for subependymal dissemination and 6.0 months for leptomeningeal dissemination (P = 0.0001).

Calculation of tumor volume was performed by multiplying the sum of enhanced regions outlined on each transverse slice by the corresponding slice thickness. An EOR ≥ 98% was defined as gross-total resection (GTR) (22). After operation, Stupp protocol was started in one month (3). Briefly, radiotherapy divided into 30 daily fractions of 2 Gy each was delivered to patients within 1-month post operation. Concomitant chemotherapy consisted of temozolomide (TMZ) at a dose of 75mg/(m^2.d) was given during the whole period of radiotherapy. After a 4-week break, patients would receive 6 cycles of adjuvant TMZ at a dose of 150–200 mg/(m^2.d), consecutive 5 days in a 28 days cycle. When tumor progressed, patients were treated at the advice of multi-disciplinary team. Reoperation was mainly considered for patients with local progression, while reirradiation was recommended to those with non-local progression. Systemic treatment could be also attempted if patients showed relatively normal laboratory tests. The most common regimen was bevacizumab (10 mg/kg in every 2 weeks) and dose-dense TMZ (100–150 mg/m^2/d, 7 days on and 7 days off).

Follow-Up

Patients were routinely followed up using MR scans with an interval of 3 months, or 1 month if there was any proof indicated disease progression. To improve the diagnostic accuracy of

recurrence, multimodal MR including perfusion, diffusion and magnetic resonance spectroscopy was used to rule out radiation necrosis and pseudoprogression. All patients were followed until death or censored at the last follow-up. Progression represented a ≥25% increase in the maximal cross-sectional tumor area, or the appearance of any new lesion, or significant increase in T2/FLAIR nonenhanced lesions. Overall survival (OS) was defined as the time period from the date of operation to the date of death or last follow-up. Timespan between tumor progression and death/last follow-up was defined as post-progression survival (PPS). The median follow-up of this cohort was 43.0 (range: 2.0–84.0) months. All the patients experienced progression and 187 (75.7%) patients died at the time of data analysis.

Statistical Analysis

Summary of data were presented as the mean ± SD for parametric variables and percentage for categorical variables. Comparisons of categorical variables between groups were performed using Chi-square test or Fisher's exact test, as appropriate. Differences in age, tumor size and preoperative Karnofsky performance scale (KPS) score were evaluated by student t-test. The variables with P values less than 0.1 were entered into the multivariate logistic regression analyses to identify the predictors of non-local progression and LTS. On the basis of the predictors screened by regression analyses, nomogram models were constructed. The performance of models was evaluated by discrimination and calibration. The calibration of models was performed by a visual calibration curve comparing the predicted and actual probability. Furthermore, the nomogram models were subjected to 1000 bootstrap repetitions for internal validation to assess the predictive accuracy. The survival rate of patients was estimated with Kaplan-Meier plot, and differences between curves were compared by log-rank test. Cox proportional hazard regression model was constructed to estimate the hazard ratio (HR) for each potential prognostic factor. All data were analyzed with SPSS software package version 22.0 (IBM Corporation, Armonk, NY, USA) and R software (http://www.r-project.org). Probability values were obtained using 2-sided tests, and a P value of <0.05 was considered to be statistically significant.

RESULTS

Demographics and Clinical Characteristics

According to the inclusion and exclusion criteria, a total of 247 patients were enrolled in the present study, including 157 (63.6%) males and 90 (36.4%) females with a mean age of 48.5 ± 11.7 years. The clinical, radiological and molecular data of our cohort were shown in **Table 1**.

Patterns of Progression and Outcomes

At presentation, there were 186 (75.3%) patients with local progression, 15 (6.1%) with distant progression, 33 (13.3%) with subependymal dissemination, and 13 (5.3%) with leptomeningeal dissemination (**Figure 1**).

Time from pathological diagnosis to development of progression was similar across different patterns. Patients with GBM experienced local progression at a median period of 8.5 months, distant progression at a median period of 7.0 months, both subependymal and leptomeningeal dissemination at a median period of 6.0 months (P = 0.422, **Figure 1F**). In contrast, the survival of patients varied by progression patterns. The most favorable OS occurred in the group of local progression (23.0 months), followed by the group of distant progression (17.0 months), then the group of leptomeningeal dissemination (14.0 months), and tailed by the group of subependymal dissemination (13.0 months) (P = 0.0001) (**Figure 1G**). Similarly, the median PPS of patients with local progression, distant progression, leptomeningeal and subependymal dissemination was 11.0, 8.5, 6.0, and 5.5 months, respectively, which imparted a significant difference (P = 0.0001) (**Figure 1H**).

Risk Factors for Non-Local Progression

Considering that there was no significant difference of survival among patients with distant progression, subependymal or leptomeningeal dissemination, these patients were reclassified into non-local group for the convenience of comparative analysis (**Figures 2A, B**). According to the results of intergroup comparisons, we found male was slightly more common in non-local group comparing with those in local group (P = 0.056). The

TABLE 1 | Comparison of baseline characteristics between different progression patterns.

Variable	Local (n = 186)	Non-local (n = 61)	*P* value
Age (years)	49.0 ± 11.2	47.2 ± 13.1	0.308
Gender (n, %)			0.056
Male	112/186 (60.2%)	45/61 (73.8%)	
Tumor size (mm)	50.1 ± 14.4	46.8 ± 16.5	0.146
Preoperative KPS	77.3 ± 15.2	75.4 ± 13.2	0.421
SVZ involvement (n, %)			**0.010**
Yes	93/186 (50.0%)	42/61 (68.9%)	
Extent of resection (n, %)			0.917
GTR	99/186 (53.2%)	32/61 (52.5%)	
Radiotherapy (n, %)			0.630
Yes	163/186 (87.6%)	52/61 (85.2%)	
Chemotherapy (n, %)			**0.028**
Yes	173/186 (93.0%)	51/61 (83.6%)	
1q polysomy (n, %)			0.152
Yes	28/158 (17.7%)	5/53 (9.4%)	
19p polysomy (n, %)			0.192
Yes	54/158 (34.2%)	13/53 (24.5%)	
MGMT promoter (n, %)			**0.016**
Methylation	59/176 (33.5%)	31/61 (50.8%)	
TERT promoter (n, %)			0.288
Mutation	42/72 (58.3%)	10/22 (45.5%)	
P53 expression (n, %)			0.533
Positive	107/157 (68.2%)	33/52 (63.5%)	
EGFR expression (n, %)			0.204
Positive	132/156 (84.6%)	40/52 (76.9%)	
Ki-67 index (n, %)			0.696
High	68/186 (36.6%)	24/61 (39.3%)	

KPS, Karnofsky performance score; SVZ, subventricular zone; GTR, gross-total resection; MGMT, O^6-methylguanine-DNA-methyltransferase; TERT, telomerase reverse transcriptase; EGFR, epidermal growth factor receptor.
In bold: p value less than 0.05.

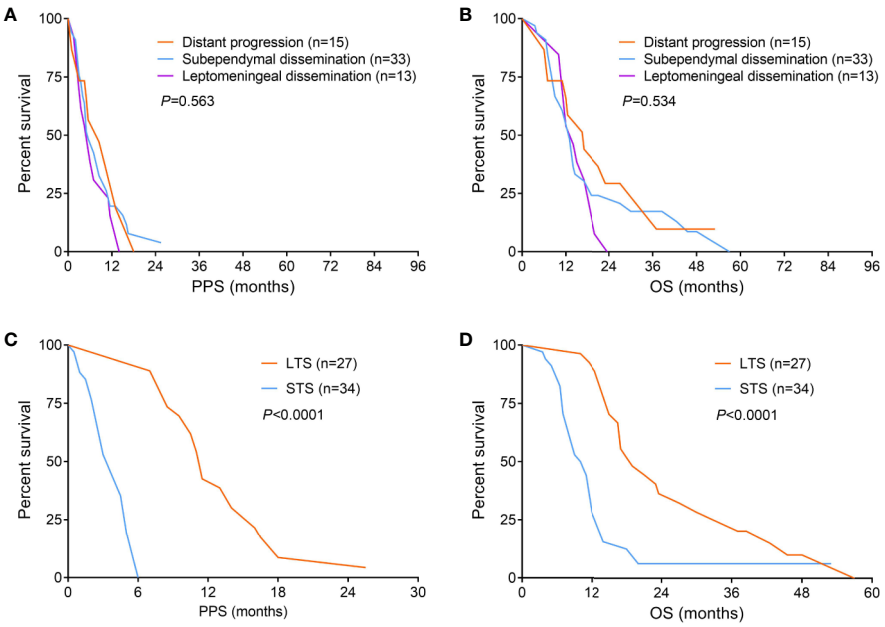

FIGURE 2 | Survival comparisons among different progression patterns **(A**, **B)** and patients with non-local progression could be divided into long-term and short-term survivors **(C**, **D)**.

frequency of SVZ involvement in local group was significantly lower than that in non-local group (50.0 vs. 68.9%, $P = 0.01$). Most of the treatments were equivalent between the two groups, except patients with non-local progression had a lower rate of chemotherapy (83.6 vs. 93.0%, $P = 0.028$). With respect to the molecular data, MGMT promoter methylation could be found in 31 of 61 patients with non-local progression, which was higher than that in patients with local progression (50.8 vs. 33.5%, $P = 0.016$) (**Table 1**).

In the multivariable logistic regression analysis, chemotherapy was the only protective factor of non-local progression (odds ratio [OR] = 0.316, 95% CI: 0.122–0.814, $P = 0.017$). Meanwhile, gender of male, SVZ involvement, and MGMT promoter methylation were confirmed as risk factors for non-local progression (OR = 2.020, 95% CI: 1.018–4.007, $P = 0.044$; OR = 2.516, 95% CI: 1.317–4.805, $P = 0.005$ and OR = 2.539, 95% CI: 1.352–4.768, $P = 0.004$, respectively) (**Table 2**). A nomogram model that integrated these independent factors was constructed. We could estimate the risk of patient developed to non-local progression after operation by adding the score of each factor which was shown in **Figure 3A**. The concordance index (C-index) for the prediction nomogram was 0.88. Calibration curve demonstrated excellent agreement between predicted and observed probability of non-local progression (**Figure 3B**).

Long- and Short-Term Survivors in Patients With Non-Local Progression

Since patients showed a median survival of 6.0 months after diagnosis of non-local progression, they were divided into long-term survivors (LTS) and short-term survivors (STS) at a cutoff of 6.0 months (**Figures 2C, D**). Compared with STS, LTS had a lower rate of SVZ involvement (44.4 vs. 88.2%, $P < 0.001$) but higher rates of reirradiation (70.4 vs. 32.4%, $P = 0.003$) and MGMT promoter methylation (81.5 vs. 26.5%, $P < 0.001$) (**Table 3**). In the multivariable analysis, all these three parameters were further confirmed as independent predictors of LTS ($P < 0.05$) (**Table 2**). According to these predictors, we built a nomogram model to predict the probability of being LTS. This model conferred a C-index of 0.70 (**Figure 4A**). The calibration curve presented a good agreement between the prediction based on our nomogram and actual observation (**Figure 4B**).

TABLE 2 | Results of multivariate logistic regression analysis.

Variables	Odds ratio	95% Confidence interval	*P* value
Predictors for non-local progression			
Gender (male)	2.020	1.018-4.007	**0.044**
SVZ involvement (yes)	2.516	1.317-4.805	**0.005**
Chemotherapy (yes)	0.316	0.122-0.814	**0.017**
MGMT promoter (methylation)	2.539	1.352-4.768	**0.004**
Predictors for LTS in non-local group			
SVZ involvement (yes)	0.124	0.022-0.690	**0.017**
MGMT promoter (methylation)	5.506	1.271-23.851	**0.023**
Reirradiation (yes)	5.238	1.106-24.807	**0.037**

SVZ, subventricular zone; MGMT, O^6-methylguanine-DNA-methyltransferase; LTS, long-term survivors.
In bold: p value less than 0.05.

Multivariate Survival Analysis

A Cox proportional hazard regression model including all recorded potential prognostic factors was established. The

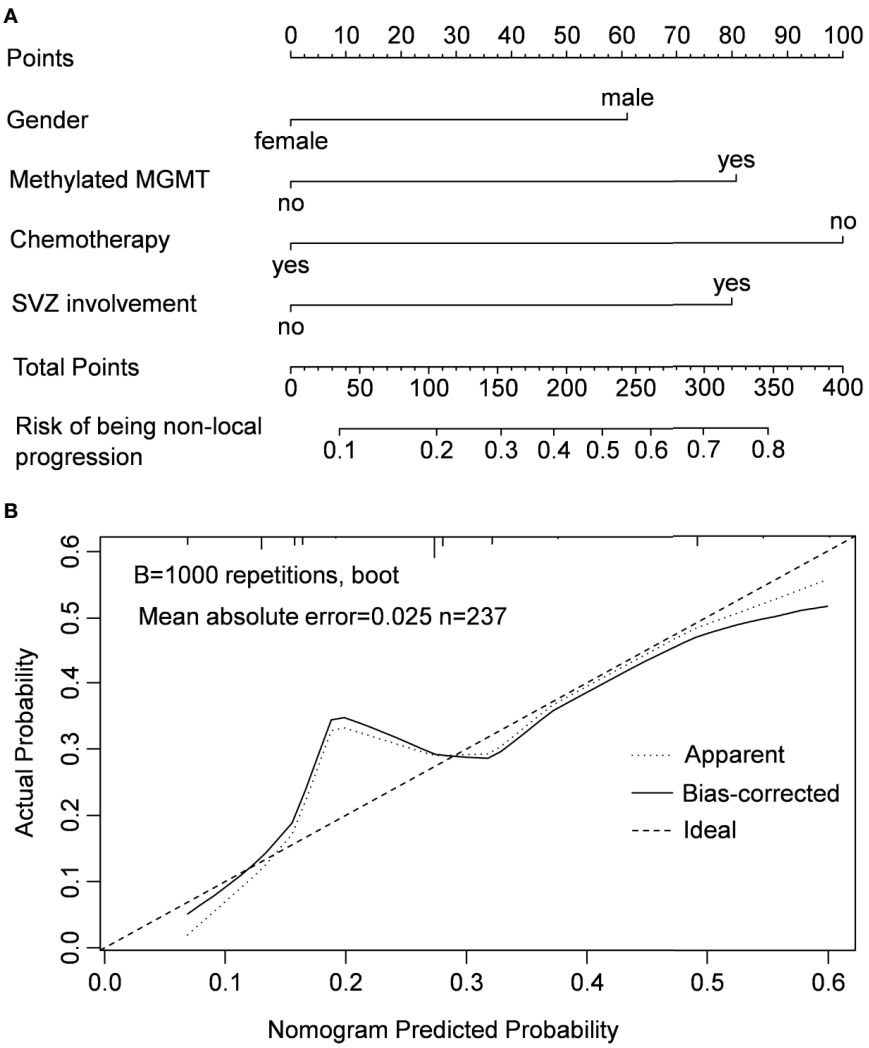

FIGURE 3 | Nomogram model for predicting the risk of being non-local progression in patients with IDH-wildtype GBM **(A)** and calibration curve of the nomogram model **(B)**.

results showed that progression pattern, TERT promoter mutation, MGMT promoter methylation, chemotherapy, radiotherapy, GTR, and SVZ involvement were identified as independent prognostic factors ($P < 0.05$) (**Figure 5**).

DISCUSSION

This study systematically elucidated the incidence and implication of different progression patterns in GBM population. We reclassified the progression pattern into two subtypes: local and non-local, which could be easily applied in routine clinical practice. In addition, the predictive factors of non-local progression and LTS have also been identified. Furthermore, nomogram models were constructed which could predict the risk of being non-local progression in patients with IDH-wildtype GBM and estimate the probability of being LTS in non-local group.

Tumor progression seems to be an inevitable outcome in terms of the current treatment status of GBM. Although the majority of GBM cases suffer local failure, non-local progression can be also encountered in clinical practice and still shows a clear upward trend in recent years (5–8, 23). The reported rate of non-local progression of GBM at first recurrence ranges from 2% to 34.5% (5–8, 24–31). In the present study, 61 (24.7%) patients developed non-local progression which was in accordance with prior studies (28, 30, 31). The variable incidence of different progression patterns is significantly correlated with classification criteria. However, no definite consensus on the definition of progression patterns so far

TABLE 3 | Comparison of baseline characteristics between long- and short-term survivors in non-local group.

Variable	Long-term survivors (n = 27)	Short-term survivors (n = 34)	*P* value
Age (years)	49.3 ± 14.1	45.5 ± 12.2	0.256
Gender (n, %)			0.071
Male	23/27 (85.2%)	22/34 (64.7%)	
Tumor size (mm)	43.0 ± 16.0	50.0 ± 16.5	0.101
KPS score	72.9 ± 14.6	77.3 ± 12.1	0.223
SVZ involvement (n, %)			**<0.001**
Yes	12/27 (44.4%)	30/34 (88.2%)	
Extent of resection (n, %)			0.933
GTR	14/27 (51.9%)	18/34 (52.9%)	
Reirradiation (n, %)			**0.003**
Yes	19/27 (70.4%)	11/34 (32.4%)	
Re-chemotherapy (n, %)			0.482
Yes	19/27 (70.4%)	21/34 (61.8%)	
Yes	2/22 (9.1%)	5/31 (16.1%)	
1q polysomy (n, %)			1.0*
Yes	2/22 (9.1%)	3/31 (9.7%)	
19p polysomy (n, %)			0.092
Yes	8/22 (36.4%)	5/31 (16.1%)	
MGMT promoter (n, %)			**<0.001**
Methylation	22/27 (81.5%)	9/34 (26.5%)	
TERT promoter (n, %)			0.383*
Mutation	2/7 (28.6%)	8/15 (53.5%)	
P53 expression (n, %)			0.117
Positive	16/21 (76.2%)	17/31 (54.8%)	
EGFR expression (n, %)			1.0*
Positive	16/21 (76.2%)	24/31 (77.4%)	
Ki-67 index (n, %)			0.210
High	13/27 (48.1%)	11/34 (32.4%)	

SVZ, subventricular zone; KPS, Karnofsky performance scale; GTR, gross-total resection; MGMT, O^6-methylguanine-DNA-methyltransferase; TERT, telomerase reverse transcriptase; EGFR, epidermal growth factor receptor.
**Fisher's-Exact Test.*
In bold: p value less than 0.05.

has been reached (9). Several studies divided the progression patterns of GBM into four types: local, diffuse, distant and multifocal (5, 27). The most common definition for local progression is recurrence contiguous with the resection cavity or original tumor site (9). There are also reports delineated local failure according to the distance between recurred lesion and original resection cavity (9, 27, 32–34). But the distance is not uniformly defined in different studies (27, 32–34). Moreover, the definition of diffuse progression varies greatly. Piper et al. even state that the diffuse progression is a kind of pattern being poorly understood (9), while we think both local and diffuse progression is the same pattern which has been detected in different time period.

The impact of progression patterns on survival is still controversial (6, 10, 29, 35–37). No significant difference has been observed in terms of the time to development of different progression patterns in this study, which was consistent with prior reports (10, 36). But Tejada et al. held that the median progression-free survival of patients underwent non-local failure was significantly longer than those with local failure (29). However, we found that the OS and PPS of patients were highly dependent on progression patterns. The most favorable prognosis occurred in local group, while no significant difference of survival was observed among patients with distant progression, subependymal or leptomeningeal dissemination. This survival advantage of local progression over non-local progression may partly result from the higher rate of reoperation (29.6 vs. 16.4%, $P = 0.043$). Brandes et al. reported that patients with recurrence out of radiation field conferred a longer survival than those showing recurrence within radiation field, which was attributed to the improved local control (6). However, non-local failure is commonly considered as a sign of advanced stage of GBM and indicates a worse prognosis (7, 10, 35). Therefore, considering the contradictory results on the definition and implication of progression patterns, we divided the progression pattern of GBM into local and non-local subtypes based on clear and easily replicable criteria, which could contribute to addressing these controversies.

We also explored factors that predisposed to the development of non-local progression in this study. In agreement with previous studies, MGMT promoter methylation and gender of male were found to be predictors of non-local progression (6, 38, 39). Methylated MGMT promoter was associated with higher chemosensitivity, which would lead to an improved local control for patients with GBM. It has also been confirmed by our Cox regression model (**Figure 5**). While male patients presented a higher risk for non-local progression might be ascribed to the unfavorable response to treatment (40). Additionally, SVZ involvement was identified as an independent risk factor of non-local progression in our study. This finding is in congruence with Lim et al. and Adeberg et al., who found an association between neurogenic niche contact, multifocal distant progression, and poor outcome (13, 21). SVZ is regarded as a neurogenic region where has been resided by neural stem cells. It is hypothesized that GBM cancer stem cells may stem from dysregulated neural stem cells (41). Given that tumors involved SVZ are in close proximity with cerebrospinal fluid (CSF), our finding can be explained by the hypothesis that CSF circulation may seed tumor cells to distant sites. This hypothesis has been proved by Shibahara et al. who found a high CD133 expression in patients with distant failure (42). Fortunately, chemotherapy can eliminate the tumor cells in the CSF and decrease the incidence of non-local failure, which has been confirmed by our results and previous studies (10, 43).

Other factors that associated with the incidence of non-local progression has also been reported, such as radical resection (44), gains of 1p36 (45), and high epidermal growth factor receptor (EGFR) expression (46). Despite prior report suggested radical resection could influence the progression pattern of GBM by improving local control (44), we found no difference in the rate of GTR between local and non-local groups. Korshunov et al. concluded that gains of 1p36 were correlated with leptomeningeal dissemination of supratentorial GBM, which might be resulted from the activation of potent oncogenes or growth-regulating genes

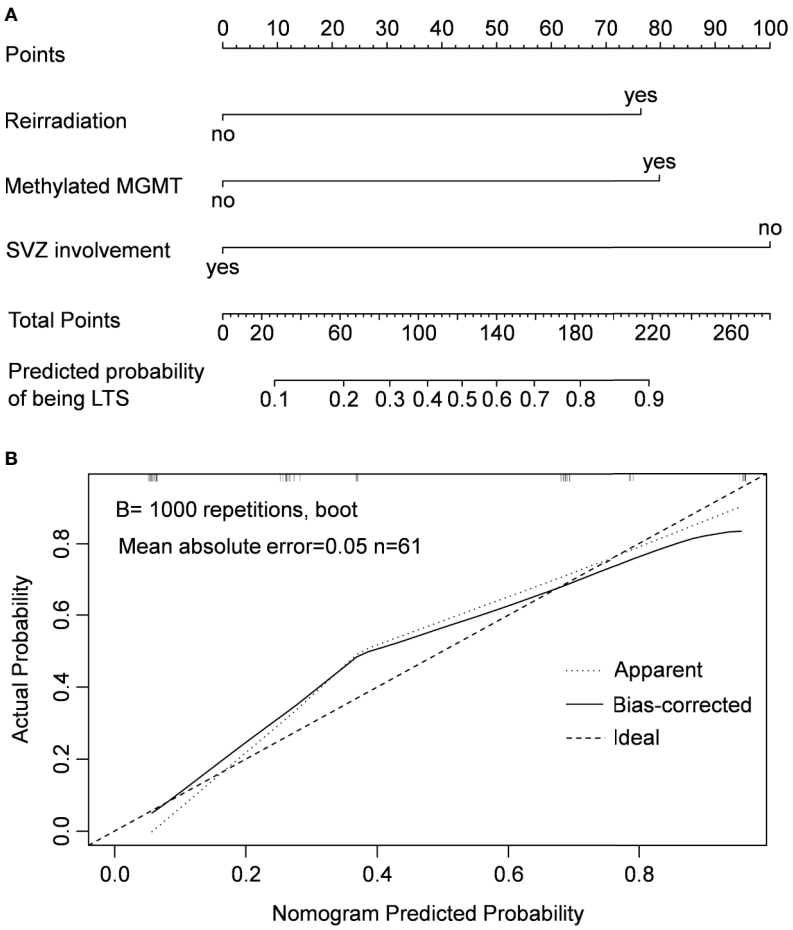

FIGURE 4 | Nomogram model for predicting the probability of being long-term survivors in patients with non-local progression **(A)** and calibration curve of the nomogram model **(B)**.

located in this chromosome region (45). Tini et al. illustrated that patients with high EGFR expression showed a higher rate of distant recurrence (46). But we did not find this trend in our cohort.

Considering non-local progression is an increasingly prominent clinical problem, we explored the features of LTS in non-local group. Final results demonstrated that tumor bearing SVZ involvement was associated with poor prognosis, while reirradiation played a vital role in prolonging the survival of patients with non-local progression. It supported the finding by Dardis et al. who concluded that radiotherapy could improve the OS of patients with leptomeningeal metastases (47). Interestingly, the rate of MGMT promoter methylation which has been identified as a predictor of non-local progression was remarkably increased in the subgroup of LTS. In order to disclose the potential reason for this phenomenon, we compared the survival of patients receiving re-chemotherapy or not based on the status of MGMT. Results showed that patients with methylated MGMT might benefit from re-chemotherapy, while this survival advantage disappeared when analysis focused on those with unmethylated MGMT (**Figure S2**). Therefore, re-chemotherapy seems to be a treatment option for patients with methylated MGMT when tumor underwent non-local progression.

Limitations do exist in this study. Firstly, it's a single-center, retrospective study which inevitably includes bias in patient selection. Secondly, the MR based definition of tumor progression may imply a misinterpretation of pseudoprogression, which can, to some extent, impact the result of this study. Additionally, although internal validation of our models confers optimal discrimination and excellent calibration, the generalizability of these nomograms still requires external validation based on additional database. Finally, as the data is collected from adult neuro-oncology department, the mean age at diagnosis of GBM in this study seems to be younger than the reported data (18). Thus, our results may not be applicable for the older patients.

CONCLUSIONS

To summarize, this study systematically analyzed the incidences, characteristics, and prognoses of different progression patterns

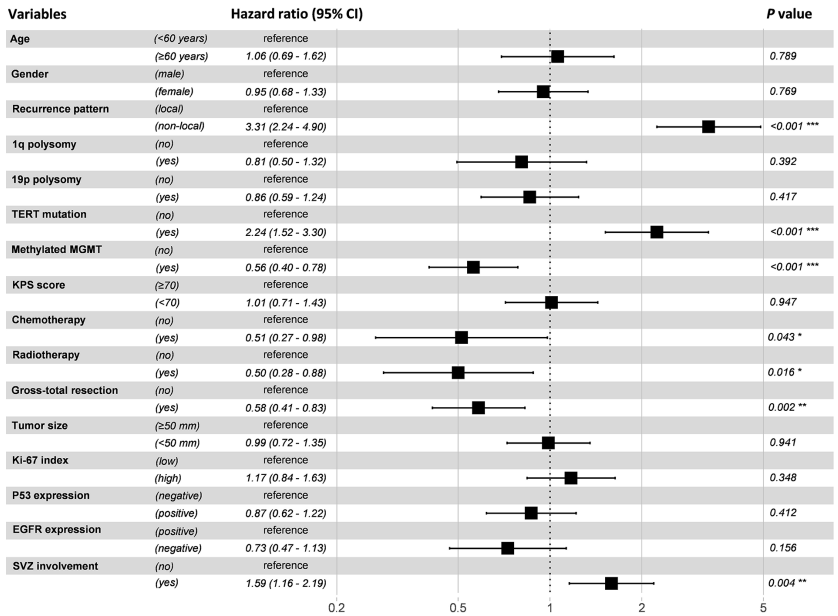

FIGURE 5 | Forest plot of multivariable Cox proportional hazard regression analysis. Variables with a hazard ratio larger than 1 were considered as risk factors, while those with a hazard ratio less than 1 were considered as protective factors.

based on a relatively larger cohort of GBM. Despite some inevitable limitations, our results suggest that gender of male, SVZ involvement and MGMT promoter methylation are correlated with higher risk for non-local progression. Our nomogram models could be used to predict the risk of being non-local progression in patients with IDH-wildtype GBM and estimate the probability of being LTS in non-local group.

DATA AVAILABILITY STATEMENT

All data supporting the conclusions of this article are available on request from any qualified investigator.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Acquisition of data: HJ, ML, CY, and XZ. Analysis and interpretation of data: HJ, KY, YC, and XR. Statistical analysis: HJ and KY. Drafting the article: HJ and SL. Funding acquisition: SL and YC. Conception and design: HJ and SL. Study supervision: SL. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.590648/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Panel I, detection result of chromosome 1p and 19q: 1p intact **(A)**, 1p deletion **(B)**, 1q polysomy **(C)**; 19q intact **(D)**, 19q deletion **(E)**, and 19p polysomy **(F)**. Panel II: Wildtype IDH1 and IDH2. Panel III: MGMT promoter unmethylation **(A)** and MGMT promoter methylation **(B)**.

SUPPLEMENTARY FIGURE 2 | In the subgroup of MGMT promoter methylation, patients with re-chemotherapy showed a trend toward better PPS comparing with those without re-chemotherapy (11.5 vs. 8.5 months, $P = 0.079$) **(A)**. While in the subgroup of MGMT promoter unmethylation, this trend disappeared (4.8 vs. 3.0 months, $P = 0.232$) **(B)**.

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REVIEW
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To Become or Not to Become Tumorigenic: Subventricular Zone *Versus* Hippocampal Neural Stem Cells

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Neural stem cells (NSCs) persist in the adult mammalian brain in two neurogenic regions: the subventricular zone lining the lateral ventricles and the dentate gyrus of the hippocampus. Compelling evidence suggests that NSCs of the subventricular zone could be the cell type of origin of glioblastoma, the most devastating brain tumor. Studies in glioblastoma patients revealed that NSCs of the tumor-free subventricular zone, harbor cancer-driver mutations that were found in the tumor cells but were not present in normal cortical tissue. Endogenous mutagenesis can also take place in hippocampal NSCs. However, to date, no conclusive studies have linked hippocampal mutations with glioblastoma development. In addition, glioblastoma cells often invade or are closely located to the subventricular zone, whereas they do not tend to infiltrate into the hippocampus. In this review we will analyze possible causes by which subventricular zone NSCs might be more susceptible to malignant transformation than their hippocampal counterparts. Cellular and molecular differences between the two neurogenic niches, as well as genotypic and phenotypic characteristics of their respective NSCs will be discussed regarding why the cell type originating glioblastoma brain tumors has been linked mainly to subventricular zone, but not to hippocampal NSCs.

Keywords: neurogenesis, glioblastoma, neural stem cells, oncogenicity, cancer-driver mutations

INTRODUCTION

Gliomas constitute the most common and lethal primary tumor in the central nervous system (CNS). The World Health Organization (WHO) classified CNS tumors by their histological origin, molecular parameters and malignancy (1, 2). Glioblastoma multiforme (GBM) is considered as the highest grade (Grade IV) astrocytoma, characterized by poorly differentiated cells with microvascular proliferation, pseudopalisading necrosis, abundant mitoses and pleomorphic cells. This type of glioma also shows a high degree of phenotypic, genomic and transcriptional heterogeneity (3, 4). Extremely invasive, GBMs cannot be completely resected by surgery, and are resistant to conventional therapies, including chemotherapy and radiation. As a consequence,

the prognosis for GBM patients is very poor, with an average survival rate of about 14–15 months even after intensive treatment (5, 6).

The vast majority of GBMs (about 90% of cases) are primary GBMs that rapidly develop *de novo* in elderly patients without radiological or histological evidence of pre-existing less-malignant precursor lesion. About 10% of the cases correspond to secondary GBMs progressing from lower grade gliomas and preferentially arise in younger patients (2). Although both GBM types are histologically indistinguishable, secondary GBMs are unequivocally characterized by the presence of *IDH1* (isocitrate dehydrogenase) mutations (7). For this reason, primary and secondary GBMs can also be named as IDH-wild type and IDH-mutant GBM, respectively (2). Primary (IDH-wild type) GBMs typically present epidermal growth factor receptor (*EGFR*) amplification and loss of the tumor suppressor phosphatase and tensin homolog (*PTEN*). Inactivation of the tumor suppressors *TP53* (coding a protein called tumor protein 53 or p53) and *NF1* (neurofibromin 1), or mutations in the promoter of *TERT* (telomerase reverse transcriptase) are also commonly identified in both GBM types (3, 8, 9).

Identification of the cell of origin for GBM, this is, the cell type that acquires the initial tumorigenic mutation, is a fundamental issue for understanding the etiology of the disease and for developing early prognostic markers and preventive therapies. Specifically, the cell of origin in IDH-wild type GBM has been much more object of debate since, in contrast to IDH-mutant GBM, the wild type arises without any precursor disease. One of the hypotheses states that neural stem cells (NSCs) remaining in the adult brain could be the cell of origin of this devastating disease. NSCs are found in two neurogenic niches: the subventricular zone (SVZ), lining the walls of the lateral ventricles, and the subgranular zone (SGZ), in the dentate gyrus of the hippocampus (10). Recent evidence has shown that SVZ-derived NSCs might be the cell type harboring the cancer-driver mutations that lead to GBMs (11). In contrast, to date, no substantial data support the contribution of hippocampal-derived NSCs in the development of these malignant tumors. Remarkably, in mouse models of malignant gliomas and in GBM patients, the hippocampus appears to be a region spared from GBM invasion whereas the SVZ is a site for preferred infiltration of this type of tumor (12).

In this article we will analyze differences in these two neurogenic niches, as well as between the NSC population residing in each of them, which might explain why the cell of origin of IDH-wild type GBM has been linked mainly to the SVZ, but not to hippocampal NSCs.

THE ADULT BRAIN NEUROGENIC NICHES IN MAMMALS: SUBVENTRICULAR ZONE AND HIPPOCAMPUS

In the majority of species of terrestrial mammals, adult CNS new neurons can be generated from NSCs residing in two specific regions: the SVZ and the SGZ in the dentate gyrus of the hippocampus (10, 13). Young neurons produced in the SVZ migrate over an extended distance along the rostral migratory stream toward the olfactory bulb, where their final differentiation takes place (14). In contrast, neuroblasts generated in the SGZ mature into granule cells within the same hippocampus (15). Below, we will describe specific features of these two neurogenic regions in rodents, since these are the mammals in which most studies have been reported.

Neural Stem Cells of the Adult Subventricular Zone

NSCs of the adult rodent SVZ have the ability to generate neurons, astrocytes, and oligodendrocyte progenitor cells (OPCs) depending on niche signals (16). They are known as type B1 cells and the cell body is located under the layer of ependymal cells (type E cells) lining the ventricle (**Figure 1A**). B1 cells are polarized cells with a basal process contacting blood vessels and a non-motile primary cilium that contacts the cerebrospinal fluid (CSF) of the lateral ventricle (17, 18). Approximately 20% of B1 cells self-renew through symmetric divisions whereas ~80% generate transit-amplifying neural progenitors called type C cells (19) (**Figure 1A**). Type C cells are located deeper within the niche, close to the vascular network, are highly proliferative, and divide symmetrically before becoming neuroblasts (type A cells) (20) (**Figure 1A**). Type A cells are highly migratory and organize into chains to leave the SVZ through the rostral migratory stream to finally reach the olfactory bulb. Once in the olfactory bulb, type A cells change their migration pattern from tangential to radial through the cellular layers of the olfactory bulb to get to their target layer, where they ultimately differentiate into mature interneurons (14, 18, 21). In addition, a small proportion of type B1 cells can generate type C cells that express the oligodendroglial lineage marker Olig2 and give rise to OPCs (**Figure 1A**) that migrate to the corpus callosum and white matter tracts in the striatum and fimbria fornix (22, 23). SVZ-derived neuroblasts and OPCs can also migrate toward sites of brain injury, where they contribute to neural regeneration (24–26).

The SVZ also contains a population of proliferative astrocytes named type B2 cells (**Figure 1A**) that are located further beneath the ventricle but do not contact it. B2 cells isolate neuroblast chains from other cell types and help to shape the niche (20). Microglia are also an integral part of the SVZ niche (**Figure 1A**). Interestingly, SVZ microglia are clearly distinguished from microglia in other brain regions both antigenically and morphologically, and intervene in the control of neurogenesis through gap junctional communication and the release of soluble factors and extracellular vesicles [reviewed in (27)].

Additional constituents of the SVZ niche with relevance in the regulation of neurogenesis are endothelial cells. They have been reported to intervene in neuroblast migration and maturation (28) and to secrete soluble factors that regulate NSCs behavior (29). Moreover, direct cell-cell contact between B1 cells and endothelial cells is involved in the maintenance of B1 cells in a quiescent state (30).

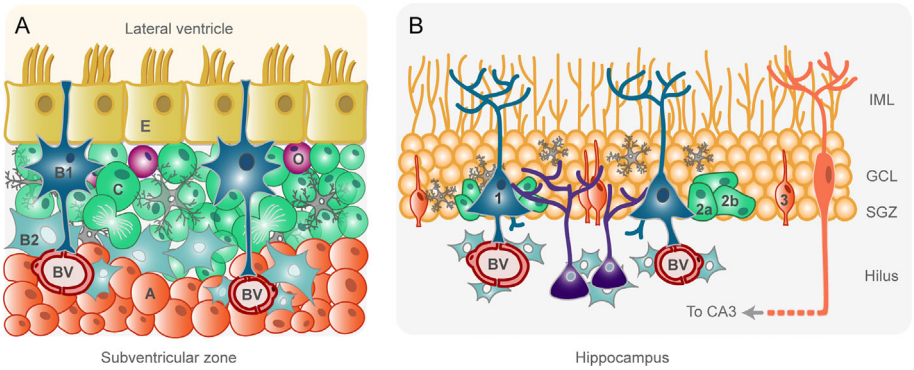

FIGURE 1 | Schematic drawings of the adult rodent subventricular zone **(A)** and hippocampus **(B)**. Equivalent cell types within each niche are represented in the same color. **(A)**. Ependymal cells (type E, in yellow) separate the cerebrospinal fluid (CSF) in the lateral ventricle from the brain parenchyma. Type B1 cells (in dark blue) are neural stem cells with a basal process in contact with blood vessels (BV) and an apical process in contact with the CSF. B1 cells generate type C cells (in green) by asymmetric divisions. Type C cells are transit-amplifying intermediate progenitors that divide rapidly and produce neuroblasts (type A, in orange). Neuroblasts migrate in chains ensheathed by astrocytes toward the olfactory bulb, where they differentiate into mature interneurons. Type C cells can also produce oligodendrocyte progenitor cells (O, in purple). Other glial cells, such as astrocytes (type B2, in sky blue), and microglia (in gray) intervene in the control of subventricular zone neurogenesis. **(B)**. Neural stem cells (type 1, dark blue), located in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus, harbor a basal process that contact BVs and numerous branches in the inner molecular layer (IML). Type 1 cells generate transit-amplifying non-radial type 2 cells (in green), which can be subdivided in type 2a and type 2b cells. Type 2 cells then give rise to a neuron-committed intermediate progenitor (type 3 cell, in orange). Type 3 cells generate fully functional granule cells in the granule cell layer (GCL) which, after maturation, develop dendritic arborization in the IML and axonal projection to the CA3. Microglia (gray) and astrocytes (sky blue) exert different roles in the control of neurogenesis.

Neural Stem Cells of the Subgranular Zone of the Dentate Gyrus of the Hippocampus

The SGZ of the adult rodent hippocampus contains NSCs in different states of proliferation or differentiation, as well as other cell types that contribute to neurogenic functions. NSCs in the SGZ are radial glia-like cells called type 1 cells (**Figure 1B**) that are highly polarized, like radial glia and B1 cells (31). Type 1 cells harbor a primary cilium that contacts blood vessels in the hilus, a long process that extends through the granule cell layer and numerous branches in the inner molecular layer (**Figure 1B**). All these processes and branches allow type 1 cells to detect local neural activity from granule cells and from synaptic terminals, as well as signals from glial cells and blood vessels (31–33). Indeed, as it happens in the SVZ, the close proximity of blood vessels within the SGZ niche provides an abundant source of extrinsic factors that regulate proliferation, neuronal differentiation and survival (34). Unlike B1 cells, type 1 cells of the SGZ do not contact the CSF (31).

Under physiological conditions, type 1 cells are multipotent cells with low rate of division that can remain for long periods out of the cell cycle in a quiescent state (35, 36). NSC activation may lead to the expansion of the stem cell pool through symmetric and asymmetric self-renewal (35). The asymmetric divisions of hippocampal NSCs generate non-radial transit-amplifying cells (type 2 cells) destined to become neurons (37). There are two subtypes of type 2 cells, a glial-like type (type 2a) and a neuronally determined type (type 2b), which are negative and positive respectively for the immature neuron marker doublecortin (**Figure 1B**). Type 2 cells are highly proliferative and comprise the transition from a glia-like precursor cell to a neuronal determination, since they give rise to more committed intermediate progenitors (type 3 cells) that are constrained to a neuronal fate (15, 35, 38). Type 3 cells are neuroblasts with little proliferative activity, which migrate radially to the granule cell layer to generate fully functional granule cells (15) (**Figure 1B**). During the maturation stage, the newborn neurons extend their dendrites into the inner molecular layer and their axon to CA3 (15) (**Figure 1B**). Type 1 cells can also give rise to astrocytes through differentiation (37) but, unlike SVZ B1 cells, SGZ type 1 cells do not have the capacity to generate OPCs under normal conditions (39).

In the transition between type 2 and type 3 cells, there is a drastic decrease in newborn neurons, mediated by apoptosis. Apoptotic cells are rapidly cleared out through phagocytosis by microglia present in the adult SGZ niche (40) (**Figure 1B**). Astrocytes are also in close contact with different components of the SGZ niche and intervene in the regulation of the neurogenic process (41, 42).

As a brief summary, in rodents, neurogenic niches for hippocampal and subventricular zone neurogenesis exhibit many similarities but also clear differences. For instance, the hippocampus lacks ependymal cells and the whole process of neurogenesis is physically localized in the dentate gyrus (15, 31). In **Table 1**, we have summarized the main differences in the progenitor cell population between these two niches.

DISTINCTIVE FEATURES OF THE HUMAN SUBVENTRICULAR ZONE AND THE HUMAN HIPPOCAMPUS

The SVZ of the adult human brain presents some peculiarities with respect to the SVZ organization of rodents described before.

TABLE 1 | Main differences between progenitor cells of the adult subventricular zone and the adult hippocampus in rodents.

	Rodent subventricular zone	Rodent hippocampus
Neural Stem Cells	B1 cells. Apical process in direct contact with cerebrospinal fluid (17, 18, 20)	Type 1 cells. Do not contact the cerebrospinal fluid (31)
Intermediate Progenitors	Type C cells. Give rise to oligodendrocyte progenitor cells and neuronal progenitors (18, 20, 23, 24)	Type 2 cells. Give rise to neuronal progenitors (15, 38)
Neuronal Progenitors	Type A cells. Migrate tangentially to the olfactory bulb to generate interneurons (14, 18, 21)	Type 3 cells. Migrate radially in the hippocampus to generate granule cells (15)

The cytoarchitecture significantly differs since, instead of a layer of abundant type C and type A cells, characteristic of the rodent SVZ, an almost acellular layer devoid of neuroblasts is located beneath the ependymal cells in the human SVZ (43, 44) (**Figure 2A**). Adjacent to this hypocellular layer there is a dense ribbon of astrocytes that extend processes across the hypocellular gap layer to maintain contact with the surface of the lateral ventricle (**Figure 2A**). Astrocyte-like NSCs are located in the hypocellular gap layer (43) (**Figure 2A**). This appearance is adopted as early as 18 months of age, when both proliferative activity and the expression of markers of immature neurons are largely depleted (49). In addition, during this limited postnatal period of neurogenesis, not all neuroblasts generated in the human SVZ are destined to the olfactory bulb, since many of them migrate tangentially to the prefrontal cortex (49). The incorporation of newborn neurons in the human olfactory bulb is nearly extinct by adulthood (50, 51), which may be related to the reduction in the dependence of olfaction manifested in humans (50). Surprisingly, the scarce neuroblasts formed in the adult human SVZ migrate to the striatum, where they differentiate into interneurons, a phenomenon that has been observed mainly in response to cell loss due to injury (49, 50, 52). Importantly, newly generated cells from NSCs in adult human SVZ are mainly oligodendrocytes, not neurons (50, 52), which suggests that the oligodendrogenic process and its corresponding myelin maintenance acquires more relevance in the human brain when compared to other mammalian brains (**Figure 2A**).

With respect to the human hippocampus, proliferating neural progenitor cells and newly generated neurons were described in the adult human dentate gyrus by the end of the last century (45) (**Figure 2B**) and their existence has been suggested, even in the aged brain, in recent studies (46, 53, 54). The preservation of neurogenesis throughout life in healthy older people seems to be important to maintain cognitive function (46, 54). Remarkably, a larger proportion of hippocampal neurons are subject to exchange in humans in comparison to the mouse (55). However, other studies have argued against the existence of adult neurogenesis in humans (47, 48) **(Figure 2B)**. Sorrells and colleagues showed that proliferating progenitors and young neurons in the dentate gyrus declines sharply during the first year of life and only a few isolated young neurons can be observed by 7 and 13 years of age (47). Therefore, an interesting debate has been established about the existence or not of neurogenesis in the adult human hippocampus. The contradictory hypothesis seems to be due to differences in the treatment of human postmortem tissue and in the neuronal

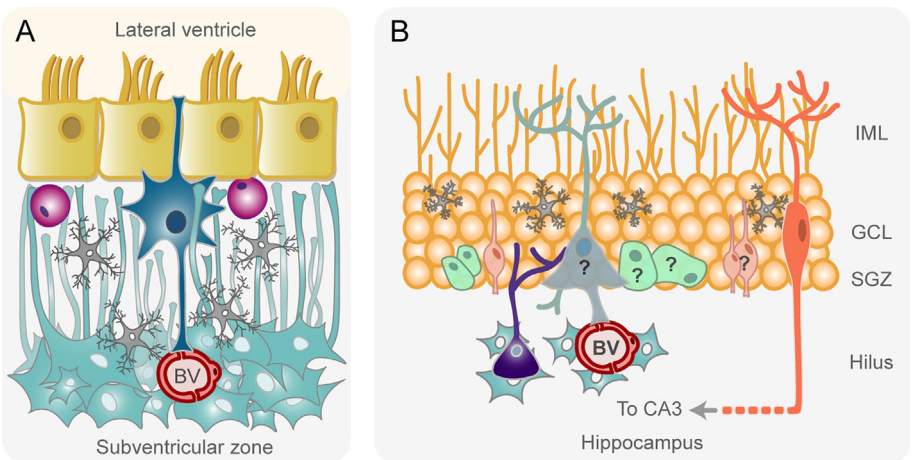

FIGURE 2 | Schematic drawing of the adult human subventricular zone **(A)** and hippocampus **(B)**. **(A)** Astrocyte-like neural stem cells (NSCs, in dark blue) are located beneath the ependymal cell layer (in yellow) lining the lateral ventricles, within a hypocellular layer devoid of neuroblasts and transit-amplifying progenitor cells. Microglial cells in this layer are represented in gray. Putative oligodendrocyte progenitor cells generated from NSCs are shown in purple. NSCs contact the cerebrospinal fluid of the lateral ventricle and blood vessels (BV) of an adjacent layer consisted of a dense ribbon of astrocytes (pale blue) with processes in the hypocellular layer. **(B)** According to some studies (45, 46) the subgranular zone of the dentate gyrus of the adult human hippocampus contains radial glia-like neural stem cells (in pale blue) that generate proliferating intermediate neural progenitors (in pale green). These intermediate progenitors form neuronal committed progenitors (in pale orange) that become mature granule neurons (in orange). In contrast, other studies (47, 48) have reported the total absence of neuronal progenitors and immature neurons in the adult human subgranular zone. A question mark has been texted on these cells to symbolize this controversy. Other cell types of the niche are microglia (in gray) and astrocytes (in sky blue). GCL, granule cell layer; IML, inner molecular layer; SGZ, subgranular zone.

markers used in diverse studies (56). Thus, while some authors used doublecortin as a marker for young neurons (54), others affirm that this marker is not specific to newborn neurons, since they continue to express it as they differentiate, and it is also expressed by non-neuronal glial cells (47). Therefore, there is a clear need to unify criteria in order to have a better understanding of the neurogenic process in the hippocampus of the adult human brain.

DIFFERENT THEORIES ON THE CELL OF ORIGIN OF GLIOBLASTOMAS

The cell type that originates this devastating tumor has been a subject of debate in the last few years, with data supporting NSCs, astrocytes or OPCs as putative candidates (57). For instance, several reports in mice have demonstrated that the knockout/knockdown of tumor suppressor genes (e.g. *PTEN*, *P53*, *NF1*, *retinoblastoma protein RB1*) in NSCs from the SVZ leads to glioma formation (58, 59) **(Figure 3)**. Genetic modifications in astrocytes or in NSC-derived astrocytes leading to combined inactivation of several tumor suppressor genes or in driver oncogenes (*EGFR)* are also capable of initiating gliomagenesis in mice (60–62) **(Figure 3)**, although other authors have reported that oncogenic mutations in mature astrocytes do not contribute to the formation of gliomas (58, 59). OPCs have also been considered as the cell population that originates this deadly cancer. Thus, inactivation of *p53* and *NF1* (63) *or p53, NF1* and *PTEN* (64) in adult OPCs induce glioma formation in mice **(Figure 3)**.

Latest data obtained from human patients have reinforced the candidacy of NSCs from the SVZ, as the possible cell type carrying the cancer-driving mutations of GBM (11). Lee et al. (11) have convincingly demonstrated that tissue from the tumor-free SVZ of IDH-wild type GBM patients contained low-level GBM driver mutations (in *TP53, PTEN* or *EGFR*), that were found in the dominant clones of its matching tumors (11). In addition, *TERT* promoter mutations were identified in all the patients with GBM that had driver mutations in tumor-free SVZ tissue. In the same publication, authors performed experiments in mice to support their findings in human tissue. Thus, they generated a mouse model with *p53*, *PTEN* and *EGFR* mutations in NSCs from the SVZ and showed that mutant cells migrated from the SVZ to distant regions of the brain and eventually developed high-grade glioma. Interestingly, mutated cells that migrated toward the olfactory bulb differentiated into mature neurons and did not lead to gliomas. The aberrant growth of the mutated OPC lineage, but not of the mutated astrocytic lineage, was found to be involved in the glioma formation (11). Previous experiments performed in a mouse model of glioma led to similar conclusions (65). In their article, Liu *et al.* induced *p53/NF1* mutations in NSCs to model gliomagenesis in mice and analyzed mutant NSCs and their progeny at premalignant stages (65). They demonstrated aberrant growth of OPCs, but not of NSCs or any other NSC-derived lineages as neurons or astrocytes. Upon tumor formation, it was confirmed the OPC nature of the tumor cells. Consistently, gliomas were also formed when these mutations were performed directly in OPCs. These results indicate that while both SVZ NSCs and OPCs could be the cell of origin of GBM, OPCs could rather constitute the

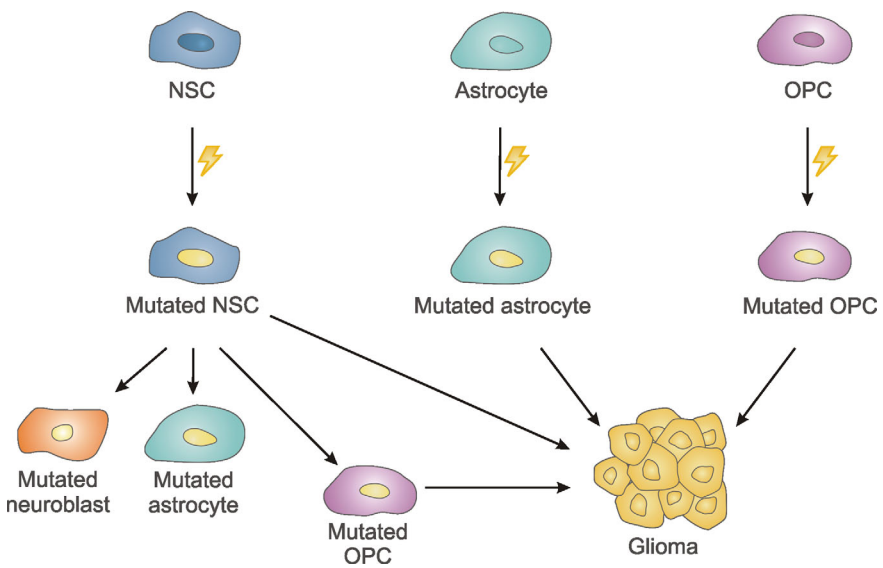

FIGURE 3 | Schematic drawing summarizing different theories on the cell of origin of glioblastomas. Neural stem cells (NSC) of the subventricular zone, astrocytes and oligodendrocyte precursor cells (OPCs) of the brain parenchyma might acquire cancer-driver mutations leading to gliomas. Additionally, NSC-derived mutations can be transmitted to their progeny generating mutated neuroblasts and mutated astrocytes, that do not likely give rise to tumor formation, or mutated OPCs with the ability to grow aberrantly.

population involved in the generation of the tumor mass, this is, the "tumor-propagating cell population".

Further evidence supporting SVZ NSCs as cell of origin of GBM comes from striking similarities in marker expression between NSCs and GBM cells. Some typical NSC markers identified in GBMs are: nestin, glial fibrillary acidic protein (GFAP), Sox2, CD44, and CD133 (prominin-1) (66–68). Nonetheless, characteristic expression profiles of OPCs such as neuronal glial antigen 2 (NG2), Olig2 and platelet-derived growth factor receptor α (PDGRα) can also be found in some GBM subtypes (69–71). This indicates that NSCs and OPCs may give rise to distinct GBM subtypes contributing to the intertumor heterogeneity of GBMs (64).

Remarkably, in spite of sharing many features with SVZ NSCs, NSCs residing in the hippocampus have barely been involved in glioma formation. Friedmann-Morvinski and colleagues showed that transduction of hippocampal NSCs with oncogenic lentiviral vectors gave rise to malignant glioma in mice (72) but, surprisingly, no further data have been published supporting a role for hippocampal NSCs in glioma formation. In addition, the hippocampus is a region spared from GBM (12). Mughal *et al.* analyzed the invasion patterns of glioma cells in two mouse models of invasive GBM and in magnetic resonance images from GBM patients, and showed that, in all cases, despite extensive tumor cell infiltration in hippocampal adjacent structures, very few tumor cells were observed within the hippocampus itself (12). The absence of NSCs in the adult human hippocampus reported by some authors (47, 48) may account for the lack of hippocampal involvement in glioma formation or invasion. But if adult human hippocampal NSCs do exist, as other authors have shown (45, 46, 53, 54), alternative explanations should be provided for the scarce literature relating hippocampal NSCs with glioma development.

POSSIBLE REASONS UNDERLYING THE LACK OF DATA RELATING HIPPOCAMPAL NEURAL STEM CELLS WITH GLIOMAGENESIS

Differences in NSC Fate in Human SVZ and Hippocampus: OPCs Are More Likely to Develop Gliomas Than Neuroblasts

OPCs, also referred to as NG2 cells, represent a major resident glial cell population in the mammalian CNS with the ability to generate myelinating and non-myelinating oligodendrocytes [reviewed in (73)]. Most OPCs are generated during development from the ventral germinal zones of medial and lateral ganglionic eminences (74). During both the postnatal period and the adulthood, additional OPCs are generated from NSCs of the SVZ (16, 22). OPCs constitute the major dividing cell population of the adult mouse and human brain (70, 75, 76). Moreover, in rodents, the proportion of NG2 cells that is actively cycling (~50%) does not decrease with age, although the cell cycle time does (less than 2 days at postnatal day 6 (P6), ~9 days at P60, and ~70 days at P240) (77). Proliferation of adult OPCs and subsequent differentiation into myelinating oligodendrocytes can be activated by stimuli such as neuronal activity or brain injury (76, 78, 79). As mentioned earlier, adult OPCs can be reactivated to a highly proliferative state by oncogenic mutations and give rise to malignant gliomas (63, 64). Alternatively, OPCs generated from SVZ NPCs carrying cancer-driver mutations can also proliferate aberrantly to generate GBM (11). Therefore, adult OPCs are cells to take into account in gliomagenesis, either for being the cell that acquires the initial oncogenic mutations or for being the cell type that propagates the tumor (**Figure 3**).

As mentioned earlier, adult human SVZ NSCs can form neuroblasts that migrate to the striatum (52), but their main progeny are OPCs (50, 52). In contrast, NSCs of the human hippocampus exclusively generate neuronal progenitors (46, 53, 54). This suggests that, if a cancer-driver mutation occurs in adult human NSCs of either origin, the mutation would be transmitted to their progeny, neuroblasts and OPCs in the SVZ, but only neuroblasts in the hippocampus. In mice, mutated OPCs can act as tumor-propagating cells (11, 65) whereas neuroblasts derived from mutated SVZ NSCs migrate to the olfactory bulb and do not develop gliomas (11). It is reasonable to think that neuroblasts born from putative mutated NSCs of the adult human SVZ would not grow aberrantly either after migration to the striatum. In support of this notion, Alcantara Llaguno *et al.* (80) assessed the tumor-initiating potential of late-stage neuronal progenitors, neuroblasts and differentiated neurons, in which the tumor suppressors genes *Nf1*, *p53*, and *Pten* were inactivated by Cre recombinase-mediated gene targeting. They showed that the susceptibility of malignant transformation decreases as the lineage restriction increases. Although cellular and molecular defects were detected as a consequence of the inactivation of the tumor suppressor genes, no evidence of glioma formation was observed in any case (80).

To sum up, if we assume that the population harboring the initial cell mutations is the same that the population that develops the tumor, then, NSCs from both SVZ and hippocampal origin might have similar chances to develop gliomas. But if the tumor-propagating cell population differs from the cell of origin, then it is more likely that OPCs originated from mutated SVZ NSCs, and not neuroblasts born from mutated hippocampal NSCs, proliferate aberrantly to form a tumor mass (**Table 2**).

Neuroblast Final Differentiation Takes Place in the Hippocampus, but Not in the SVZ

As explained in detail in a former section, one of the most remarkable differences between the SVZ and the hippocampus neurogenic niches is that in the SVZ, neuroblasts generated from transit amplifying progenitors do not differentiate into neurons within this region. Instead, they migrate tangentially toward the olfactory bulb where their final differentiation occurs (14, 18, 21). In contrast, neuroblasts generated from intermediate progenitors in the hippocampus differentiate into mature granule cells within

TABLE 2 | Analysis of possible factors determining why neural stem cells of the adult human subventricular zone might be more susceptible to malignant transformation than neural stem cells of the adult human hippocampus.

Human subventricular zone	Human hippocampus
NSCs mainly produce OPCs (50, 52)	NSCs mainly produce neuroblasts (45–46)
Niche factors favor neuroblast migration (18, 21)	Niche factors favor neuroblast differentiation and neuronal maturation (15, 18)
Chemoattractive for glioma cells (81)	Chemorepellent for glioma cells (12)
Microglia are supportive for neurogenesis (82, 83)	Microglia phagocyte apoptotic neuroblasts (40)
NSCs contact the CSF (17, 18, 84, 85)	NSCs do not contact the CSF (31)
NSCs do not express the tumor suppressor gene *HOPX* (86)	NSCs express *HOPX* (86)
Mutations in *TERT* might increase senescence (11, 87)	Mutations in TERT might not be relevant to senescence (88)

CSF, cerebrospinal fluid; HOPX, homeodomain-only protein; NSCs, neural stem cells; OPCs, oligodendrocyte precursor cells; TERT, telomerase reverse transcriptase.

the same niche (15, 18). There is a high variety of factors that control the important step of final differentiation, ranging from intrinsic factors (specific to the progenitor cell population) to extrinsic factors (as the result of the surrounding microenvironment) (18). This cocktail of elements together with their induced molecular responses will determine whether a neuroblast initiates tangential migration toward the olfactory bulb (as it happens in the SVZ) or differentiates (as it happens in the hippocampus). The existence of factors promoting neuronal differentiation in the hippocampus might constitute another possible explanation for the lack of literature reporting tumorigenicity of hippocampal NSCs (**Table 2**). In support of this notion, two recent studies have demonstrated that reprogramming GBM cells into neurons suppresses tumor growth and prolongs survival in mice implanted with human GBM cell lines (89) or with patient-derived GBMs (90). Strikingly, the reprogramming of GBMs into neurons achieved by treatment with mTOR (mammalian target of rapamycin) and ROCK (Rho-associated kinase) inhibitors prevented GBM local recurrence (89). These are important findings in terms of therapeutics development for GBM patients.

The Hippocampal Niche Does Not Favor GBM Invasion

Tumoral cells both in GBM animal models and in patients, follow preferred migratory paths (e.g. optic and pontine white matter structures) and avoid the hippocampus despite being closely located to the tumor (12). However, the SVZ is revealed as one of the most tumor-infiltrated regions in the same study. Other reports have demonstrated that the SVZ is a region of preferred migration for both IDH1-wild type and IDH1-mutant GBMs (91). The presence of molecular cues derived from the specific composition of the extracellular matrix of these regions might guide tumor cell migration toward or away from them (**Table 2**). It is reasonable to think that factors released by the SVZ neurogenic niche might be chemoattractive for glioma cells. Indeed, Qin *et al.* have shown that NSCs of the SVZ secrete pleiotrophin, which forms a chemoattractant complex with other proteins that promote glioma invasion (81). Interestingly, these authors described that pleiotrophin is undetectable in the hippocampus. Therefore, pleiotrophin might be an important molecule involved in the preferred migration of GBM cells toward the SVZ. Another SVZ-released molecule implicated in the stimulation of glioma invasion is CXCL12 (C-X-C motif chemokine 12) (92). In addition, in the SVZ, CXCL12 can mediate GBM resistance to radiation therapy (93). However, this chemokine is also expressed in the hippocampus (94), where it intervenes in the support of newborn neuron maturation (95). Thus, it is less likely that this chemokine might act as a preferred cue for glioma cell migration. Besides, in adult human SVZ, the layer where NSCs reside (hypocellular gap layer) is almost an acellular layer filled with astrocytic processes that provides and environment with fewer hindrances to invasion.

Therefore, the difference for preferred migration exists, but is it connected to a higher lethality of the tumor? In a retrospective study with a cohort of 207 adult patients who underwent cytoreductive surgery for GBM followed by chemotherapy and/or radiation, the authors showed that GBMs contacting the SVZ show earlier recurrence and lower survival than those contacting the SGZ of the hippocampus (96). This work concluded that the SVZ has unique properties that contribute to GBM pathobiology.

On the other hand, the fact that the hippocampus is a region spared from GBM invasion indicates that molecular cues within this niche are not favorable either for tumor chemoattraction or for tumor support (**Table 2**). Identification of signals from the hippocampus that do not support tumor formation as well as the chemoattractants from the SVZ might be of great importance for the development of novel therapeutic strategies for GBM.

Microglia in the Hippocampus Do Not Provide Trophic Support to NSCs

Microglial cells are a main constituent of the adult neurogenic niches (**Figure 1**) although the role exerted by these immune cells differs between the SVZ and the hippocampus. Thus, microglia in the postnatal and adult SVZ provide trophic support for newly-generated neuroblasts and promote their migration toward the olfactory bulb (82, 83), whereas microglia in the hippocampus are involved in the control of neurogenesis through phagocytosis of newborn cells that become apoptotic (40). This process of phagocytosis of apoptotic cells is mediated by purinergic "find me, eat me" signals (97). Interestingly, microglia in the SVZ and in the rostral migratory stream show very low level of purinergic receptors, which allows them to avoid inappropriate activation in response to locally active purines that might result in undesired phagocytosis of neuroblasts before they reach the olfactory bulb (83). Therefore, microglia have a "classical" immune function in the hippocampus whereas in the SVZ acquire a different phenotype to support neurogenesis. It is important to highlight that microglia/macrophages of the GBM microenvironment adopt a tumor-supportive phenotype characterized by the

release of anti-inflammatory molecules, trophic factors and metalloproteinases [reviewed in (98)]. It is therefore reasonable to think that, if a driver mutation occurs in SVZ NSCs, adjacent microglia might act as supporter cells favoring the proliferation and migration of these cells. However, if this were the case in the hippocampus, niche microglia would not have a permissive phenotype for tumor evolution (**Table 2**).

NSCs of the Hippocampus Do Not Contact the CSF

As mentioned before, NSCs in the SVZ are in direct contact with the CSF through a small apical process harboring a primary cilium (**Figures 1A** and **2A**) that is likely involved in signal transduction (84, 85). In contrast, in the SGZ of the hippocampus, type 1 cells do not contact the CSF (31). This noticeable difference between the two adult neurogenic niches might determine a higher susceptibility of NSCs of the SVZ to malignant transformation or uncontrolled proliferation (**Table 2**).

Soluble factors in the CSF (which are released by epithelial cells of the choroid plexus) are important not only for the maintenance of NSC quiescence in the SVZ, but also for the regulation of multiple aspects of the adult NSC behavior and their progeny (84, 99–101). Recently, de Sonnaville et al. have shown that human ventricular CSF increases proliferation of SVZ NSCs (102). Some of the soluble molecules contained in the CSF are growth factors which have been reported to be involved in the stimulation of NSC proliferation, such as insulin-like growth factor 2 (IGF-2) (103), transforming growth factor-β (TGF-β) (104), vascular endothelial growth factor A (VEGFA) (105), leukemia inhibitory factor (LIF) (106) or endogenous ligands of the EGFR (107). Noteworthy, these factors have also been shown to contribute to glioblastoma growth or support (103, 108–111). Consequently, the direct contact of SVZ NSCs with these mitogens might confer them growth advantages if the concentrations of these factors were increased or if NSCs had acquired somatic mutations which made them more responsive to these proliferative signals.

Lately, other constituents of the CSF have gained much importance in intercellular communication: the extracellular vesicles (EVs). EVs are small membrane vesicles (30 nm–10 μm) secreted by almost all cell types that are implicated in the transfer of mRNAs, microRNAs, proteins and lipids between cells and thus are able to modify the function of recipient cells. Isolation of membrane vesicle-enriched fractions and further proteomic studies have demonstrated the presence of EVs in the human CSF (112, 113). This might be relevant since EVs can transfer oncogenic cargo to recipient cells. For instance, EGFRvIII contained in EVs released by glioma cells has been shown to be transferred to indolent glioma cells in which they induce oncogenic activity (114). Similarly, Gutkin *et al.* reported EV-mediated horizontal transfer of hTERT mRNA from cancer to non-cancer cells (115). These observations raise the hypothesis that NSCs may be susceptible to malignant conversion *via* EV-mediated molecular transfer.

Therefore, NSCs in the SVZ, due to their direct contact with the CSF, are exposed to growth factors and EVs that might increase their susceptibility to aberrant growth or malignant transformation (**Table 2**).

Adult Hippocampal, but Not Adult SVZ NSCs, Express the Tumor Suppressor Gene *HOPX*.

SVZ and hippocampal NSCs share astroglial features and expression of numerous molecular markers (18). However, there are some differences that might be relevant in terms of susceptibility to malignant transformation. One of these differentially-expressed proteins is HopX (homeodomain-only protein), an atypical homeodomain protein that cannot bind DNA and exerts its actions by interacting with serum responsive factor (SRF) and blocking its transcriptional activity (116). *HOPX* gene is selectively expressed by quiescent NSCs of the adult hippocampus, but not by adult SVZ NSCs (86). Specifically in the dorsal SVZ, this protein is present during embryonic and postnatal stages in NSCs primed toward astrocytic fates but declines to nearly undetectable levels in adulthood (117). In the adult hippocampus, HopX has been described to intervene in the regulation of neurogenesis by promoting apoptosis of NSCs. Remarkably, *HOPX* expression is down-regulated in GBMs and on the other hand, a cell-permeable version of HopX protein with gain of function characteristics causes an increase in apoptosis in a subset of GBM cells and a decrease in clonogenicity (118). It is worth mentioning that *HOPX* expression is lost or down-regulated in other cancers as well (119, 120). Hence, we hypothesize that the restricted expression of HOPX in adult NSCs of the hippocampus might confer tumor-suppressive properties to this population of cells (**Table 2**).

Mutations in the Catalytic Subunit of Telomerase Reverse Transcriptase (TERT) Might Have Different Implications in SVZ NSCs Than in Hippocampal NSCs

Telomere length is essential for the prolonged persistence of stem cell functions in organs with extensive cell turnover (121, 122). The maintenance of telomere length is mediated by telomerase, an enzyme that adds nucleotides to the end of the chromosomes and prevents the replication-dependent loss of telomere and cellular senescence (122, 123). The active telomerase enzyme consists of a protein component TERT that serves as catalytic subunit, and a telomerase RNA component (TERC) (124, 125). Human somatic cells lack telomerase activity, which can be considered as a tumor suppressor mechanism since it prevents unlimited clonal expanding. Accordingly, 90% of human tumors are telomerase positive (126).

In the context of GBM, Lee and colleagues demonstrated that all the IDH-wild type GBM patients with driver mutations in tumor-free SVZ tissue also presented mutations in the *TERT* promoter in this tissue (11). Indeed, they suggested that mutation-driven activation of *TERT* in SVZ NSCs might be the earliest event by which these cells, having limited self-renewal capability, are able to avoid replicative senescence thereby increasing their chances of acquiring other driver mutations over time (11). Hippocampal NSCs might also acquire mutations

in *TERT* that could increase their senescence and the subsequent possibility of becoming tumorigenic. However, current data do not support this hypothesis, as will be discussed below.

First, in humans, *TERT* mutations have been reported to occur in NSCs of normal or non-cancer aged hippocampus at much lower rates than those found in SVZ NSCs of IDH-wild type GBM (11). Second, overexpression of TERT in mouse hippocampal NSCs does not lead to tumor formation (88). Third, in rodents, the SVZ and olfactory bulb have significantly higher levels of telomerase activity than the hippocampus (87). And fourth, in the mouse hippocampus, TERT exerts additional roles independent of its telomerase activity (88). Thereby, by knockdown and overexpression of TERT, Zhou and colleagues (88) demonstrated that TERT is required for neural circuit integration of hippocampal newborn neurons, as well as for spatial memory processing. TERT actions through non-canonical pathways have not yet been described in SVZ NSCs.

These data suggest that putative mutations in *TERT* leading to increased telomerase activity would have more chances to induce glioma formation in SVZ NSCs than in hippocampal NSCs (**Table 2**).

CONCLUSIONS

Experiments performed in mice have revealed that oncogenic mutations in NSCs of the SVZ, astrocytes or OPCs can all lead to glioma formation. However, recent data obtained from GBM patients have reinforced the hypothesis that NSCs of the SVZ are the cell of origin of IDH-wild type GBM. Since NSCs are anatomically restricted in the SVZ whereas astrocytes and OPCs are widely distributed in the brain, the clinical implications on the diagnosis and therapy for these lethal tumors may considerably vary depending on the cell of origin.

NSCs of the hippocampus, however, have not been associated either to the origin or to the propagation of GBM. Throughout this article we have provided some possible explanations for this fact that are summarized in **Table 2**.

One of the reasons supporting SVZ-derived NSCs as cell of origin of IDH-wild type GBM rely on the specific progeny of NSCs of every neurogenic niche. SVZ-derived NSCs mainly produce OPCs in the human brain, whereas hippocampal-derived NSCs produce neuroblasts. Progenitors committed to a neuronal fate are less prone to develop gliomas than those committed to the oligodendroglial lineage. In addition, factors within the SVZ niche might be more permissive for aberrant tumoral cell migration and growth than those present in the SGZ niche. Other possibilities such as differential expression of tumor suppressor genes and differential effects of *TERT* mutations and roles between SVZ and SGZ NSCs can be taken into account to find possible explanations for the higher susceptibility of malignant transformation of SVZ NSCs.

AUTHOR CONTRIBUTIONS

EM conceived and designed the manuscript. ÁF-L, SM, and EM wrote the manuscript. BB-T and MD-L designed and edited the figures. RM revised the manuscript. All authors contributed to the article and approved the submitted version.

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The Epigenetics of Glioma Stem Cells: A Brief Overview

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Glioma stem cells (GSCs) are crucial in the formation, perpetuation and recurrence of glioblastomas (GBs) due to their self-renewal and proliferation properties. Although GSCs share cellular and molecular characteristics with neural stem cells (NSCs), GSCs show unique transcriptional and epigenetic features that may explain their relevant role in GB and may constitute druggable targets for novel therapeutic approaches. In this review, we will summarize the most important findings in GSCs concerning epigenetic-dependent mechanisms.

Keywords: glioblastoma, histone, DNA, methylation, acetylation, Polycomb, H3.3, HDACi

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INTRODUCTION

GB is the most common and aggressive primary brain cancer in adults. Despite the combined clinical therapy of surgical resection, radiotherapy and chemotherapy with the first-line agent temozolomide (TMZ), the prognosis is still unfavorable, with a median overall survival of 15 months and a high risk of recurrence (>90%) (1). This ability to resist chemo- and radiotherapy can be explained by the presence of a subpopulation of cells within the perivascular and hypoxic niches of the tumor known as GSCs or brain tumor-initiating cells. The subventricular zone (SVZ) is a neurogenic niche containing NSCs and progenitor cells and is suspected to be the origin of different brain tumor types due to the generation of GSCs (2–4). GSCs share functional characteristics with NSCs, including the capacity for self-renewal and long-term proliferation required to maintain and propagate the tumor, respectively (5). In addition, GSCs exhibit other properties of cancer cells, such as angiogenesis, invasion and immunosuppression, that promote disease progression and complicate treatment (6). Cells positive for stemness markers (*e.g.*, CD133) have the ability to form tumors *in vivo* and oncospheres *in vitro* (reminiscent of neurosphere-derived NSCs) (6). In fact, understanding the hallmarks of GSCs can offer novel therapeutic strategies targeted at these cells to achieve an effective treatment for this disease.

Abbreviations: BMP, Bone morphogenic protein; BRD, Brodomain; CNTF, Ciliary neurotrophic factor; DIPG, Diffuse intrinsic pontine gliomas; EED, Embryonic ectoderm development; EMT, Epithelial–mesenchymal transition; ESC, Embryonic stem cell; EZH2, Enhancer of Zeste homolog 2; GB, Glioblastoma; GSC, Glioma stem cell; HDAC, Histone deacetylase; HDACi, HDAC inhibitor; HOTAIR, HOX transcript antisense RNA; KAT, Lysine acetyltransferase; lncRNA, Long Non-coding RNA; MELK, Maternal embryonic leucine zipper kinase; MGMT, O-6-methylguanine-DNA methyltransferase; NEK2, NIMA-related kinase 2; NSC, Neural stem cell; PRC1/2, Polycomb repressive complex 1/2; SUZ12, Suppressor of Zeste 12; SVZ, subventricular zone; TMZ, Temozolomide; TUG1, Taurine upregulated gene 1; VPA, Valproic acid.

THE RELEVANCE OF EPIGENETICS IN THE REGULATION OF GENE EXPRESSION IN GSCs AND NSCs

The nucleosome is the structural unit of chromatin and is composed of 147 bp of DNA wrapped around an octamer of histones (H2A, H2B, H3, and H4). The chromatin organization and its degree of compaction are modulated by DNA and histone covalent modifications, ATP-dependent chromatin remodeling and certain non-coding RNAs (ncRNAs). Epigenetic mechanisms contribute to the cellular hierarchy of tumoral tissue in GB (7) and are crucial to understanding tumorigenesis and response to treatment in gliomas. For example, promoter hypermethylation of the O-6-methylguanine-DNA methyltransferase (*MGMT*) gene can predict good outcomes in TMZ treatment (8, 9). Additionally, mutations in arginine 132 of the tricarboxylic acid cycle component IDH1 (or in arginine 172 of IDH2), which are associated with longer survival, induce the overproduction of the 2-hydroxybutyrate metabolite that inhibits the α-ketoglutarate-dependent activity of epigenetic enzymes such as JumonjiC histone demethylases and TET hydroxymethylases, affecting both histone and DNA methylation (10).

The gene expression profiles of GSCs resemble those of normal NSCs (11, 12), but differential gene expression patterns between both types of cells can identify a transcriptional signature that is correlated with patient survival (13); however, copy number variations only explain a small portion of such gene expression alterations and other mechanisms (*e.g.*, epigenetics) should be more relevant. For instance, changes in the patterns of DNA methylation, H3K27me3 and H3K4me3 are important in neural lineage differentiation (14–16), and a comparison of the genome-wide distribution of these and other epigenetic marks revealed important differences between GSCs and normal NSCs, affecting genes involved in neural differentiation and cancer processes (17, 18). These glioma-specific patterns of epigenetic marks can be found in DNA elements that are important for gene regulation:

- Bivalent promoters are considered a feature of embryonic stem cells (ESCs) due to their high prevalence in these cells (16, 19) and are characterized by the coexistence of epigenetic marks associated with active and repressed genes (generally H3K4me3 and H3K27me3). Genes under the control of such promoters are poised, *i.e.*, maintained in silent state but ready to be activated under appropriate external or developmental stimuli (20). Genome-wide analyses identified a high diversity of bivalent regions within GSCs, which were shown to have significantly distinct patterns compared to NSCs and ESCs (17, 21). Loss of bivalency in GSCs affected a very low number of promoters but associated with the potential activation of proto-oncogenes and genes related to transcription, and the potential repression of genes linked to cell adhesion and ion channels (17). Moreover, consistent bivalent genes across several GSCs were members of the Wnt pathway and HOX family as well as potassium channels and solute carriers that can be associated with overall survival (21).
- Enhancers often regulate cell-specific gene expression and are defined by the simultaneous occupancy of H3K27ac and H3K4me1. Although enhancer patterns are relatively conserved between GSCs and NSCs, unique GSC patterns are mainly linked to genes with functions in DNA damage response, p53 signaling and angiogenesis; prominent examples are HOX cluster genes, which acquire enhancer histone modifications in GSCs and become highly expressed despite promoter methylation (22). In contrast, NSC-specific enhancers are more associated with stem cell differentiation, apoptosis and epigenetic regulation (22).

Overall, GSCs are characterized by an impairment of differentiation due to a permanent epigenetic block that maintains the self-renewal capacity of these cells (18, 23). Nonetheless, GSCs can rapidly adapt to diverse microenvironments by modulating their transcriptomes and DNA methylomes (24), indicating that such alterations are at least partially reversible, contrary to genetic variations. Reversibility of epigenetic marks was demonstrated in reprogramming experiments of glioma cells: with the appropriate combination of transcription factors they can be reversed into an early embryonic state that was accompanied by a widespread resetting of cancer-associated DNA methylation (23). Still, this resetting was not sufficient to abolish the malignant behavior of these cancer cells, indicating that we need to decipher how epigenetic-related activities work in GSCs to explaining their malignancy. In the following sections we review the experimental evidences found in GSCs about the role of epigenetics in malignancy and potential treatments.

THE ROLE OF POLYCOMB REPRESSIVE COMPLEXES IN THE MAINTENANCE OF THE GSC PHENOTYPE

The Polycomb repressive complexes, essential for normal developmental processes, have been the most studied epigenetic modulators in GSCs. The most relevant findings are summarized in **Figure 1A**. Polycomb repressive complex 2 (PRC2) is necessary for neurogenesis at the SVZ (25, 26) and regulates the trimethylation of H3K27 thanks to the catalytic activity of Enhancer of Zeste Homolog 2 (EZH2), which transfers a methyl group from S-adenosyl methionine, in cooperation with Suppressor of Zeste 12 (SUZ12) and Embryonic Ectoderm Development (EED). Overexpression of EZH2 has proto-oncogenic implications in several cancers, including glioma, in which elevated EZH2 expression has been associated with high-grade disease and poor overall survival (27, 28). Moreover, EZH2 activity is required for GSC maintenance by targeting MYC expression (29). Even in cells derived from diffuse intrinsic pontine gliomas (DIPG), a brain pediatric cancer that can also affect young adults, in which the actions of EZH2 are inhibited by the H3K27M mutation, residual EZH2 activity is still retained at strong PRC2 targets to drive GSC proliferation (30). Therefore, it is not surprising that selective EZH2 inhibition can constitute a promising therapeutic approach, as treated GSCs can reduce the

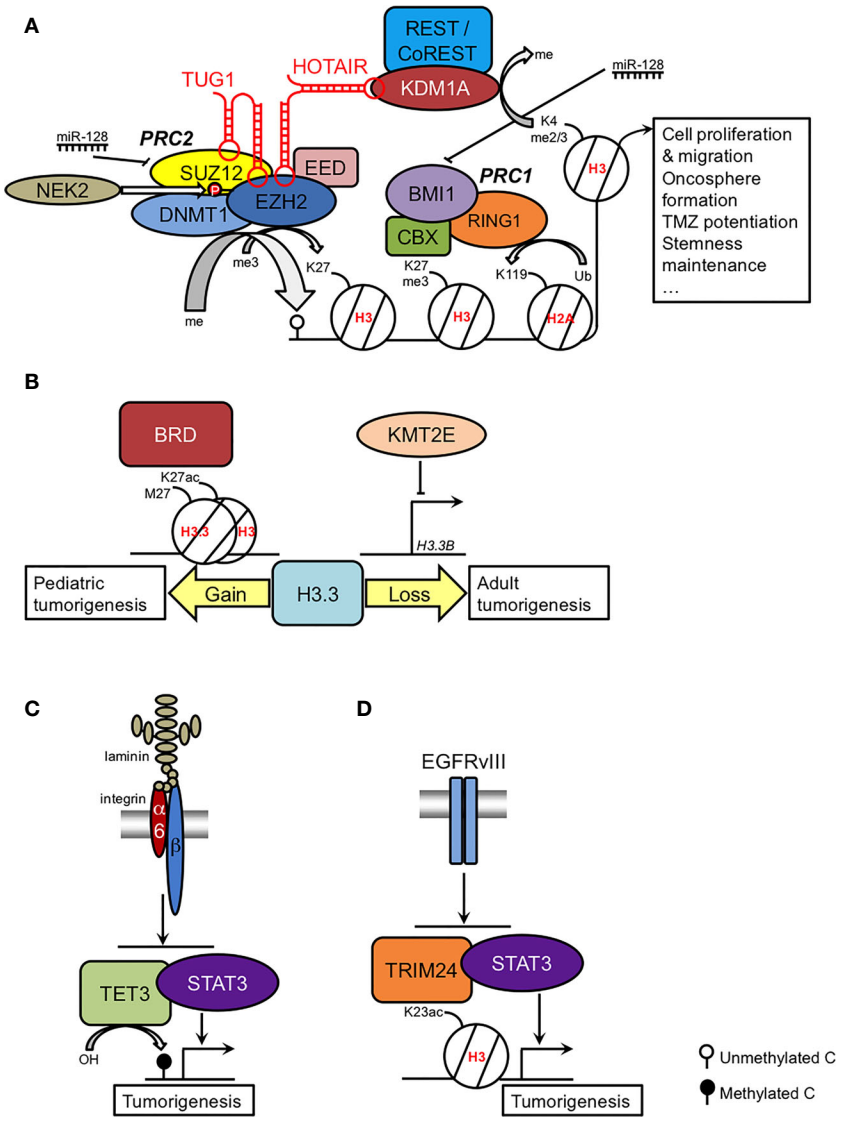

FIGURE 1 | Summary of the epigenetic regulation of the GSC phenotype. **(A)** Coordinated actions and regulation of Polycomb complexes; **(B)** role of H3.3 in pediatric and adult GSCs as a result of gain and loss-of-function, respectively; **(C)** TET3-STAT regulation by the laminin-integrin signaling pathway; **(D)** H3K23ac-TRIM24-STAT regulation by the EGFRvIII signaling pathway. See main text for further details.

levels of EZH2 and H3K27me3, cell proliferation and migration, the number and diameter of oncospheres, and the growth of intracranial xenotransplanted cells in mice, reverse epithelial-mesenchymal transition (EMT), potentiate the effects of TMZ and downregulate stem cell markers while increasing the expression of differentiation markers (29, 31–33).

PRC2 activity is important for other epigenetic modifications. First, trimethylation of H3K27 is a prerequisite for histone H2A monoubiquitylation by Polycomb repressive complex 1 (PRC1) (34). Within this complex, the ring finger protein BMI1 is also a glioma stemness marker, and interference of its activity affects GSC malignancy *in vitro* and in xenotransplanted mice and enhances radiosensitivity (35–37). Second, EZH2 can recruit DNA methyltransferases (38), which explains the hypermethylation of PRC2 targets in primary GB (39, 40).

GSC characteristics display regional variations depending on the tumor niche. Whereas the regions defined by the disruption of the blood-brain barrier in angiogenesis foci were characterized by a high expression of proneural genes, an enrichment of EZH2/SUZ12/H3K27me3 targets and GSCs primarily positive to the proneural markers SOX2 and OLIG2, the hypoxic necrotic regions contained high expression of mesenchymal genes, a strong association with H2A119ub, an enrichment of BMI1 targets and GSCs primarily positive to the mesenchymal markers CD44 and YKL40 (41). Selective inhibition of either EZH2 or BMI1 was highly effective against the survival of

proneural and mesenchymal GSCs, respectively. Thus, the combined strategy to abolish the activity of both PRCs can target different tumor compartments, increasing the efficacy of the therapy (41).

Research on GSCs is starting to disentangle EZH2-dependent oncogenic mechanisms. In certain GSCs, astroglial differentiation mediated by the bone morphogenic protein (BMP) and ciliary neurotrophic factor (CNTF) signaling pathways is impaired due to the silencing of the BMP receptor subtype gene *BMPR1B* by hypermethylation of its promoter, mediated by the EZH2-dependent recruitment of DNMT1 (42). Whereas incubation with BMP2 or CNTF can induce an increase in the differentiation markers GFAP or β-III tubulin in cultured NSCs and GSCs, in GSCs with impaired expression of *BMPR1B*, these trophic factors enhance proliferation (42). These pleiotropic actions are reminiscent of the role of the BMP signaling pathway in embryonic NSCs to promote either NSC proliferation or neuronal differentiation, depending on the expression of the BMP receptor subunit (43). To add more complexity to the EZH2 involvement in gliomagenesis, EZH2 can methylate non-histone proteins such as oncogenic STAT3. This association leads to enhanced activation of STAT3 to positively regulate GSC self-renewal and survival (44).

How PRC2 activity is deregulated in GSCs has been intensively explored. For instance, EZH2-dependent resistance of GSCs to radiotherapy can be explained by the transcriptional upregulation of EZH2 induced by maternal embryonic leucine zipper kinase (MELK) and activation of EZH2 through phosphorylation by NIMA-related kinase 2 (NEK2) (33, 45). Moreover, it has been proposed that dysfunction of miR-128 is an early event of gliomagenesis that increases the levels of both SUZ12 and BMI1, augmenting the histone modifications they regulate: H3K27me3 and H2AK119ub. These observations suggested a coordinated regulation of PRC1 and PRC2 activities. Therefore, restoring miR-128 expression diminishes proliferation and confers radiosensitivity (46). Additionally, EZH2 activity can be regulated by the lncRNA HOX transcript antisense RNA (HOTAIR), which is associated with poor survival in diverse cancers (47). In $CD133^+$ cells, HOTAIR recruits both EZH2 and the lysine demethylase KDM1A/LSD1 to repress the tumor suppressor gene *PDCD4* (48). In addition, another lncRNA, taurine upregulated gene 1 (TUG1), also binds to EZH2 and SUZ12 to repress neuronal differentiation genes such as *BDNF*, *NGF*, and *NTF3* (49).

THE HISTONE VARIANT H3.3 IN PEDIATRIC AND ADULT GSCS

The histone H3 variant H3.3 can play a determinant role in pediatric GB. H3.3 is an independent replication variant that replaces the canonical histones H3.1 and H3.2 during brain development, becoming predominant in adulthood (50). H3.3 is encoded by two genes: *H3F3A* (*H3.3A*) and *H3F3B* (*H3.3B*). Mutations in *H3F3A* are present in approximately one-third of pediatric gliomas, affecting either lysine 27 (H3K27M) or glycine 34 (H3G34R/V), although the former mutation can also be found to a much lesser extent in the *HIST1H3B* (*H3C2*) gene (51–53). H3K27M is a relevant driver mutation in the pathogenesis of DIPG and is sufficient to immortalize NSCs from human embryo pons (54). In DIPG-derived cell lines, H3K27M specifically increases the acetylation of H3K27 and creates heterotopic H3K27M/H3K27ac nucleosomes that can be targeted by inhibitors of bromodomain (BRD) proteins to modulate the expression of the cell cycle arrest gene *CDKN1A*, the neuronal mature markers *TUBB3* and *MAP2*, and the Zn finger protein *GLI2* (55), a relevant downstream target of the Sonic Hedgehog pathway that is implicated in the etiology of DIPG (56) (**Figure 1B**).

In adult GB, dominant negative mutations in histone H3 are extremely rare. Instead, downregulation of the *H3F3B* gene has been reported to lead to a deficit of H3.3 function in GSCs as a result of the action of the lysine methyltransferase KMT2E (myeloid/lymphoid leukemia MLL5), maintaining the self-renewal capacity of GSCs and interfering with their differentiation (57) (**Figure 1B**). These findings suggest that H3.3 impairment in adult GB may produce similar chromatin rearrangements as the H3.3 mutation in pediatric GB, given the similar DNA methylation patterns in both types of tumors (57).

OTHER EPIGENETIC MODULATORS

In addition to PRCs and H3.3, other epigenetic-related factors have been implicated in the GSC phenotype and are listed in **Table 1**.

HDAC INHIBITORS AS THERAPEUTIC AGENTS IN GSCs

Considering that altered gene expression levels have been reported for histone deacetylases (HDACs) in GB (66, 67), most therapeutic approaches have been focused on histone deacetylase inhibitors (HDACis) due to their recognized antiproliferative effects in multiple cancer models and their benefits and tolerability in the amelioration of several neurological conditions *in vivo* at the preclinical stage; in addition, some of these compounds have been approved as therapeutic agents in other types of cancers. Histone acetylation is regulated by the opposing enzymatic activities of lysine acetyltransferases and HDACs: whereas the former enzymes transfer the acetyl group from an acetyl-CoA molecule to the lysines of the protruding histone tails (an activity that is associated with active genes), HDACs catalyze this removal, which is associated with gene repression. Inhibition of HDACs can induce cell cycle arrest, apoptosis and cellular differentiation and can interfere with cancer angiogenesis (68). One interesting target of HDACis is the phosphatase DUSP1, an inhibitor of the JNK, ERK1/2 and p38 MAPK pathways that is associated with GSC differentiation and good prognosis (69).

Among the tested HDACis in clinical trials, vorinostat/SAHA, romidepsin/FK228/FR901228 and panobinostat/LBH-589

TABLE 1 | List of other epigenetic-related factors in GSC studies.

Modulator	Epigenetic action	Role and mechanisms
Helicase, lymphocyte-specific HELLS	Member of the ATP-dependent chromatin remodeling SWI2/SNF2 complexes	Maintenance of proliferation and self-renewal of GSCs through binding to the promoters of cell cycle genes, including E2F3 and MYC targets (58).
Lysine-specific demethylase KDM1A/LSD1	Demethylation of mono- and di-methylated lysines 4 and 9 of histone H3	Cell viability, oncosphere formation and tumorigenesis of intracranial xenografts. Rescue by novel inhibitors (59).
Lysine demethylase with Jumonji domain KDM6B/JMJD3	Demethylation of mono- and di-methylated lysine 27 of histone H3	Cell growth and tumorigenesis of intracranial xenografts of pediatric brainstem GSCs. Rescue by treatment with GSKJ4 (60)
Lysine methyltransferase KMT2A/MLL1	Methylation of lysine 4 of histone H3	Upregulation in GSC and in hypoxic GB. GSC growth and self-renewal (61).
Ten–Eleven Translocation TET3	Conversion of 5 mC to 5 hmC	Inhibition of self-renewal and tumorigenesis after downregulation of its repressor, the nuclear receptor NR2E1/TLX (62). In highly aggressive GSCs, maintenance of laminin-integrin α6 signaling pathway-dependent cell survival through TET3 interaction with STAT3 at methylated loci, leading to global increase of 5hmC levels and the upregulation of oncogenes (*e.g.*, c-Myc, surviving, BCL2-like protein BCL-XL (63) (**Figure 1C**). Inhibition of the differentiation marker GFAP (22) after TET3 translocation into the GSC nucleus.
Tripartite motif-containing protein TRIM24	Reader of histone H3 with unmethylated K4 and acetylated K23	Association with tumor grade and GB recurrence (64). In EGFRvIII-expressing glioma cells, association with increased H3K23ac and recruitment of STAT3 to promote GSC proliferation and oncosphere formation (**Figure 1D**). Rescue by treatment with EGFR inhibitor erlotinib (65).

demonstrated very limited efficacy as therapeutic agents in single therapies in both newly diagnosed and recurrent GB. However, the most promising effect of HDACis is as sensitizers to current therapeutic approaches such as radiotherapy and TMZ therapy [see (70) for a review on this topic]. Some considerations should be kept in mind to understand the potential benefits and limitations of HDACi-based treatment *in vivo*. First, acetylation increase by HDACis is not exclusive of histones (71); second, antineoplastic actions of HDACis can be achieved independently of (or in addition to) HDAC inhibition (72); third, the solubility of HDACis in water is usually poor, resulting in inefficient transport through the blood-brain barrier with oral administration (73); last, chemoresistance has been reported in long-term treatments (74). In any case, the prospects of using HDACis are still promising, and research on GSCs can help in elucidating the underlying anticancer mechanisms of HDAC inhibition and in proposing novel formulations to improve drug delivery (*e.g.*, loading these hydrophobic compounds into nanomicelles) (75). Efforts are being mainly focused on valproic acid (VPA), with proven antitumoral effects (72, 76, 77). Often administered as an anticonvulsant agent to treat epilepsy in brain tumors (78), retrospective clinical studies reported that treatment with this compound increased the overall survival of GB patients (79) although this effect was not found in other reports and still remains controversial (80, 81). VPA is capable of inducing a predifferentiation state in GSCs (74) and can be combined with other antineoplastic compounds for synergistic effects, as reported for the antimitotic paclitaxel (82). However, VPA failed to sensitize GSCs to TMZ (74), although another study reported sensitization to both TMZ and nimustine (ACNU), especially in MGMT-expressing cells (83). VPA is able to modify the DNA methylomes of GSCs (74), leading to the activation of the Wnt/β-catenin pathway which was related with growth inhibition, reduced migration and EMT impairment (84). This is in conflict with the suppression of the Wnt/β-catenin pathway by SAHA, which partially rescues the downregulation of histocompatibility complex class I and antigen-processing machinery genes, as a plausible strategy to potentiate the activation of cytotoxic T cells *in vivo* (85). Side effects have also been reported as VPA can exacerbate the unfolded protein response program, leading to protein homeostasis dysregulation and proteostasis stress in GSCs (86).

CONCLUDING REMARKS

Research on the epigenetics of GSCs has the potential to elucidate the self-renewal and perpetuation mechanisms of these cells through the identification of the epigenetic program that governs aberrant gene activation and repression in cancer. Less known epigenetic modifications should be further explored, as they can provide further insights into tumorigenesis, as in the case of 5'-formylcytosine (5fC) and 5′-carboxylcytosine (5caC) (22). In addition, a systematic and detailed description of direct target genes of epigenetic activities is required to understand the complex mechanisms of epigenetic dysregulation in gliomas. As we have seen through this review, multiple epigenetic activities can be involved in glioma malignancy in a complex manner; therefore, the simultaneous modulation of various epigenetic activities may be highly effective, as demonstrated by the dual inhibition of HDACs and KDM1A/LSD1 (87, 88).

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Glioblastoma Distance From the Subventricular Neural Stem Cell Niche Does Not Correlate With Survival

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Objective: To determine the relationship between survival and glioblastoma distance from the ventricular-subventricular neural stem cell niche (VSVZ).

Methods: 502 pre-operative gadolinium-enhanced, T1-weighted MRIs with glioblastoma retrieved from an institutional dataset (n = 252) and The Cancer Imaging Atlas (n=250) were independently reviewed. The shortest distance from the tumor contrast enhancement to the nearest lateral ventricular wall, the location of the VSVZ, was measured (GBM-$VSVZ_{Dist}$). The relationship of GBM-$VSVZ_{Dist}$ with the proportion of glioblastomas at each distance point and overall survival was explored with a Pearson's correlation and Cox regression model, respectively, adjusting for the well-established glioblastoma prognosticators.

Results: 244/502 glioblastomas had VSVZ contact. The proportion of non-VSVZ-contacting glioblastomas correlated inversely with GBM-$VSVZ_{Dist}$ (partial Pearson's correlation adjusted for tumor volume R=-0.79, $p=7.11x10^{-7}$). A fit of the Cox regression model adjusted for age at diagnosis, Karnofsky performance status score, post-operative treatment with temozolomide and/or radiotherapy, *IDH1/2* mutation status, *MGMT* promoter methylation status, tumor volume, and extent of resection demonstrated a significantly decreased overall survival only when glioblastoma contacted the VSVZ. Overall survival did not correlate with GBM-$VSVZ_{Dist}$.

Conclusions: In the two independent cohorts analyzed, glioblastomas at diagnosis were found in close proximity or in contact with the VSVZ with a proportion that decreased linearly with GBM-$VSVZ_{Dist}$. Patient survival was only influenced by the presence or absence of a gadolinium-enhanced glioblastoma contact with the VSVZ. These results may guide analyses to test differential effectiveness of VSVZ radiation in VSVZ-contacting and non-contacting glioblastomas and/or inform patient selection criteria in clinical trials of glioblastoma radiation.

Keywords: glioblastoma, subventricular zone, survival, stem cell, glioma

INTRODUCTION

The median survival of patients with glioblastoma is 16 months (1, 2). Survival is even lower in those patients in whom the glioblastoma has invaded or contacted the ventricular-subventricular zone (VSVZ) at diagnosis (3, 4). The VSVZ is a neural stem cell niche in the lateral walls of the lateral ventricles in the brain (5–7). Recent results have supported the hypothesis that VSVZ houses the cellular origins of some human glioblastomas (7, 8).

Glioblastomas with and without VSVZ invasion or contact are not genomically distinct in bulk profiling (9, 10). Therefore, the more severe clinical phenotype of those patients is likely due to the microenvironment of the VSVZ (9). The microenvironment is especially attractive to glioblastoma cells (11, 12), and factors released by the VSVZ can mediate resistance to radiation therapy in glioblastoma cells (13). Consistent with these reports, recurrence is earlier in those patients in whom the initial glioblastoma invaded or contacted the VSVZ (3, 4).

Research efforts in this area have been conducted by dichotomizing glioblastomas as having VSVZ contact or not, demonstrating survival is lower with contact. Whether clinical outcome is related continuously with glioblastoma distance from the VSVZ (GBM-VSVZ$_{Dist}$) is unknown. Therefore, in this brief report, we conducted survival analyses to uncover the relationship of patient survival with glioblastoma distance from the VSVZ that yielded consistent findings in two distinct datasets.

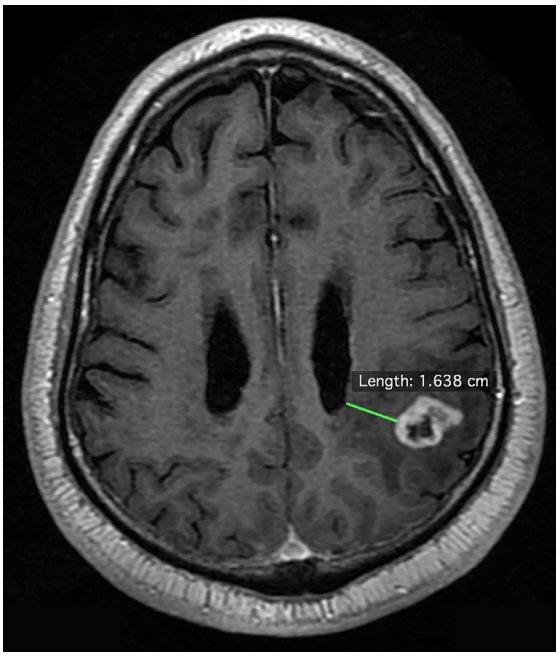

FIGURE 1 | Example measurement of the shortest distance between the enhancing edge of a glioblastoma to the lateral wall of the lateral ventricle on an axial pre-operative gadolinium-enhanced, T1-weighted magnetic resonance image.

METHODS

A total of 502 patients with histologically confirmed glioblastoma were analyzed: 252 from a prospectively maintained single-institution (VUMC) registry and 250 from The Cancer Imaging Atlas (TCIA) (14). In the institutional dataset, the median year of diagnosis was 2012 [interquartile range: 2009-2015] and the patients were followed to 2017. Institutional review board approval was obtained; patient consent was waived. Two independent, outcome-blinded reviews of pre-operative gadolinium-enhanced, T1-weighted MRIs were conducted. One of these assessments was made by a board-certified neuro-radiologist. All institutional MRIs were obtained using a 1.5 or 3-tesla magnetic field, and details of the MRIs in the TCIA are published (14). As per standard assessment, all MRIs were first dichotomized as demonstrating contact of the tumor contrast enhancement with the lateral ventricular ependyma —the location of the VSVZ—or not. GBM-VSVZ$_{Dist}$ was then measured for all non-VSVZ-contacting glioblastomas. Specifically, the shortest distance from the tumor contrast enhancement to the nearest lateral ventricular wall visible on any one of three MRI dimensions (axial, coronal, or sagittal) was measured to the nearest millimeter (mm) (**Figure 1**). All disagreements were resolved by group consensus. Glioblastoma volume contained within contrast enhancement was also calculated. All of these radiological assessments were performed using OsiriX Lite software (version 9.4, Pixmeo, Geneva, Switzerland).

Corresponding clinical data were obtained from the institution registry and The Cancer Genome Atlas (9). Patient survival was recorded as the time from initial diagnosis to death or to the last time known to be alive (censored survival). The relationship between GBM-VSVZ$_{Dist}$ and survival was assessed using the Cox regression model, adjusting for well-established glioblastoma prognosticators. These included age at diagnosis (treated as a continuous variable), pre-operative Karnofsky performance status score (KPS; categorized into 100-80, 70-50, 40-0), postoperative treatment with temozolomide and/or radiotherapy (yes or no), *IDH1/2* mutation status (wildtype, mutant, or unassessed), *MGMT* promoter methylation status (methylated, unmethylated, or unassessed), tumor volume (continuous variable), and extent of resection of the enhancing portion of the glioblastoma. The extent of resection was categorized as biopsy (<50%), subtotal (50%–95%), near-total (95%–99%) and gross total (100%) based on independent assessments by a neurosurgeon and neuroradiologist of the postoperative, post-contrast MRI obtained within 24 h after the operation. The adjusted Cox models were confirmed to meet the proportional hazards assumption, which was tested by assessing the significance of the relationship between Schoenfeld residuals and time for the overall model. The results of the Cox models are reported as hazard ratios with 95% confidence intervals. Finally, the relationship between GBM-VSVZ$_{Dist}$ and adjusted hazard ratios were depicted using a quadratic spline fit.

Standard descriptive statistical methods were used to report variables and compare distributions of continuous and proportions of categorical variables. Correlation between two variables was assessed using Pearson's correlation treating both variables continuously. Statistical significance was claimed with a

two-sided p-value of ≤ 0.05 or 95% confidence intervals that did not span 1. All analyses were conducted using R version 3.4 (R Foundation for Statistical Computing, Vienna, Austria) and the Survminer package (Version 0.4.0).

RESULTS

Patient and glioblastoma characteristics are listed in **Table 1**. Out of 502 glioblastomas, 244 (48.6%) had VSVZ contact (GBM-$VSVZ_{Dist}$=0). Interestingly, the number of non-VSVZ-contacting glioblastomas correlated inversely with GBM-$VSVZ_{Dist}$ (R=-0.85, p=3.8x10^{-9}; **Figure 2A**). This correlation was partly confounded by the inverse correlation of tumor volume with GBM-$VSVZ_{Dist}$ (R=-0.44, p=2.2x10^{-16}; **Figure 2B**); therefore, it was adjusted for the median tumor volume at each GBM-$VSVZ_{Dist}$ value and remained significant (partial Pearson's correlation R=-0.79, p=7.11x10^{-7}). KPS and extent of resection significantly correlated with GBM-$VSVZ_{Dist}$ in the institutional VUMC dataset (R=0.19, p=0.002; R=0.28, p=5.7x10^{-6}, respectively), but not in the TCIA dataset (in which extent of resection data are not available; **Figure 2C**).

Next, we conducted survival analyses by fitting a Cox regression model, adjusting for the available co-variables (**Table 1**). The adjusted fit demonstrated a significantly decreased survival, or increased hazard, when glioblastoma contacted the VSVZ relative to the hazard at GBM-$VSVZ_{Dist}$ of 5mm (**Figures 2D, E**). An increased hazard was also noted within the immediate proximity (≤1–3 mm) of VSVZ. However, it was concluded to be an artifact of the fit manifested by the greatly increased hazard associated with VSVZ contact, because in an adjusted Cox regression analysis of only the non-VSVZ-contacting glioblastomas, the hazard ratio remained non-significantly altered along the range of GBM-$VSVZ_{Dist}$ values (**Figures 2F, G**).

TABLE 1 | Patient and tumor characteristics of the two cohorts analyzed.

	VUMC (n=252)	TCIA (n=250)	P-value
VSVZ-contacting (%)	120 (47.6)	124 (49.6)	0.723
Age [years; median (IQR)]	61.63 [51.93, 69.44]	59.50 [52.00, 69.00]	0.324
Tumor volume [median (IQR)]	30.20 [14.84, 51.77]	33.09 [17.77, 60.59]	0.082
KPS (%)			<0.001
100-80	123 (48.8)	157 (62.8)	
70-50	111 (44.0)	43 (17.2)	
40-10	18 (7.1)	10 (4.0)	
Unknown	0 (0.0)	40 (16.0)	
IDH1/2 mutation status (%)			0.001
WT	222 (88.1)	189 (75.6)	
Mutant	8 (3.2)	12 (4.8)	
Unknown	22 (8.7)	49 (19.6)	
MGMT promoter methylation status (%)			<0.001
Unmethylated	166 (65.9)	82 (32.8)	
Methylated	55 (21.8)	82 (32.8)	
Unknown	31 (12.3)	86 (34.4)	
Temozolomide (%)	202 (80.2)	165 (66.0)	0.001
Radiotherapy (%)	223 (88.5)	188 (75.2)	<0.001
Extent of Resection (%)			
Biopsy	17 (6.7)	N/A	
STR	126 (50.0)	N/A	
NTR	64 (25.4)	N/A	
GTR	45 (17.9)	N/A	

DISCUSSION

Recent studies in mouse models and human intraoperative glioblastoma samples suggest that genetically altered neural stem cells can migrate out of the VSVZ and ultimately generate glioblastomas (8). Select glioblastoma cells can, in turn, be attracted to the VSVZ (12, 13). Complimenting these discoveries, our results revealed that about half of glioblastomas radiographically contact the VSVZ at diagnosis. The remaining tumors were found near the VSVZ with a frequency that decreased linearly with GBM-$VSVZ_{Dist}$.

The decreasing number of patients with greater GBM-$VSVZ_{Dist}$ added a limitation for the analysis, as it resulted in increasing confidence intervals around the hazard ratio when examining individual datasets (**Figure 2E**). Hence, we pooled the datasets to address this limitation, yielding a more constant and precise range of confidence interval around the hazard ratio, which remained steady around 1 (**Figures 2D, F**). Our results are in accordance with MRI probabilistic maps of glioblastoma that highlight areas associated with lower survival (15, 16).

Patient survival was only influenced by the presence or absence of a gadolinium-enhanced glioblastoma contact with the VSVZ (3). Glioblastoma volume, depth, and any potential influence of these variables on the extent of resection may not solely explain the lower survival associated with VSVZ-contacting glioblastomas.

Several studies have sought to understand the molecular basis for the increased malignancy of glioblastomas with VSVZ contact (17–19). Two of them demonstrate a correlation with increased glioblastoma expression of CD133, a glioma stem cell marker, with proximity to the VSVZ (20, 21). However, large bulk tissue analyses have not revealed a consistent molecular signature of VSVZ-contacting glioblastomas (9, 10). Therefore, the role of the microenvironment of the VSVZ is also being probed to understand the increased malignancy of glioblastomas with VSVZ contact. Changes in the disease course that can occur once glioblastoma cells invade the VSVZ, whereupon they are theorized to become therapy-resistant (13), drive recurrence, and disseminate further (22), are hypothesized to explain the lower survival associated with VSVZ-contacting glioblastomas.

This work has some limitations. First, we solely used uniaxial T1-weighted MRIs that did not uniformly include 3D MRI-based sequences, which could lead to biased conclusions. Second, we acknowledge that the enhancing edge of glioblastoma may not represent the true edge of glioblastoma. Therefore, it is critical to rely on a proper combination of MRI studies, and additional histological validation studies are required.

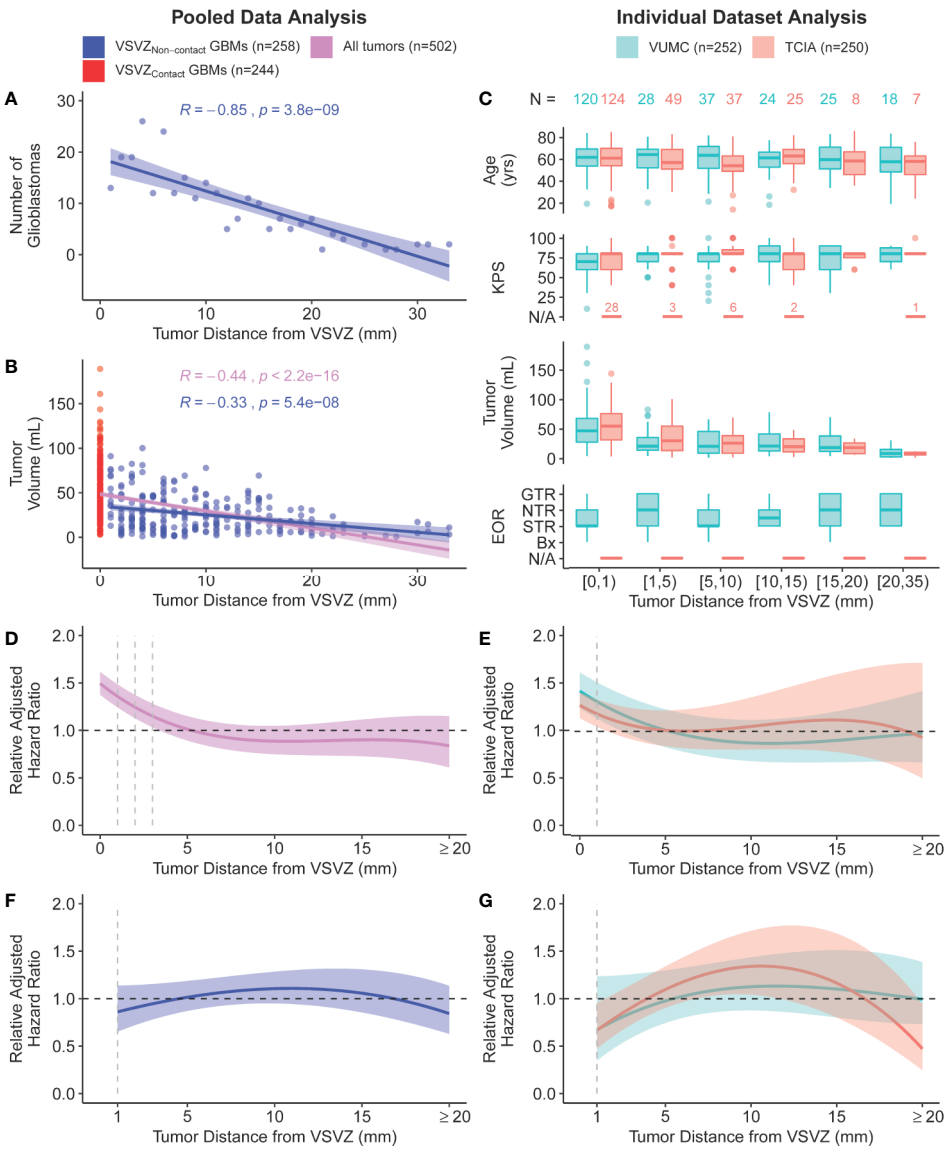

FIGURE 2 | Scatter plot of **(A)** the number of non-VSVZ-contacting glioblastomas ($VSVZ_{Non\text{-}contact}$ GBMs) and **(B)** tumor volume plotted in relation to their distance to the VSVZ (GBM-$VSVZ_{Dist}$). The relationships are quantified with a Pearson's correlation. **(C)** Boxplot distributions of age, Karnofsky performance status score (KPS), glioblastoma volume, and extent of resection of enhancing glioblastoma [EOR; <50% (biopsy, bx); 50%–95% (subtotal, STR); 95%–99% (near total, NTR); 100% (gross total, GTR)] plotted against GBM-$VSVZ_{Dist}$ grouped into select ranges in the single-institution (orange) and The Cancer Imaging Archive (TCIA; teal) datasets. The relationship between GBM-$VSVZ_{Dist}$ and adjusted hazard ratio relative to the hazard at GBM-$VSVZ_{Dist}$ of 5mm in **(D)** pooled data and **(E)** within each dataset is depicted using a quadratic spline fit. The size of the colored ribbon represents the 95% confidence interval. The hazard ratio is adjusted for the following: age at diagnosis (continuous), Karnofsky performance status score (KPS: 100-80, 70-50, 40-0, or not available, as in the case of few TCIA patients denoted in **(C)**, post-operative treatment with temozolomide and/or radiotherapy (yes or no), *IDH1/2* mutation status (wildtype, mutant, or unassessed), *MGMT* promoter methylation status (methylated, unmethylated, or unassessed), tumor volume, and EOR (not available in TCIA hence unadjusted in the TCIA and pooled analyses). **(F, G)** Prior analyses repeated with only non-VSVZ-contacting glioblastomas.

There is a heightened interest in assessing the effectiveness of VSVZ radiation in addition to the standard of care treatment for glioblastoma. For example, one randomized trial is underway (ClinicalTrials.gov Identifier: NCT02177578). Our results may guide analyses of such trials. For example, in existing trials, it may be beneficial to test the differential effectiveness of VSVZ radiation in niche-contacting and non-contacting glioblastomas. Our results may also be used to inform patient selection criteria of future trials in this area; for example, a trial of VSVZ radiation focused only on patients with niche-contacting glioblastoma.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Vanderbilt University Medical Center. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AM conceptualized and designed the study, acquired the data, analyzed the data, drafted the manuscript, and revised the manuscript for intellectual content. NM acquired and analyzed the data, and drafted the manuscript. SS acquired the data. LD acquired and interpreted the data. RI conceptualized the study, interpreted the data, and revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Strange Case of Jekyll and Hyde: Parallels Between Neural Stem Cells and Glioblastoma-Initiating Cells

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During embryonic development, radial glial precursor cells give rise to neural lineages, and a small proportion persist in the adult mammalian brain to contribute to long-term neuroplasticity. Neural stem cells (NSCs) reside in two neurogenic niches of the adult brain, the hippocampus and the subventricular zone (SVZ). NSCs in the SVZ are endowed with the defining stem cell properties of self-renewal and multipotent differentiation, which are maintained by intrinsic cellular programs, and extrinsic cellular and niche-specific interactions. In glioblastoma, the most aggressive primary malignant brain cancer, a subpopulation of cells termed glioblastoma stem cells (GSCs) exhibit similar stem-like properties. While there is an extensive overlap between NSCs and GSCs in function, distinct genetic profiles, transcriptional programs, and external environmental cues influence their divergent behavior. This review highlights the similarities and differences between GSCs and SVZ NSCs in terms of their gene expression, regulatory molecular pathways, niche organization, metabolic programs, and current therapies designed to exploit these differences.

Keywords: glioblastoma stem cells, neural stem cells, neurogenic niche, tumor microenvironment, tumor metabolism

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INTRODUCTION

Glioblastoma [GBM, International Classification of Diseases for Oncology (ICD-O) code 9440/3] is the most common and aggressive primary CNS malignancy in adults. The short median survival of 9–18 months in patients with GBM has been attributed to the highly invasive nature of the disease with rapid cell infiltration, frequent relapses, and therapy resistance (1–5). Anatomically, GBMs arise predominantly in the cerebral cortex (40%), followed by temporal lobe (29%), the parietal lobe (14%), deeper brain structures (14%), and the occipital lobe (3%) (6). The current therapy for GBM, consisting of maximal surgical resection followed by radiation and temozolomide (TMZ), a cytotoxic chemotherapy (7), has yielded minimal survival benefit with a vast majority of GBM patients presenting with tumor recurrence. Recently, the addition of tumor-treating fields (TTFs) to the standard chemoradiotherapy regimen has extended survival of patients from 16 to 20.9 months (8).

Limited therapeutic options, poor survival, and the universally fatal nature of the disease have fueled research efforts to uncover novel molecular vulnerabilities within GBM. However, despite the best efforts, the discovery of novel effective treatments remains elusive. GBM tumors exhibit a large degree of intra- and inter-tumoral heterogeneity, which frequently renders majority of targeted therapies ineffective (9). In an attempt to deconvolute this heterogeneity, an increasing body of scientific work has been established to identify the cell-of-origin in GBM to shed light on the hierarchical organization of GBM tumors and identify vulnerabilities to target the tumor at its roots. Historically, two major candidate cells of origin of GBMs have been proposed, neural stem cells (NSCs) and oligodendrocyte precursor cells (OPCs). The supporting evidence and shortcomings of the two hypotheses have been recently reviewed in detail by Fan et al. (10). The initial identification of a subpopulation of GBM cells with multilineage potency, increased self-renewal ability, proliferation, and migration, termed glioma stem cells (GSCs) (11–13) has provided correlative evidence for the possibility of GBMs arising from transformed neural stem cells (NSCs). Through analysis of patient samples and genetically engineered mouse models of GBM, several studies have subsequently provided molecular evidence suggesting that GBM arises from migration of mutated, astrocyte-like NSCs from the subventricular zone (SVZ) (11–15). In this review, we describe the intrinsic and extrinsic regulations of SVZ NSCs and GSCs including molecular pathways, microenvironment, and metabolic activity to further evaluate how the differences can be exploited in the next generation of targeted therapies for GBM.

SUBVENTRICULAR ZONE NEURAL STEM CELLS IN ADULT NEUROGENESIS

Neural stem and progenitor cells are a specialized population of multipotent cells that contribute to lifelong neural plasticity. During embryonic neurogenesis, NSCs are spatiotemporally regulated to generate multiple neural populations including neurons and glial cells (16). Beyond development, a small pool of NSCs are maintained and become spatially restricted to two neurogenic niches in the brain; the dentate gyrus of the hippocampus known as the subgranular zone (SGZ), or the ventricular-subventricular zone (SVZ) (17). NSCs were long believed to be a retained pool of self-renewing stem cells as suggested by long-term expansion and retention of differentiation potential by neurosphere culturing (17). Much of our current understanding of the human SVZ has been derived from studies in other mammals, namely mice. While mice display robust SVZ neurogenesis, humans have shown an increased preponderance of SGZ or hippocampal neurogenesis (18). Nonetheless, studies in other mammals provide deep insight into comparable NSC regulation and differentiation, highlighting significant complexity and heterogeneity in the adult brain. The SVZ is the largest germinal center in the adult human brain found on the walls of the lateral ventricles (19). SVZ-NSCs, also known as B1 cells, are displaced and surrounded by bi- or multi-ciliated ependymal cells to form a pinwheel-like structure, in which the NSC apical surface contacts the cerebrospinal fluid (CSF) and some the ventricle, while the basal process terminates vascular vessels and the extravascular basal lamina (17, 20). The morphology of B1 cells is reminiscent of radial glia in the embryonic ventricular zone from which they are hypothesized to originate (20, 21). These NSCs, which express GFAP and CD133 at quiescence, can become activated, express Nestin and EGFR, and become highly proliferative (22). Activation of these NSCs ultimately gives rise to EGFR+ transient amplifying cells, which in turn differentiate into progenitors and finally, neuroblasts. These cells follow a specialized migratory route known as the rostral migratory stream to the olfactory bulb in which they disperse radially and differentiate into GABAergic interneurons, or form corpus callosum oligodendrocytes (23, 24). Purification and subsequent single-cell transcriptomics have revealed that SVZ-NSCs exhibit a phenotypic continuum between quiescence and activation suggesting a high degree of transcriptional dynamics (21, 25, 26). NSCs present a heterogeneous profile of multiple activation states in the adult SVZ niche regulated by various molecular programs affected by both intrinsic and extrinsic programs (21, 23).

Intrinsic Regulation

Adult NSC self-renewal and multipotency have been proposed to be regulated by various transcriptional factors. One such factor is the orphan nuclear receptor TLX, which has been shown to be an essential transcriptional regulator of NSC maintenance and proliferation in the adult brain (27). Transcriptional regulation has also been demonstrated to be controlled by arsenite-resistance protein 2, a critical activator of the Sox family of DNA binding proteins, particularly Sox2 (28). TLX has also been suggested to regulate NSC maintenance by repression of cell-cycle inhibitory factors and recruitment of a host of tumor suppressor genes including Bmi1 (29), p53 (30), and the PTEN pathway (31) which regulate stem cell maintenance (27). Adult SVZ NSCs have also been shown to be regulated by basic helix-loop-helix (bHLH) transcription factors, which inhibit differentiation and maintain stemness. BHLH genes, particularly of the Hes family have also been implicated as Notch signaling effectors, which inhibit neuronal differentiation, and maintain NSCs by inducing quiescence (17, 32, 33). Beyond transcriptional regulators, other nuclear receptors such as estrogen receptors (34), thyroid hormone receptors (35), and peroxisome proliferator activated receptor-gamma (36), have been shown to regulate NSC proliferation and differentiation (17, 32).

Cell-intrinsic regulation is also maintained through epigenetic modification and chromatin remodeling. Epigenetic control has been demonstrated to be regulated by the aforementioned polycomb repressor Bmi1 by methylation of the histone tail H3K27 to promote self-renewal (37). SVZ-NSC differentiation is alternatively regulated by methylation of the histone tail H3K4 by the TrxG family of proteins (38). The balance between self-renewal and differentiation is subsequently

mediated by switches from a polycomb-repressor driven chromatin remodeling to that of the TrxG family (39, 40). Epigenetic regulation has also been shown to work through histone acetyltransferase (HATs) and deacetylases (HDACs) in NSCs (41). HDACs promote the silencing of key neurogenic transcription and cell-cycle factors in a comparatively more dynamic fashion relative to the polycomb family of epigenetic regulators to tightly regulate fate specification, differentiation, and cell-cycle exit (32, 37).

Epigenetic mechanisms in SVZ-NSCs are also regulated by a network of miRNAs and non-coding RNAs, which play an additional regulatory role in adult neurogenesis. Many members of the small RNA family have been implicated in modulating neuronal differentiation by binding to the RE1-silencing transcription factor (REST), a crucial regulator of neuronal gene expression (42). Together, small non-coding RNAs fine tune epigenetic programs to regulate cell states of SVZ-NSCs.

Extrinsic Regulation

NSC intrinsic programs are also regulated by signals from the neurogenic niche (37, 43). The NSC niche is an extensive microenvironment that hosts cell-cell and cell-microenvironment interactions (44). Here, NSC proliferation and fate determination is facilitated by various cell-extrinsic molecular signals.

The extracellular matrix is a critical component of the SVZ niche that has been identified as a regulator of NSC proliferation (**Figure 1A**). It is composed of vessel basal lamina rich in laminin, collagen-I, and other molecules including metalloproteinases, brevican, tenascin-C, growth factors, and proteoglycans (44, 45). Unlike embryonic development, the adult SVZ also consists of a unique extravascular component consisting of ECM aggregates near the ventricular surface known as fractones (46). Fractones play an important role in facilitating the binding of growth-factors, cytokines, and chemokines from the circulating CSF to fractone-associated heparan sulfate proteoglycans (HSPGs) (47). Fractones then present these molecules to their cognate receptors on NSCs. Some of these molecules include fibroblast growth factor-2 (FGF-2) (48) and bone morphogenetic proteins (BMP) to influence NSC proliferation (49).

Molecular signals in the ECM regulating adult NSC activity can originate from multiple cell types. In assessing the cellular composition of the SVZ microenvironment, endothelial, pericyte and vascular cells, as well as immature and mature lineages of NSCs are found (50, 51). Most significant are the ependymal cells which uniquely line the ventricular surface in a pinwheel formation around single NSCs (20, 52). Ependymal cells secrete local signaling factors into the circulating CSF, which include noggin, a BMP signaling inhibitor, to activate adult human NSCs and promote fate commitment (53). Endothelial cells in the SVZ also secrete factors including vascular endothelial growth factor (VEGF) and neurotrophin-3 which promote self-renewal and quiescence, respectively (54). NSCs and their immediate progeny also self-regulate through autocrine and paracrine mechanisms (55, 56). This regulation is particularly controlled by diffusible factors transmitted through gap junctions such as the neurotransmitter GABA which modulates quiescence (57), and cytokines such as IL-1β and IL-6 which promote NSC differentiation (58). Cell-cell interactions of adult NSCs are also observed in the SVZ microenvironment through signaling molecules such as ephrin B2 and Jagged1 on endothelial cells which promote quiescence and cell cycle suppression (59). Direct cell-cell interactions have also been demonstrated, such as that with endothelial cells through α6β1 integrins which modulate NSC proliferation (60). Thus, multiple cell types contribute to the signaling *milieu* of the SVZ niche.

Extrinsic factors that affect adult NSC regulation can also be derived from the cerebrospinal fluid (CSF) and blood-derived systemic signals. NSCs have direct access to the CSF which provides a rich supply of various additional mitogens such as PDGF, and morphogens including the Wnt ligands which particularly promote proliferation and self-renewal of adult NSCs *via* canonical Wnt signaling (61). Peripheral circulating morphogens have also been implicated in modulating mouse NSC behavior such as GDF11 which induces vascular remodeling leading to NSC proliferation (62, 63).

NSC multipotency and stemness is also modified by endogenous and niche-derived metabolic factors. Adult NSCs are known to rely on aerobic glycolysis prior to differentiation. Changes in metabolic activity affects adult NSC differentiation and cell-fate commitment, particularly by activation of mitochondrial respiration and reactive oxygen species production (64). Oxygen tension or hypoxia in the microenvironment also stimulates proliferation within the SVZ and migration into the hypoxic region (65). Metabolomic analyses of NSCs has also revealed that lipid metabolism can induce changes in NSC state. Adult NSCs have been shown to require lipogenesis for proliferation to ensure quiescence (66, 67). Extrinsic insulin/insulin-like growth factor signaling has also been shown to stimulate NSC reactivation and proliferation through regulation of CDK4 activity (68, 69).

While other niche-mediated cues such as regional identity (70) and positional information (71) modulate adult NSC activity, it is the combination of molecular stimuli, cytoarchitecture, and structural components of the SVZ niche that continually regulate NSC state and function.

CANCER STEM CELL HYPOTHESIS AND GLIOBLASTOMA

The cancer stem cell (CSC) hypothesis has been used as a framework describe and provide explanation for the high degree of molecular heterogeneity, cellular plasticity, and the molecular divergence of recurrent GBM. CSCs were observed to share many of the similar properties to the healthy stem cells including multipotent differentiation and self-renewal (72), low frequency and low proliferative rate (73–76), ability to regulate the surrounding microenvironment (77), strict re-regulation of proliferation and cell death, and reliance of similar molecular pathways (78). The initial evidence of cancer stem cell-driven tumorigenesis came through studies involving serial re-transplantation of a specific subpopulation of leukemic cells in

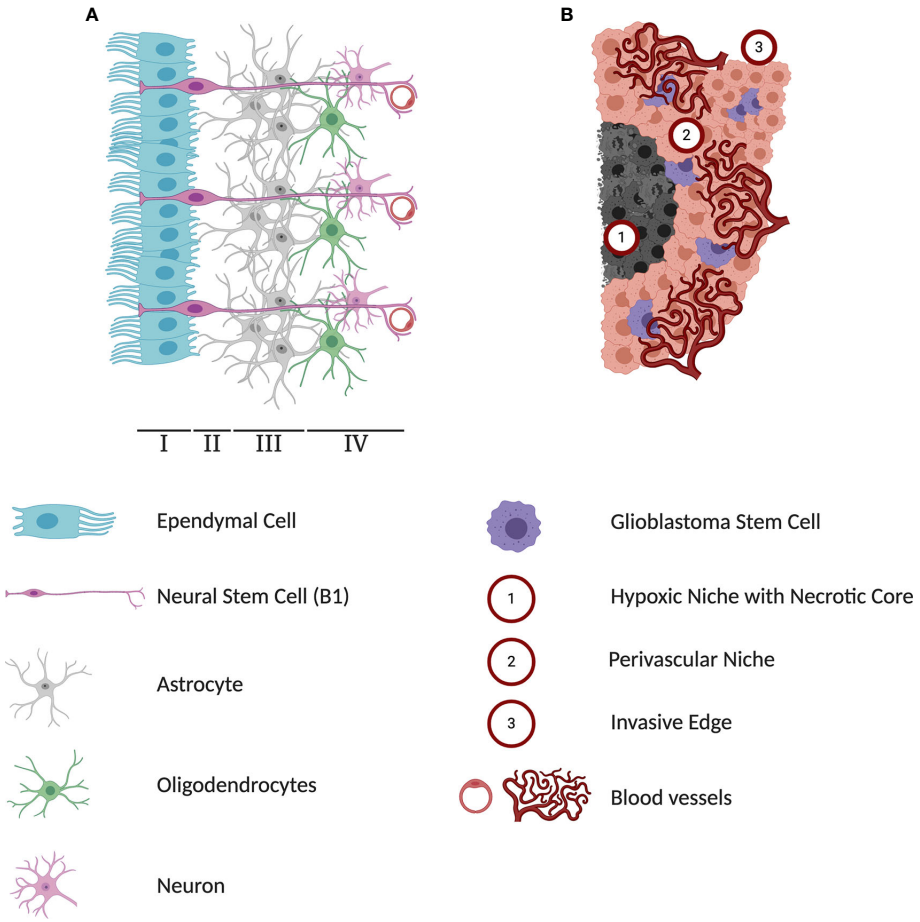

FIGURE 1 | Differences in cytoarchitectures surrounding subventricular zone (SVZ) neural stem cells (NSCs) and glioblastoma stem cells (GSCs). **(A)** A schematic representation of adult human SVZ. Human SVZ is composed of four distinct layers. The superficial ependymal monolayer, Lamina I, is in contact with ventricular lumen. The second layer, Lamina II is a vastly acellular layer formed by the neuroblast depletion, containing ependymal expansions and numerous astrocyte processes. Lamina III, is a region known as astrocytic ribbon, containing densely packed astrocytes. Lastly, Lamina IV is a transitional zone rich in oligodendrocytes and myelinated neurons. The NSC niche is an extensive microenvironment that hosts cell-cell and cell-microenvironment interactions that contribute extensively to the extrinsic regulation of NSC proliferation and self-renewal. **(B)** A schematic representation of three major microenvironments within glioblastoma (GBM) tumors. The hypoxia region formed in the course of tumor growth lacks any blood and oxygen supply and has been implicated in playing a protective role against chemoradiotherapy. Perivascular niches exist along capillaries or arterioles where endothelial cells come into direct contact with glioblastoma cells. In addition to producing high levels of pro-angiogenic factors driving tumor vascularization, cells in the perivascular contribute to activation of pathways regulating self-renewal and proliferation of GSCs. As a highly invasive tumor, GBMs can infiltrate into healthy brain tissue and limit the effectiveness of surgical interventions.

immunodeficient mice (74, 79). Since the early 2000s, CSCs have successfully been identified in numerous solid tumors including breast cancer (80), colorectal cancer (81, 82), and brain cancers including GBM (12) in which they are specifically termed GBM stem cells (GSCs). GSCs have demonstrated chemo- (83, 84) and radiotherapy (85) resistant, while contributing to invasion (86), angiogenesis (87) and tumor recurrence (87). Comparison of underlying molecular mechanisms within GSCs to those in NSCs will allow for development of selective therapies to target the rare cell population responsible for tumor initiation, propagation, and evasion of current therapies.

While the precise identification of the GBM cell of origin remains elusive, two major hypotheses have been explored over the years. In one theory, GBM arises from a transformation events in differentiated astrocytes, while others have suggested that a GBM pathogenesis begins with a transformed NSC [Comprehensive review by Fan et al. (10)]. Previously, astrocyte progenitor cells were believed to be the sole proliferating cells in the adult brain (88) and were hypothesized to drive GBM tumorigenesis due to extensive expression of the marker GFAP in both healthy astrocytes and glioma samples (89). This would require a fully committed astrocytes to acquire mutations, de-differentiate and become tumorigenic. Other attempts to identify the cell-of-origin in GBM using lineage tracing experiments in mouse models have suggested oligodendrocyte precursor cells (OPCs) (90, 91). The similarity in expression levels of PDGFRα and NG2 in OPCs and GBM provided further support to the notion of OPC-derived GSC (92–94), and in a study by Hide et al., the authors have proposed a

model where a transformation of both OPCs and NSCs is required for generation of GSCs (90).

Early mouse models exploring effects of genetic alterations in either NSCs or differentiated astrocytes have failed to provide definitive resolution to the cell-of-origin question. Some reports suggested that overexpression of Ras and Akt signaling in neural progenitor cells but not in more differentiated astrocytes was sufficient to induce formation of GBM-like lesions (95). On the other hand, other groups have provided evidence that genetic alterations in either population is sufficient to induce GBM formation (96). Mounting evidence of the hierarchical organization of GBM tumors and the upregulation of developmental pathways in GSCs became the principal evidence for the notion of transformation of NSCs from SVZ as the initial stage of gliomagenesis. In addition to evident functional overlap, similarities in expression patterns of a number of genes including CD133 (97, 98), Sox10 (99), Nestin (100, 101), Musashi (101, 102), GFAP (103), and Olig1/2 (104, 105) highlight shared molecular programs between NSCs and GSCs. Through deep sequencing of isocitrate dehydrogenase wild-type GBM patient samples and normal SVZ tissue, researchers observed similar expression of driver mutations in both the SVZ and patient matched-tumor tissue (15). Intriguingly, multiple studies have reported shorter survival of GBM patients in cases where tumors were in contact with SVZ (106–109). The comprehensive profiling and understanding of GBM cell of origin may pave the way for identification of prognostic markers along with targeted preventative and curative therapies.

INTRINSIC DEREGULATIONS OF GLIOMA STEM CELLS COMPARED TO NEURAL STEM CELLS

Like all cancers, GBM exhibits behavioral hallmarks that distinguish it from healthy tissue (110). Compared to NSCs, GSCs are self-sufficient in providing growth signals, resistant to growth inhibition, evade programmed cell death, have limitless replicative potential, sustain angiogenesis, and invade surrounding tissue (**Figure 2**). Markers of interest to explain these phenotypes have been extensively studied and while some have been exploited in clinical settings, no single one is responsible for GBM's relentless growth.

Aberrant Growth Signals

Epidermal growth factor (EGF), is a critical regulator in the proliferation of normal NSCs in mice (111). Aberrant EGF signaling prevents mouse NSC differentiation while increasing proliferative capacity and invasiveness, properties that closely resemble those of high-grade gliomas (112, 113). As epidermal growth factor receptor (*EGFR*) amplification is one of the most frequent mutations in GBM patients (114), and has been implicated in human gliomagenesis (115), its targeting in the clinical setting has been extensively investigate (116). Even in the absence of EGF, aberrant behavior of the EGFR pathway maintains stemness properties and promotes self-sufficient growth in tumors human (117).

Along with EGF, fibroblast growth factors (FGF) play an important role in the regulation of stemness in GSCs *in vitro* (118, 119). The FGF superfamily consists of 22 genes with various isoforms (120). Of particular interest is FGF-2, which does not follow conventional secretion (121) and was found to increase proliferation of NSCs in rat SVZs (122). The low molecular weight isoform of FGF-2 can be excreted and internalized for autocrine or paracrine signaling *via* fibroblast growth factor receptors (FGFRs), or be translocated directly to the cytoplasm and nucleus (123). The transcription factor ZEB1, which has previously been implicated in regulation of glioma stemness (124), has been found to regulate FGFR1 expression (125), suggesting that FGFRs could also be associated with GSCs. Indeed, FGFR1 was found to be preferentially expressed on GSCs, and regulated stem cell transcription factors SOX2, OLIG2, and ZEB1 to promote GBM growth *in vivo* (126). While FGF is a large and cumbersome family to investigate,

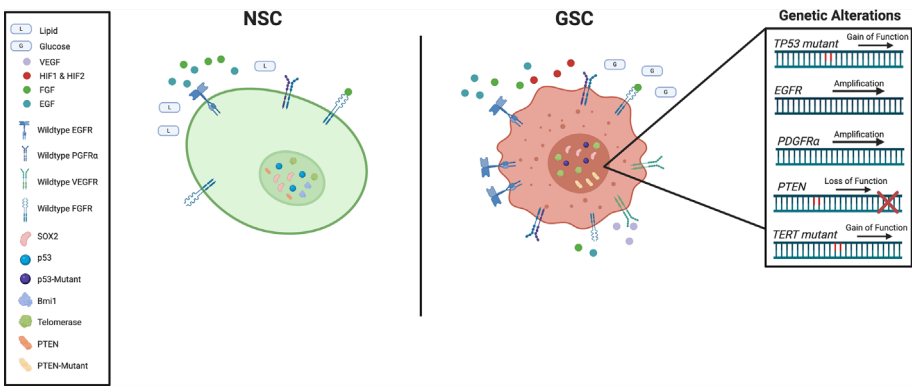

FIGURE 2 | Schematic representation of key genetic and signaling difference between subventricular zone (SVZ) neural stem cells (NSCs) and glioblastoma stem cells (GSCs).

further inquiries into their functions and potential redundancies may offer insights into preferential novel therapeutic vulnerabilities of GSCs compared to NSCs.

Platelet-derived growth factor (PDGF) is another important regulator of NSCs in the SVZ. When neural stem cells were identified to express PDGF-receptor-alpha (PDGFRA) in the adult mouse SVZ, supplemental PDGF alone was sufficient to induce hyperplasia with some features of GBM (127). These receptors show functional significance by promoting evasion of radiotherapy (128). Amplification of *PDGFRA* is one of the most common mutations in GBM and is most commonly associated with the proneural subtype (129). As PDGFRs are important in the healthy development of the normal central nervous system (130), its increased activity has led to important relationships between subclasses of GBM (131). While these receptors provide another intriguing avenue to potentially eradicate GBM, selective inhibition to avoid targeting of surrounding healthy tissue requires further research and clinical investigation.

Insensitive to Growth Inhibition

As one of the most studied tumor suppressors in cancer, TP53 has been shown to regulate a wide range of cellular functions of various cancers including GBM. As a key stem cell maintenance regulator, TP53 is expressed in proliferating cells within the SVZ and has been implicated in controlling cell division and differentiation (132). The key regulatory role of TP53 is not limited to development as it has been shown to regulate proliferation and self-renewal of NSCs in adult mice (133, 134). While deletion mutations in TP53 are predominant in several cancers, in the context of GBM, mutations in TP53 are often gain-of-function, resulting in a wider range of downstream effect (135). Mutations in TP53 may cause healthy NSCs to prematurely migrate out of the SVZ effectively seeding the brain with pre-cancerous stem cells (136, 137).

Another common loss of tumor suppressor in GBM is phosphatase and tensin homolog (PTEN). The role of PTEN in the regulation of mouse NSCs in SVZ is extensively reviewed in Li et al. (138). Together with TP53, PTEN also regulates the self-renewal and differentiation of both NSCs and GSCs (139). Loss of PTEN was observed to represses GSC differentiation (140), and similarly promote NSC conversion to a GSC-like phenotype (141). Unsurprisingly, mutational status of PTEN positively correlates with a worse overall prognosis for GBM patients (142).

Evasion of Programmed Cell Death

The PI3K/AKT/mTOR intracellular pathway is a critical pathway for cell cycle regulation, proliferation, and it is directly antagonized by PTEN (143). The pathway exerts direct influences on cell quiescence, proliferation, longevity by acting predominately through phosphorylation and subsequent activation of AKT/mTOR driving downstream effects (144). Although the PI3K/AKT/mTOR pathway is found throughout the body, its role in promoting growth and proliferation and preventing differentiation in adult NSCs (145) make it an important area of research in GBM and GSCs (146). In addition to being a key pathway in preventing GSC differentiation (147), PI3K/AKT/mTOR further contributes to GBM growth by blocking apoptosis signaling (146).

Signal transducer and activator of transcription-3 (STAT3) is a transcription factor whose activity is directly regulated by PI3K/AKT/mTOR (148). A variety of cytokines, growth factors and interferons converge to regulate STAT3, but its influence on an array of pathways within normal and cancerous stem cells is well documented as it is highly conserved (149). Most importantly, STAT3 plays a major role in maintaining stemness and promoting tumor survival and invasion while suppressing anti-tumor immunity (150). Several studies have shown that reducing levels of STAT3 can lead to a reduction of CD133 and other stemness markers while increasing the propensity for apoptosis and differentiation (151, 152). Inhibition of STAT3 in recurrent GBM has also been shown to reduce levels of BCL-XL and survivin, leading to caspase-3 activation and apoptosis in GSCs (153).

Limitless Replicative Potential

Healthy replication of cells requires proper activity of telomerase enzymes to ensure the end of chromosomes do not shorten or fuse with other chromosomes (154). Telomerase activity becomes restricted to the SVZ as mammals age (155) but proper maintenance allows NSCs to remain present into adulthood (156). Likewise, increased activity of telomerase leads to replicative immortality within GSCs and is one of the most frequent mutations in GBM (157, 158). The most common gain-of-function mutation of telomerase is located in the promoter region of *TERT* (159) and is predictive of shorter survival times (160).

Sustained Angiogenesis

Angiogenesis is a tightly controlled pathways in normal tissue and is initiated in response to injury (161). However, several of the receptors discussed before that are upregulated in GSCs are also involved in angiogenic pathways. EGFR, PDGFR, FGFR, and VEGFR have all been shown be involved in angiogenesis with GBM cells and GSCs themselves being major producers of the signaling molecules and their respective receptors (162–167).

Similar, to the intrinsic regulation seen in NSCs, GSCs themselves can directly promote their own survival by modulating the microenvironment. In a study by Takahashi et al., mice engrafted with OCT3/4 overexpressing GBM cells were observed to have larger tumors and increased number of blood vessels (168). Furthermore, tumor-conditioned media accelerated capillary formation *in vitro* and elevated mRNA levels of VEGF in OCT3/4-overexpressing cells providing additional evidence of tumor cell contributing to angiogenesis (168). GSCs have also been shown to directly secrete VEGF-A in extracellular vesicles (169). Along with their influence on surrounding cells, when GSCs asymmetrically divide to self-renew, the differentiated daughter cell is capable of forming blood vessel structures (170).

Increased Invasiveness

Although GBM cells rarely metastasize to other organs, they do demonstrate a highly invasive growth pattern. Once a GBM is established, the infiltrative edge presents a challenge for surgical resection as the edge is enriched with chemoradioresistant GSCs,

meaning the remaining cells are poised to drive tumor relapse upon removal of therapeutic pressures (171, 172). The invasive nature of GBM cells is further exuberated by surrounding non-malignant cells, such as astrocytes secreting cytokines and chemokines (173). Additionally, GBM tumors can degrade the extracellular matrix *via* metalloproteinases (174) and cathepsins (175) to modulate their cell structure for efficient cell movement (176).

Altered Cellular Metabolism

Compared to the rest of the body, the brain naturally has a higher dependence on glucose as a source of energy, consuming 60% of our daily intake (177). In normal cells, catabolism *via* glycolysis/oxidative phosphorylation and anabolism *via* gluconeogenesis pathways achieve a glucose homeostasis. However, a phenomenon known as the Warburg effect describing the preferential usage of anaerobic glycolysis in CSCs, even in the presence of sufficient oxygen, heightens the dependency of differentiated GBM cells on glucose (178). However, in a side by side comparison, GSCs consumed less glucose and produced less lactate while maintaining higher ATP levels than their differentiated counterparts (179). GSCs are therefore thought to rely mainly on oxidative phosphorylation, however, if challenged, are capable of using other metabolic pathways (179).

Metabolic flexibility and plasticity of cellular states influence each other. In GBM cells, functional p53 leads to increased glutaminase 2 (GLS2) under stress which increases oxidative metabolism and ATP generation, by catalyzing the conversion of glutamine to glutamate and increasing α-ketoglutarate (α-KG) levels (180). This metabolic shift, known as glutaminolysis, is also observed in freshly resected GSCs (181). Glutaminolysis produces precursors for macromolecules (nucleic acids, amino acids, fatty acids), regulates redox homeostasis (*via* NADH, NADPH, and ROS levels) and contributes to immunosuppression by glutamate production to ensure pro-tumor survival (182). As an abundant non-essential amino acid, glutamine is transported through the blood and capable of crossing the blood-brain barrier, making it a particularly useful energy source by tumors (183).

Glutamate can be converted to α-KG by either glutamate dehydrogenase 1 (GDH1) or transaminases such as glutamate pyruvate transaminase 2 (GPT2) and glutamate oxaloacetate transaminase 2 (GOT2) (184). Conversions fluctuate according to nitrogen and carbon availability. In addition to a metabolite, α-KG behaves as a cofactor (along with oxygen) in the activity of α-ketoglutarate-dependent hydroxylases. These are non-heme, iron-containing enzymes that catalyze a wide range of oxygenation reactions including biosynthesis (ex. collagen and L-carnitine), post-translational modifications (ex. protein hydroxylation), epigenetic regulations (ex. histone and DNA demethylation), as well as sensors of energy metabolism. So far majority of these processes in GBM have been restricted to isocitrate dehydrogenase mutants of GBM, however, collagen prolyl hydroxylases were found to induce metastasis of breast cancer (185) by mechanistically stabilizing HIF-1α in chemoresistance (186). While the importance of HIF-1α in the conversion of GSCs in different tumor niches has been mentioned above, only recently was it reported that collagen-prolyl hydroxylases promote proliferation and invasion in GBM. Interestingly, mouse embryonic stem cells were found to maintain high αKG/succinate ratios *via* glucose and glutamine catabolism that promoted histone/DNA demethylation and maintenance of pluripotency (187). By altering intracellular αKG/succinate ratios, multiple chromatin modifications such as H3K27me3 and ten-eleven translocation-dependent (26) DNA demethylation were shown to regulate genes associated with pluripotency (187). In addition to glucose metabolism, GSCs were observed to have higher expression of genes involved in iron trafficking and metabolism when compared to healthy astroglial and neural progenitor cells, presenting an opportunity for targeted therapeutic intervention (188).

THE EXTRINSIC GLIOMA STEM CELL MICROENVIRONMENT

Unlike the NSC microenvironment in the SVZ, the GBM microenvironment is defined by three unique regions, the hypoxic-necrotic core, the perivascular niche, and the invasive edge, each with distinct contribution to the tumor progression (**Figure 1B**) (189). Each niche influences and activates different cellular programs in GSCs to express distinct markers and transcriptional profiles. This plasticity allows cells to change states and adapt to stressors as needed. The interconnected relationship between GBM and their environment maintains stemness and contributes to heterogeneity which is why emphasis to target the tumor microenvironment has gained traction is recent years, and why more advanced *in vitro* experimental methods such as 3D-culture methods and cerebral organoids are becoming more prevalent (190, 191).

Hypoxia and necrosis are defining features of GBM, caused by the tumor's exceeding growth requirements on available blood flow to supply oxygen and nutrients. This subsequent lack of oxygen protects cells from irradiation, the most effective treatment modality against GBM, by limiting the amount of molecules capable of turning into cytotoxic free radicals (192). Restricted blood flow also limits the delivery of chemotherapies such as temozolomide to the tumor cells (193). In both cases, the hypoxic environment forces tumor cells into a quiescent state, where the lack of cell division prevents cytotoxic DNA damage induced by chemo-radiotherapies (194). Effects of hypoxia on stemness and tumor survival are largely mediated through hypoxia-inducible factors (HIF-1 and HIF-2), which upregulate signaling pathways including Klf4, Sox2, Oct4, CD133, and VEGF (195, 196). Cell death in the center of the hypoxic region leads to formation of the necrotic core and contributes to the release of pro-inflammatory signals, IL-1β, IL-6, and IL-8, into the surrounding microenvironment. This signaling in turn, contributes to the conversion of tissue-associated macrophages and neutrophils into immune-suppressive and angiogenesis-promoting cells, allowing for continued GBM progression and expansion (197–200). Similar to NSC metabolism, hypoxia forces a metabolic shift in GBM toward aerobic glycolysis and

fatty acid metabolism rather than oxidative phosphorylation. Together, the hypoxic niche plays key regulatory roles leading to heterogeneity and cancer progression.

Interestingly, hypoxia can influence GBM cells to transdifferentiate into endothelial-like cells (201) which contribute to feedback loops of the second major tumor environment-the perivascular niche (202, 203). This niche most closely resembles the SVZ where NSCs reside (20, 50, 60). Perivascular niches exist along capillaries or arterioles where endothelial cells come into direct contact with GSCs (204). GSCs in the perivascular niche in turn remodel the microenvironment by producing high levels of pro-angiogenic factors, such as VEGF, that drive endothelial cell proliferation, survival, migration, and blood vessel permeability. This is critical for angiogenesis as GBM is one of the most vascularized human tumors and requires a supply of nutrients for tumor progression. The perivascular niche thus regulates stemness and induces pathways enriching for GSCs, namely nitric oxide and NOTCH (205), TGF-β (206, 207), as well as sonic hedgehog signaling pathways (208). Other perivascular cell populations in this niche, such as tumor-associated macrophages (TAMs) secrete chemokines to promote GSC growth and expansion.

The infiltrating (or "invasive") edge is the third and final major GBM niche. As a highly invasive tumor, GBMs can infiltrate into healthy brain tissue and limit the effectiveness of maximal surgical resection. To circumvent and eradicate infiltrative GSCs, patients receive whole-brain radiation therapy. However, GBM cells, and most notably GSCs, have been shown to adapt and resist the applied environmental stress of irradiation (85). Once exposed to radiation, cells undergo a process known as the proneural-mesenchymal transition, similar to the metastatic cascade known as the epithelial-mesenchymal transition (EMT). In this process, cells lose cell polarity, cell-cell adhesions, and alter their cytoskeletal organization for migration. GSCs are observed to invade along white matter tracts of the human brain through a NOTCH1-Sox2 mediated feedback loop (209). Mesenchymal GSCs are regulated by STAT3, N-cadherin, NF-κB, and integrins (210–215). These phenotypes exhibited within the infiltrative niche are also influenced by the hypoxic and perivascular niches.

LEVERAGING DIFFERENCES BETWEEN NEURAL STEM CELLS AND GLIOBLASTOMA STEM CELLS FOR DEVELOPMENT OF NOVEL TARGET THERAPIES

While understanding the similarities between GSCs and NSCs is instrumental for contextualization of gliomagenesis and underlying mechanisms driving GBM progression and therapy resistance, it is leveraging the differences between two cell populations that may offer avenues for generation of novel targeted therapies. Unlike other solid tumors, brain tumors present a unique set of challenges for development of new treatment options. First, the blood-brain-barrier (BBB), which normally protects the brain from harmful toxins, can also hinder the access of targeted therapies against GBM. Although BBB permeability can be theoretically increased through chemical modification of small molecule-based inhibitors, such approach does not expand to more precise modalities including antibody-drug conjugates (ADCs) and adoptive cell transfer therapies. In both mouse models (216, 217) and patient studies, locoregional delivery of the therapeutic offers distinct advantages. In addition to expanding the range of possible treatment modalities, while minimizing systemic toxicities, the locoregional delivery route allows for direct targeting of potential source of GSCs residing in SVZ at the border of lateral ventricle.

One of the most widespread strategies in targeting GSCs is through identification and subsequent development of targeted therapies against cell surface markers. Several different targeting approaches have been investigated in the recent years including antibody-drug conjugates (ADCs) and chimeric antigen receptor T cells (216, 218). And while researchers were able to demonstrate efficacy in mouse models, it is likely that additional combinatorial strategies will be needed to yield complete tumor clearance. Anti-angiogenic therapies have become an attractive modality to prevent tumor progression by cutting off the tumor's supply of nutrients and oxygen (219). Bevacizumab and other anti-angiogenic therapies showed great promise, but repeated failures show the adaptability of tumors to overcome single agent therapies (220). Reducing the invasion of GBMs has been tested to reduce overall tumor progression, but further therapies would be required to fully eradicate the tumor (221). The use of ibrutinib, and FDA-approved drug to treat lymphoma and leukemia, was shown to suppress the BMX-STAT3 axis in GSCs making them vulnerable to radiation therapy (222). This signaling axis was previously shown to maintain self-renewal in GSCs (223) and mitigate apoptosis (224). Additionally, because BMX is not expressed in neural progenitor cells, ibrutinib may be a selective and beneficial therapy for GBM patients (222).

While there is biological overlap between NSCs and GSCs, promising research is exposing differences and vulnerabilities of each, presenting an avenue for novel therapeutic interventions. Research on telomerase activity in a variety of tumors has resulted in development of distinct therapeutic approaches including small-molecule inhibitors, plant-derived compounds, gene therapy, and immunotherapy. Although they remain to be tested in the clinical setting, several of these therapies have demonstrated promising efficacy in mouse models of GBM (225–228). More recently, CRISPR/Cas9 technology has been tested pre-clinically as a modality to repair mutations in cancers to induce cell cycle arrest with few off-target effects in GBM (229). Moving forward, it will be vital to further interrogate the therapeutic window of telomerase activity modulating treatments by comparing their effects on GSCs and SVZ NSCs. The proximity of SVZ NSCs to the lateral ventricles allows for intracerebroventricular (ICV) delivery of potential therapeutic interventions, bypassing the challenges presented by the BBB

while increasing penetrance and distribution. Delivery of cell and gene therapies intracranially and intracerebroventricularly has been tested in both human clinical trials and mouse models of GBM and was shown to reduce tumor progression and invasion (230–232). It is important to note, that due to the extensive intra- and inter-tumoral heterogeneity between GBM tumors, it is unlikely that a single intervention will be sufficient to eradicate the tumor or control its progression, requiring more research into combinatorial approaches in both mouse models and clinical trials. Further research profiling the mechanisms by which SVZ NSCs and the surrounding microenvironment contribute to the chemoradiotherapy evasion of GBM is needed to identify therapies that will synergize with the current SoC. For example, in several *in vitro* and *in vivo* pre-clinical studies, inhibiting CXCL12/CXCR4 signaling in the mouse SVZ promoted radiosensitization and reduced GBM tumor cell proliferation (233, 234). Finally, in the past few years, the difference in the metabolic flexibility between GSCs and NSCs has become more apparent and is now being extensively investigated for the therapeutic potential.

CONCLUDING REMARKS

The aggressive growth characteristic, resistance to therapies and poor clinical outcome have been attributed to the extensive intra- and intertumoral heterogeneity within GBM tumors. Over the years, the observed similarities between GSCs and NSCs of the SVZ, have led to the hypothesis of a transformed NSCs cell presiding at the apex of GBM cytoarchitecture. Although the recent findings have corroborated this hypothesis, it has become evident that that understanding both similarities and differences between GSCs and the healthy NSCs of the SVZ is essential in the search for novel targeted therapies. The comparison of similarities can allow for improved understanding of the molecular mechanisms driving GBM formation, while the comparison of the differences can allow in identifying unique molecular vulnerabilities for development of targeted therapies with a large therapeutic index.

AUTHOR CONTRIBUTIONS

All authors (DB, NS, SSa, CV, SSi) contributed to article writing and editing. Figures were generated with BioRender.com. All authors contributed to the article and approved the submitted version.

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Genetic Architectures and Cell-of-Origin in Glioblastoma

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An aggressive primary brain cancer, glioblastoma (GBM) is the most common cancer of the central nervous system in adults. However, an inability to identify its cell-of-origin has been a fundamental issue hindering further understanding of the nature and pathogenesis of GBM, as well as the development of novel therapeutic targets. Researchers have hypothesized that GBM arises from an accumulation of somatic mutations in neural stem cells (NSCs) and glial precursor cells that confer selective growth advantages, resulting in uncontrolled proliferation. In this review, we outline genomic perspectives on IDH-wildtype and IDH-mutant GBMs pathogenesis and the cell-of-origin harboring GBM driver mutations proposed by various GBM animal models. Additionally, we discuss the distinct neurodevelopmental programs observed in either IDH-wildtype or IDH-mutant GBMs. Further research into the cellular origin and lineage hierarchy of GBM will help with understanding the evolution of GBMs and with developing effective targets for treating GBM cancer cells.

Keywords: glioblastoma, somatic mutation, neural stem cells, subventricular zone, genetically engineered mouse model

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INTRODUCTION

Glioblastoma (GBM) is a common, but aggressive, primary brain cancer of the central nervous system in adults and is associated with poor prognosis due to its invasiveness and resistance to therapy. According to 2016 WHO classification of glioma, GBMs are divided into: 1) IDH-wildtype (about 90% of cases), 2) IDH-mutant (about 10% of cases), and 3) IDH not otherwise specified (1). Molecular genetic features have emerged as fundamental factors contributing to its prognosis, particularly isocitrate dehydrogenase (IDH) mutation, which is considered a favorable factor. Whereas patients with IDH-wildtype GBM show a low median rate of survival of 14 to 16 months, patients with IDH-mutant GBM exhibit prolonged survival (median survival up to 31 months) and slower progression (1, 2). Over the past two decades, extensive and comprehensive genetic analysis of GBM has improved our understanding of GBM pathogenesis, and researchers have hypothesized that GBM arises from an accumulation of somatic mutations (3, 4). However, redundant signaling pathways and intratumoral heterogeneity underlie treatment failure and tumor recurrence (5–7). Thus, identifying the cellular origin of GBM would help with further understanding of tumor initiation/propagation and effective targets of use in treating GBM cancer cells. Regarding the cellular origin of cancer, cell-of-origin refers to normal cells in which oncogenic mutations first

Abbreviations: GBM, Glioblastoma; NSCs, Neural stem cells; IDH, Isocitrate dehydrogenase; GEMM, genetically engineered mouse model; RTK, receptor tyrosine kinase; PI3K, Phosphatidylinositide 3-kinase; *TERTp*, telomerase reverse transcriptase promoter; SVZ, Subventricular zone; ALT, alternating lengthening of telomeres; OPC, Oligodendrocyte precursor cell; SGZ, subgranular zone; GPC, glial precursor cell; APC, astrocyte precursor cell; NPC, neural progenitor cell.

occur and accumulate to initiate tumor formation, while cancer stem cells (CSCs) refers to a subset of proliferating cancer cells that sustain tumor growth (8). The CSCs in GBM have been well-reviewed in many other papers (9–11). In this mini review, we mainly focus on the cell-of-origin in GBM and discuss the recent genomic analyses of GBM and genetically engineered mouse models (GEMMs) investigating tumorigenesis of GBM.

GENETIC ALTERATIONS IN GLIOBLASTOMA

Recent large-scale sequencing analyses have uncovered molecular alterations in somatic single nucleotide variants, copy number variations, gene expression profiles, and epigenetic signatures in GBM (3, 4, 12, 13). In addition, longitudinal genetic characterization of GBM has supported predictions of the order of mutation events and patterns of tumor evolution (14–18). Reviewing these studies, we summarize in the following paragraphs key somatic mutations, known as driver mutations, frequently occurring in IDH-wildtype and IDH-mutant GBM, respectively (**Figure 1**).

IDH-Wildtype Glioblastoma

Although GBM is genetically and transcriptionally heterogeneous, previous studies have demonstrated concordant genetic alterations, including those in *TP53*, *PTEN*, *EGFR*, *PIK3CA*, and *PIK3R1*, *NF1*, and *RB1*, in human GBM samples (3, 4, 12). These mutations represent a set of deregulated signaling pathways, including growth factor (receptor tyrosine kinase [RTK]/phosphatidylinositide 3-kinase [PI3K]/Ras), p53, and retinoblastoma (Rb) signaling pathways. In the growth factor signaling pathway, *EGFR* is frequently activated with variant III deletion of the extracellular domain in GBM. Additionally, activating mutations in PI(3)K complex and inactivating mutations or deletions in tumor suppressor genes, such as *PTEN* and *NF1*, lead to uncontrolled proliferation. In the p53 pathway, inactivating mutations in *TP53*, along with *CDKN2A* (ARF) deletion, have been reported. Finally, deletions in *CDKN2A*/*CDKN3B* and amplifications of CDK4 have been found to result in Rb pathway inactivation, along with mutation or deletion of *RB1* itself. The majority of GBMs harbor genetic alterations in multiple signaling pathways, suggesting that these pathways are required for GBM pathogenesis.

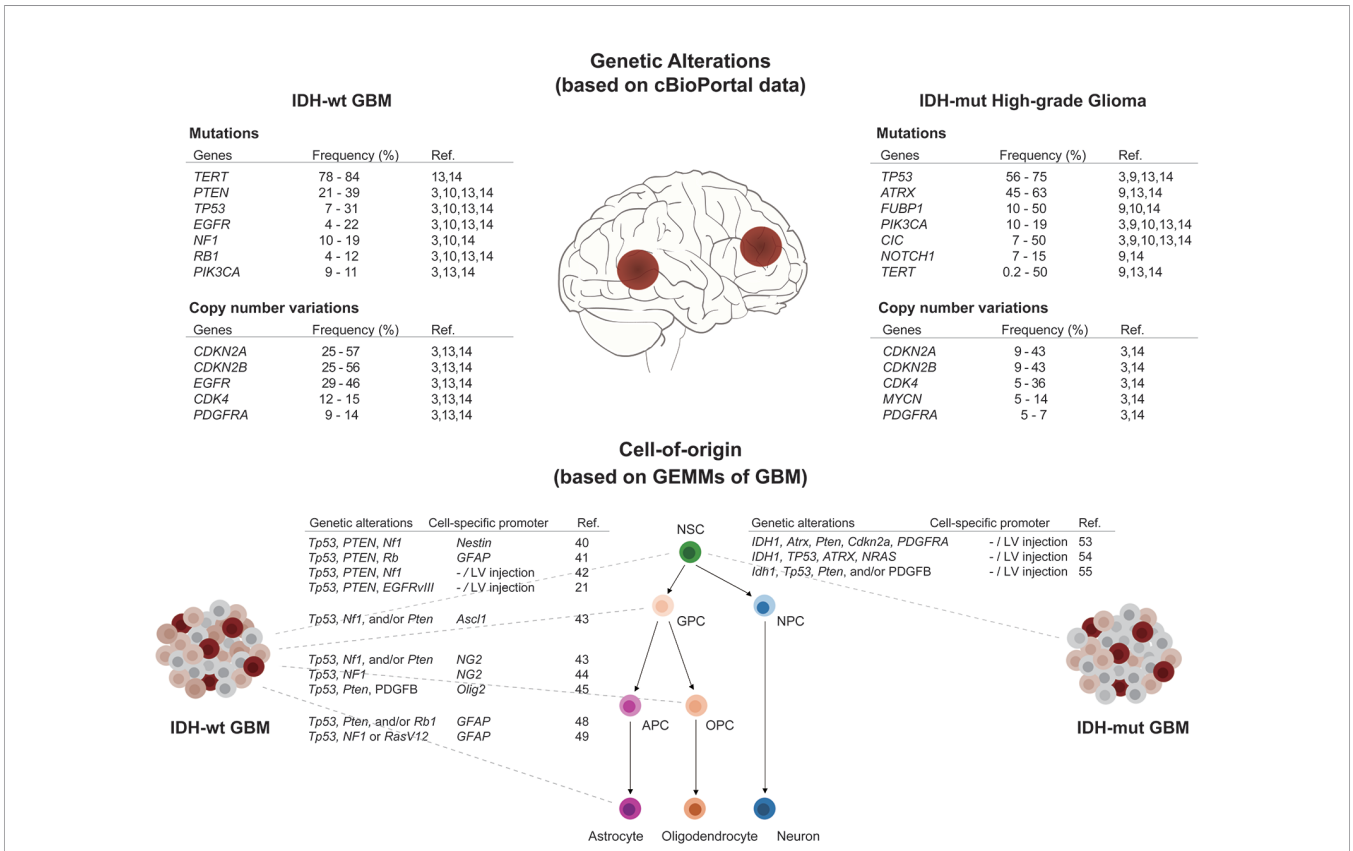

FIGURE 1 | Overview of genetic alterations and cell-of-origin in IDH-wildtype and IDH-mutant GBMs. (Upper panel) Frequently occurring driver mutations and CNVs in IDH-wildtype and IDH-mutant high-grade gliomas (WHO grade 3 & 4) were listed as above. The frequencies were obtained from published data using cBioPortal (19, 20). (Lower panel) Multipotent neural stem cells (NSCs) have capability to self-renewal and differentiate into progenitors with restricted potential including glial precursor cells (GPCs), oligodendrocyte precursor cells (OPCs), astrocyte precursor cells (APCs), and neural progenitor cells (NPCs). Using specific genetic alterations and cell-specific promoters, NSCs and progenitor cells can be transformed to generate either IDH-wildtype or IDH-mutant GBMs in GEMMs.

Interestingly, up to 83% of IDH-wildtype GBMs exhibit telomerase reverse transcriptase promoter (*TERTp*) mutations (3, 21). The *TERTp* mutations, at positions 124 bp (C228T) and 146 bp (C250T) upstream of the *TERT* ATG site, generate *de novo* transcriptional factor binding sites leading to increased expression of *TERT* and subsequent telomere activation (21, 22). A recent study has demonstrated that IDH-wildtype GBM patients carry a high frequency of *TERTp* mutations in the astrocytic ribbon, the neurogenic niche of the postnatal human brain (23). This suggests that mutation of *TERTp* is an early shared event through which NSCs in the SVZ avoid replicative senescence, thereby increasing the possibility that these cells acquire GBM driver mutations (24). On the other hand, Körber and colleagues argued that *TERTp* mutations are subsequent mutations following copy number changes in *EGFR*, *PTEN*, or *CDKN2A* (14). These studies imply that among many GBM driver mutations, *TERTp*, *EGFR*, *PTEN*, or *CDKN2A* mutations seem to play a key role in the early stage of IDH-wildtype GBM formation.

IDH-Mutant Glioblastoma

IDH-mutant GBM accounts for about 12% of all GBMs, with an occurrence rate of *IDH1/2* mutations of approximately 73% to 83% in secondary GBMs (12, 25). *IDH1/2* mutations have been observed in the vast majority of astrocytomas and oligodendrogliomas, and have been described as early molecular events during gliomagenesis (25, 26). Mutated *IDH1* elicits altered catalytic functions in metabolic, epigenetic, and reactive oxygen species managing pathways (27). GBMs with *IDH1* mutations show a higher frequency of loss-of-function mutations in *TP53* (3, 28). Based on a recent longitudinal study on IDH-mutant glioma, mutations in *IDH1* and/or *TP53* occur prior to *ATRX* alteration on the evolutionary trajectories of IDH-mutant gliomagenesis (24, 29). In addition, IDH-mutant GBMs exhibit alternating lengthening of telomeres (ALT) due to concurrent *ATRX* mutations, which are mutually exclusive with *TERTp* mutations (30, 31). Thus, genetic alteration enabling telomere maintenance are likely to be critical steps in GBM tumorigenesis.

Researchers have attempted to classify GBMs with similar molecular genetic characteristics into proneural, classical, and mesenchymal subtypes (32, 33). Each of these subtypes show an enrichment of lineage-specific gene signatures from distinct neural-glial lineages; for example, proneural GBMs show enrichment in oligodendrocyte precursor cell (OPC) genes (34). This implies that gene expression patterns in different subtypes may reflect the phenotype of their specific cell-of-origin.

CELL-OF-ORIGIN IN GLIOBLASTOMA

To identify the cell-of-origin in GBM, understanding of normal cellular hierarchy is required. NSCs are ubiquitously found in all regions of the central nervous system during embryonic development and are capable of initiating cell lineages, leading to the formation of differentiated neurons and glial cells (35). NSCs give rises to intermediate progenitor cells with more restricted potential, which can proliferate and further differentiate into the three major cell types of the central nervous system. A subset of NSCs and lineage-restricted progenitor cells continue to reside in restricted regions of the postnatal and adult brain: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (36, 37).

Considering that multiple oncogenic mutations are necessary for gliomagenesis, the self-renewal and proliferative properties of NSCs ensure appropriate conditions for endogenous accumulation of somatic mutations. Moreover, research has indicated that most driver mutations in cancer are attributable to DNA replicative errors, which are correlated with the total number of divisions of stem cells (38). Based on this notion, it has been hypothesized that the NSCs in the ventricular-subventricular zone is the main source of *de novo* somatic mutations throughout one's lifetime. A recent study indeed showed that 55.5% of tumor-free SVZ tissue contains low-level mutations, such as *TP53*, *EGFR*, *RB1*, *PDGFR*, or *TERT* variations shared by matching tumor tissue in IDH-wildtype GBM patients, but not in IDH-mutant GBM patients (23). However, this study did not show any evidence of which cell type is the cell-of-origin in IDH-mutant GBM. Knowing now that human genetic studies provide the evidence of the cellular origin of IDH-wildtype GBM, we can recapitulate human GBM in mouse models, which are an invaluable tool with which to study the processes of tumorigenesis from originating cells (39–41). Below, we give an overview of GEMMs reflective of specific cell lineages and different combinations of GBM driver mutations, with or without IDH mutation (**Figure 1**).

Animal Modeling of IDH-Wildtype Glioblastoma

To target NSCs in the adult brain, researchers utilized Cre recombinase-expressing adenovirus injected into the SVZ of mutant mice with conditional *Tp53*, *Pten*, and *Nf1* or *Rb* knockout, which resulted in the development of GBM (42, 43). Induction of the same tumor suppressor mutations in mice with *Nestin*-CreER transgenes also led to GBM formation (42). In addition, GBM has been successfully generated from NSCs harboring somatic mutations in *NF1*, *TP53*, and *PTEN* using *in utero* electroporation of CRISPR/Cas9 system (44). Similarly, *TP53* and *PTEN* mutations were introduced into the SVZ of conditional *EGFRvIII* transgenic mouse to generate a GBM (23).

Another model has suggested that GBM arises from committed precursor cells, such as glial precursor cells (GPCs), OPCs, and astrocytes. Researchers have used mice with an *Ascl1*-CreER transgene to target bipotential progenitors expressed in both adult neural and oligodendrocyte lineage progenitors (45). Bipotential progenitors carrying *NF1*, *TP53*, and/or *PTEN* mutations give rise to GBM, as do *NG2*-expressing OPCs (45–47). Several studies have suggested OPCs as the prominent cell-of-origin in GBMs, because of their aberrant growth prior to malignancy (23, 34, 47, 48). In contrast to glial lineage,

susceptibility to malignant transformation declines with neural lineage restriction (49). Researchers utilized cell-specific promoters such as *Dlx1*, *Neurod1*, and *Camk2a* to introduce oncogenic mutations at specific time points during neural lineage specification.

There were several studies showing that mature astrocytes are also capable of tumor formation through de-differentiation. Loss-of-function mutations in *TP53*, *PTEN*, and/or *RB1* in GFAP-CreER mice (50) and injection of shNF1-shp53- or H-RasV12-shp53 lentivirus in the cortex of GFAP-cre resulted in glioma formation (51). The oncogenic virus induced astrocytes to de-differentiate into NS/PC-like state, by expressing the transcriptional factors Sox2, c-myc, and Nanog. The manipulation of pluripotency regulators are capable of inducing de-differentiation or cellular reprogramming (52, 53); however, the above studies have a limitation that GFAP-cre does not discriminate $GFAP^+$ astrocytes from $GFAP^+$ NSCs.

Animal Modeling of IDH-Mutant Glioblastoma

The expression of $IDH1^{R132H}$ mutation in SVZ NSCs led to a proliferating phenotype, but it was insufficient to generate glioma (54, 55). Therefore, researchers have examined tumor-forming capacity by induction of additional oncogenic mutations in conjunction with *IDH1* mutation. Researchers utilized the RCAS-TVA system to express $IDH1^{R132H}$ and *PDGFA* in *Cdkn2a*, *Pten*, *Atrx* conditional knockout mice, thereby showing high-grade IDH-mutant glioma formation (55). Similarly, IDH-mutant glioma also was successfully generated by $IDH1^{R132H}$ and *NRAS* knock-in and sh*p53* and sh*ATRX* knockout in neonatal mice lateral ventricle using the Sleeping Beauty transposon system (56). Induction of $Idh1^{R132H}$ mutation with the loss of *p53* and *Pten* led to GBM formation using retrovirus expressing PDGFB-IRES-Cre recombinase and adenovirus expressing Cre recombinase (57). To date, all of IDH-mutant animal models mainly target NSCs in the SVZ; thus, additional animal studies need to be done to carefully examine the tumorigenic potential of other lineage-restricted cell populations following $IDH1^{R132H}$ and co-occurring oncogenic mutations.

Collectively, the cell-of-origin and subsequent mutant cell behavior appear to underlie different biological and genomic phenotypes in GBM. A recent study demonstrated that distinct characteristics in transcriptome profiles, obtained from GBM animal models targeting either NSCs or oligodendrocyte lineage cells, can be used to classify IDH-wildtype GBMs into two subtypes based on the cellular origin (58). However, individual cells from the same tumor harbor different mutations and exhibit diverse transcriptional patterns and phenotypes (59), making it difficult to completely unravel cellular origins and tumor evolution processes.

DISSECTING CELLULAR HIERARCHY IN GLIOBLASTOMA

With advances in single-cell sequencing, brain tumors have been examined at the single-cell level in an attempt to document developmental programs in GBM. Using single-cell whole-genome sequencing, researchers noted intratumoral clonal evolution based on *EGFR* aberrations (60). Patel and colleagues also showed the mosaic pattern of *EGFR* and other RTK signaling molecules (59). Despite the observed clonal heterogeneity in GBM, researchers have attempted to identify key neurodevelopmental programs from transcriptional profiles. Hierarchical clustering revealed that a subset of genes regulating oligodendrocyte function are important in primary GBM, along with genes related to the cell cycle, hypoxia, and complement/immune responses (59). Müller and colleagues also demonstrated that PDGF-driven GBMs exhibit a progressive induction of OPC-like cells (61). Additionally, several studies have recently indicated that IDH-wildtype GBM recapitulates a normal neurodevelopmental hierarchy (62, 63): malignant cells exist in four cellular states of distinct neural cell types, including NPC-like, OPC-like, astrocyte-like, and mesenchymal-like cells (62). Meanwhile, Couturier and colleagues demonstrated that putative originating cell populations share similar expression profiles of glial progenitors and that tumor cells are organized into the normal neural lineage hierarchy observed in fetal brain (63).

Although single-cell RNA sequencing of IDH-mutant GBM has not been conducted due to a small number of patients, several studies of IDH-mutant glioma have shed some light on the cellular hierarchy of IDH-mutant GBM. Therein, most malignant cells are differentiated into and are reminiscent of glial lineages (oligodendrocyte-like and astrocyte-like), while a small subset of cells remain undifferentiated, exhibiting features of NSCs (64, 65). Overall, aberrant differentiation toward glial lineage cells and developmental programs appears to dominate the cellular diversity in IDH-mutant glioma. These studies suggest that IDH-mutant GBM might originate from progenitor cells with more restricted potential.

DISCUSSION

A number of studies have described the cellular origin and hierarchy of IDH-wildtype GBMs in humans, and accumulating evidence from genome, transcriptome, and animal studies suggests that IDH-mutant GBMs have characteristics distinct from those in IDH-wildtype GBMs. This raises the hypothesis that IDH-mutant GBMs may arise from a different cell-of-origin that undergoes malignant transformation. Based on the hypothesis, we may consider another possible candidates for the cell-of-origin of brain tumor such as glial progenitor cells (66). Accordingly, additional genetic analysis and animal modeling of IDH-mutant GBM should be performed to identify the cell-of-origin. Furthermore, future research should seek to carefully characterize the underlying mechanisms of which cells initially acquire mutations and how mutation-harboring cells evolve and undergo lineage specification during gliomagenesis. Such research may benefit from focusing on influences from the tumor microenvironment (e.g., immune cell infiltration) on the

fate of tumor initiating cells and subsequent expression-based subtypes in GBM.

AUTHOR CONTRIBUTIONS

HJK, JWP, and JHL conceived the topic for the mini review and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Subventricular Zone, a Hideout for Adult and Pediatric High-Grade Glioma Stem Cells

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Both in adult and children, high-grade gliomas (WHO grades III and IV) account for a high proportion of death due to cancer. This poor prognosis is a direct consequence of tumor recurrences occurring within few months despite a multimodal therapy consisting of a surgical resection followed by chemotherapy and radiotherapy. There is increasing evidence that glioma stem cells (GSCs) contribute to tumor recurrences. In fact, GSCs can migrate out of the tumor mass and reach the subventricular zone (SVZ), a neurogenic niche persisting after birth. Once nested in the SVZ, GSCs can escape a surgical intervention and resist to treatments. The present review will define GSCs and describe their similarities with neural stem cells, residents of the SVZ. The architectural organization of the SVZ will be described both for humans and rodents. The migratory routes taken by GSCs to reach the SVZ and the signaling pathways involved in their migration will also be described hereafter. In addition, we will debate the advantages of the microenvironment provided by the SVZ for GSCs and how this could contribute to tumor recurrences. Finally, we will discuss the clinical relevance of the SVZ in adult GBM and pediatric HGG and the therapeutic advantages of targeting that neurogenic region in both clinical situations.

Keywords: glioblastoma, recurrence, subventricular zone, glioma stem cell, cancer stem cell, diffuse intrinsic pontine glioma, high grade glioma, diffuse midline glioma

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INTRODUCTION

Gliomas are the most frequent primary tumors of the central nervous system, both in adults and children. Among them, glioblastoma (GBM), classified as grade IV by the World Health Organization (WHO), is the most frequent in adults, with an overall average annual age-adjusted incidence rate of 3.2 per 100,000 (1, 2). In children, WHO grades III and IV gliomas are generally grouped together as high grade gliomas (HGGs), and are less common, with an annual age-adjusted incidence rate of 0.08 and 0.15 per 100,000, respectively (1). Diffuse intrinsic pontine glioma (DIPG) is the second most common HGG of childhood (3). This tumor has a diffuse growth pattern and is localized in the brainstem. Tumors with identical characteristics are found in other midline structures such as the thalamus and the spinal cord, reason why it has been re-classified as diffuse midline glioma (DMG) in the last WHO 2016 classification (2). In addition, these are phenotypically and molecularly distinct

from the other types of HGGs with the characteristic of having histone H3 mutations (2, 3). However, for the interest of this review, DMGs will be included with HGGs in our discussion.

Despite clear molecular and genetic differences between pediatric and adult HGGs, as reviewed by Sturm (4), there seems to be a continuum between these two age groups. For examples, DMG with H3K27M mutations, initially thought to exclusively occur in children, can also be seen in adults (5, 6), while adult GBM characteristics, such as epidermal growth factor receptor (EGFR) amplification, can be seen in adolescents (7). Furthermore, pediatric and adult HGGs share a poor prognosis due to an almost systematic relapse of the tumor despite a multimodal therapy which classically consists of the tumor resection, whenever possible, followed by radiotherapy plus concomitant and adjuvant temozolomide (TMZ) (2, 8). Recurrences most likely emanate from tumor cells which have infiltrated the parenchyma and are practically impossible to successfully eradicate through a complete surgical resection (9). Moreover, some tumor sites are not reachable by surgery which is classically the case for DMG (3).

In addition to cancer cells which have intruded the tumor surroundings, recurrences can be explained by the existence of cancer stem cells (CSCs) or glioma stem cells (GSCs). CSCs-based tumor initiation, growth and maintenance was first proposed over 150 years ago by Virchow, who first suggested that a quiescent sub-population of embryonic stem cells was able to generate tumors (10). Since then, the existence of CSCs in tumors has been demonstrated in various types of cancers (11–13), including in adult GBM (14–17) and pediatric HGG (18–20). Several GSC features supports the hypothesis that these cells contribute to recurrences (16). Indeed, GSCs adapt and survive environmental stresses, present increased resistance to standard therapies and are able to form a novel tumor (21–27). GSCs are mainly present in the tumor mass but have also been detected in the subventricular zone (SVZ), a neurogenic niche persisting after birth and containing resident neural stem cells (NSCs) (19, 21, 23).

The present review will describe the similarities between GSCs and NSCs. The migratory routes of GSCs from the tumor mass to the SVZ and the signaling pathways involved in their migration will also be described hereafter. In addition, we will debate the advantages of the microenvironment provided by the SVZ for GSCs and how this could contribute to tumor recurrences. Finally, we will discuss the clinical relevance of the SVZ in adult GBM and pediatric HGGs and the therapeutic advantages of targeting the SVZ in both clinical situations.

GLIOMA STEM CELLS SHARE FEATURES WITH NEURAL STEM CELLS

Gimple and colleagues recently suggested the following definition for GSCs; "GSCs are defined by tumor-initiating capacity following serial transplantation, self-renewal, and the ability to recapitulate tumor heterogeneity" (16). These functional characteristics are currently the only tools available for the identification of GSCs as none of the stem marker expression shows sufficient sensitivity and specificity. Indeed, GSCs markers widely overlap with NSC specific ones, thus rendering the identification of GSCs within a heterogeneous tumor rather difficult. Thus, so far, GSCs are still solely recognized based on their functional properties (11, 15).

Multiple signaling pathways involved in normal NSC biology also play a role in GSCs. For example, the Notch pathway is implicated in the maintenance of NSCs in an undifferentiated state *via* the repression of proneural gene expression and is frequently upregulated in GSCs (28, 29). The Bone Morphogenetic Protein pathway inhibits neurogenesis and promotes gliogenesis in NSCs, while it stimulates astrocyte-like differentiation and reduces proliferation in GSCs (30, 31). The Wnt pathway regulates NSC and GSC proliferation *via* the accumulation of β-catenin (32, 33). The Sonic Hedgehog pathway is involved in self-renewal of NSCs and GSCs *via* Gli1 (34, 35). STAT3 is needed for NSC and GSC proliferation and the maintenance of multipotency (36, 37). Finally, EGFR, classically expressed by NSCs and promoting their proliferation, is often overexpressed in GBM and has been associated with tumor initiation, tumor growth, cell invasion, angiogenesis, and resistance to chemo- and radiotherapy (38).

In the same way, multiple transcription factors are common between NSCs and GSCs. Bmi1, a component of the Polycomb Repressive Complex 1, is classically found in undifferentiated NSCs and is involved in the maintenance of their multipotency. It also contributes to glioma aggressiveness *via* NF-κB and matrix metalloproteinase-9 (39). In addition, the inhibition of c-Myc, a transcription factor involved in the regulation of stem cell renewal and proliferation, triggers GSC apoptosis and reduces neurospheres formation (40). Sox2 which protects NSCs from apoptosis *via* survivin overexpression (41), is essential for the stemness maintenance of GSCs, together with Oct4 and Nanog (18). Finally, Olig2, a key transcriptional factor normally required for neural progenitor cell (NPC) proliferation (42), is able to reduce the suppressive action of p53 which regulates the proliferation in GSCs (43).

The identification of markers permitting the detection of GSCs is important since it has been estimated that approximately only one GSC every 1,000 tumor cells is present in a GBM tumor (44). Despite this low number of GSCs in the tumor mass, there is now evidence that these cells might contribute to tumor recurrences. Indeed, GSCs can leave the tumor mass, invade the parenchyma and migrate to further locations, including the neurogenic zones, where they escape a surgical intervention (23, 45–47). These GSCs can then remain quiescent until a still unknown mechanism triggers the development of a new tumor (48).

The Subventricular Zone, a Hideout for Glioma Stem Cells

The Architectural Organization of the Subventricular Zone

In the adult human brain, there are two well-described neurogenic zones: the SVZ, situated in the walls of the lateral

ventricles (LV), and the subgranular zone (SGZ) of the hippocampal dentate gyrus. Although controverted, some evidence suggest that the presence of NSCs is not limited to these two well-known neurogenic niches but could extend to other parts of the human central nervous system (49, 50). In addition, multiple other niches of NSCs have been reported in other mammalians (51). As the SVZ is the largest neurogenic region of the adult brain and because a link between the SGZ and brain tumors is less clear (52), this review will focus on the role of the SVZ in HGG recurrences.

The human SVZ is composed of four distinct layers going from I to IV from the innermost toward the outermost layer. Layer I runs alongside the ventricular cavity and is a monolayer of ependymal cells responsible for the production and secretion of cerebrospinal fluid. Layer II is known as the hypocellular space as it contains cellular processes with only very few cell bodies. Layer III is a cellular ribbon mainly comprising cells expressing glial fibrillary acidic protein (GFAP). Finally, layer IV, the outermost layer adjacent to the parenchyma, is a transition zone mainly composed of myelinated axons and oligodendrocytes (**Figure 1**) (53). Describing the exact localization of NSCs within the different layers of the SVZ is difficult as it depends on which criteria have been used to define or identify these cells. Recent single-cell RNA sequencing studies helped classifying NSCs into four main populations: quiescent NSCs, activated NSCs, NPCs and neuroblasts (54). Most NSC seem to be quiescent and positive for GFAP and CD133 (55). Mammalian NSCs resembling glial cells and sharing common characteristics and markers including GFAP, are mainly detected in layer III of the SVZ (56). The transcription factor Sox2 has been validated for the detection of NSCs in the human fetal brain (57). Sox2 is expressed by quiescent and activated NSCs, but not by NPCs (57, 58). In adults, Sox2 can be detected in the different layers of the SVZ with decreasing numbers toward the parenchyma indicating the presence of NSC in the adult SVZ (59). However, when considering the expression of the immature neuronal markers such as doublecortin, only rare NSCs are found in the adult human brain and only in layer III (60). Finally, in the SVZ, the proliferative marker Ki67 decreases during aging (61) with a limited number of Ki67 positive cells detected in layer III in the adult SVZ, reflecting very few cycling cells (60). However, it is to note that whereas proliferative cells in juvenile SVZ correspond to different cell types including immature cells, in the adult SVZ, Ki67 is exclusively expressed by microglia (60), the primary resident immune cells of the brain (62).

Differences in the cellular composition exist between the SVZ found in children and the adult counterpart, with more proliferative cells in children, indicating higher neurogenesis under the age of four (60, 63). It is also worth to note that in addition to a continuum between children and adult HGGs, there is a progression in the cellular and molecular properties of NSCs hosted in the SVZ from the embryo, through childhood to adulthood (64).

Variations also exist in the organization of the different SVZ layers between species. In adult rodents, NSCs, also called type B1 cells, are separated from the LV lumen by ependymal cells. Type B1 cells undergo asymmetrical cell divisions to give rise to a new type of B1 cell population with self-renewal properties (one of the hallmarks of a stem cell), as well as transit amplifying progenitor cells, also known as type C cells. Type C cells then migrate to become neural precursor cells including migrating

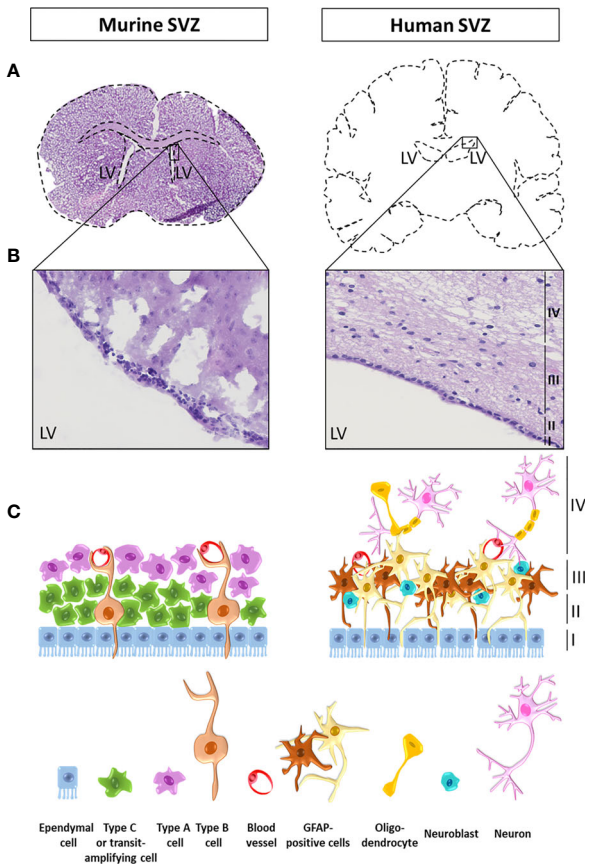

FIGURE 1 | Illustration and schematic representation of a mouse and a human subventricular zone. **(A)** Coronal sections of a mouse (left) and a human (right) brain at the level of the lateral ventricles (LV). **(B)** Zoomed images of a mouse and a human brain subventricular zone stained with haematoxylin and eosin. The four human layers are indicated as layers I to IV from the lumen of the LV toward the parenchyma. **(C)** Schematic representation of the cellular composition of the mouse and the human subventricular zone (SVZ). In adult rodents, neural stem cells (NSCs), also called type B cells, are separated from the LV lumen by ependymal cells. Type B cells undergo asymmetrical cell divisions to give rise to a new type of B cell population with self-renewal properties (one of the hallmarks of a stem cell), as well as transit amplifying progenitor cells, also known as type C cells. Type C cells then migrate to become neural precursor cells including migrating neuroblasts (type A cells) or oligodendrocytes precursor cells. In the SVZ, type B cells display a double contact, one with the ventricle and one with the basal lamina of blood vessels, where the blood-brain barrier (BBB) is not complete. The human SVZ is composed of four distinct layers going from I to IV from the innermost toward the outermost layer. Layer I runs alongside the ventricular cavity and is a monolayer of ependymal cells responsible for the production and secretion of cerebrospinal fluid. Layer II is known as the hypocellular space as it contains cellular processes with only very few cell bodies. Layer III is a cellular ribbon mainly comprising cells expressing glial fibrillary acidic protein (GFAP) and neuroblasts. Finally, layer IV, the outermost layer adjacent to the parenchyma, is a transition zone mainly composed of myelinated axons and oligodendrocytes.

neuroblasts (type A cells) or oligodendrocytes precursor cells. In the SVZ, type B cells display a double contact, one with the ventricle and one with the basal lamina of blood vessels, where the blood-brain barrier (BBB) is not complete. B cells are able to form C cells, which in turn divide in A cells that finally migrate and integrate in the olfactory bulb in mice (**Figure 1**). Note that, in human, the SVZ present a dense layer of B cells while there are just a few of A and C cells. For a complete review on neural stem cells in the adult mammalian brain and a good schematic representation of the SVZ in rodent, we would refer readers to the article from Obernier and Alvarez-Buylla (64, 65).

Glioma Stem Cells Take Different Routes to Reach the Subventricular Zone

In non-tumoral brains, white matter tracts can act as a guide, or a motorway, for the migration of NSCs or glial progenitor cells. Multiple evidence highlights a similar pattern of migration for GBM cells. The first evidence of GBM invasion through the white matter tracts was formulated by Scherer and collaborators in 1938 after they studied 100 patients with gliomas. They also demonstrated GBM cell migration through other routes like blood vessels, the neural parenchyma and the subarachnoid space (66). Later, NSCs were transformed to gain tumorigenic capacities before being implanted in mouse brains to model GBM tumor growth and brain invasion. Grafted mice successfully recapitulated GBM tumor development four weeks post-injection. More importantly, two weeks after injection, few GBM cells were detected in the corpus callosum, consisting of white matter tracts connecting the two cerebral hemispheres (67). As reported by our team, the injection of human GSCs in the mouse striatum led to the formation of a tumor. Furthermore, some GSCs left the tumor mass and migrated through the corpus callosum to reach the ipsi- and controlateral SVZ (**Figure 2**). GSCs were also detected in the olfactory bulb, demonstrating their migration from the SVZ through the rostral migratory stream (45), a structure containing a high density of parallel blood vessels classically used as a scaffold for neuroblasts (68). Another interesting study by Kakita et al. revealed the migration of labeled glial progenitors from the neonatal SVZ through the corpus callosum to the contralateral hemispheres, which correlates with the pattern of migration described by Kroonen et al. (45, 69). More recently, diffusion and magnetic resonance performed on seven glioma and six control patients showed that the human corpus callosum also act as a GBM cell migration track (70). In children, Caretti et al. analyzed a series of autopsies from 16 patients with DMG and observed that tumoral cells spread to the SVZ in 63% of the cases (19). For the last ten years, neurogenic niches have received more and more attention as not only it is the largest site for stem cells persisting in adulthood, but also, as discussed above, it can be a preferred destination site for GSCs (23, 45–47).

Chemoattractants Secreted by the Subventricular Zone Contribute to Glioma Cell Migration

The SVZ secretes various factors including chemokines and other proteins regulating cell migration (71). Some of the pathways involved in the migration pattern of GSCs from the tumor mass toward the SVZ have already been identified (46, 47).

The first axis, CXCL12/CXCR4, has been identified by our team in 2015 (46). CXCL12 is a chemokine acting on two main receptors, CXCR4 and CXCR7 (72–75). We have demonstrated that CXCL12 is a key player in the migration of CXCR4 positive adult GSCs from the tumor mass toward the SVZ (21). In addition to its chemokine activity, CXCL12 is involved in many biological activities (76) including the regulation of cell proliferation and tumor growth (75), favors an epithelio-mesenchymal transition, regulates the expression of GSC cell markers (77), and increases resistance to both radiotherapy and chemotherapy (21, 22, 78, 79). Furthermore, CXCL12 increases cell survival and facilitates DNA double strands break repairs through the recruitment and phosphorylation of nuclear MAP kinase phosphatase 1. It is also interesting to note that both effects induced by CXCL12 (oriented migration and DNA repair) are dependent on a CXCR4 signalization (78). A potential role for CXCR7 in the mediation of CXCL12 effects on GSCs remains to be investigated since these cells also express that receptor (76, 78).

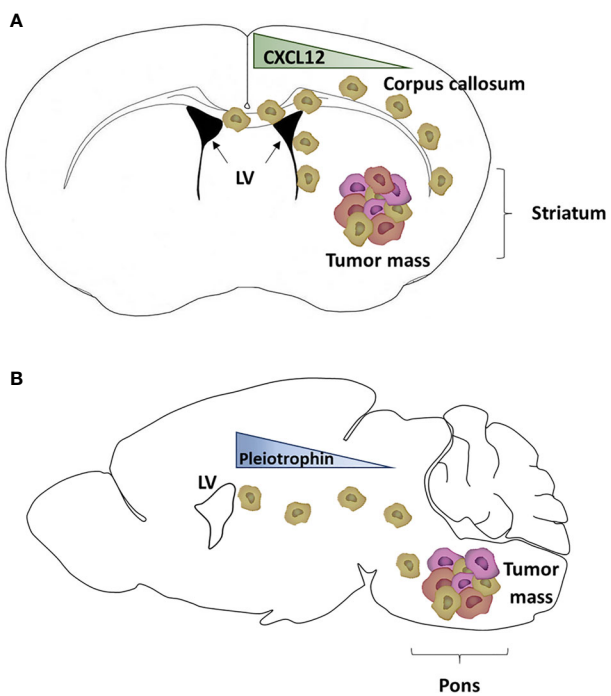

FIGURE 2 | Mouse model of glioblastoma cell invasion into the subventricular zone. **(A)** Schematic representation of adult glioblastoma (GBM) cells grafted into the right striatum of a mouse brain (schematically drawn as a coronal section ahead of the hippocampus) and generating a tumor mass. Some GBM cells expressing CXCR4 (light brown cells) migrated through the corpus callosum to reach the subventricular zone (SVZ) of the lateral ventricles (LV), following a CXCL12 gradient (45, 46). **(B)** Schematic representation of pediatric diffuse midline glioma (DMG) cells grafted into the pons of a mouse brain (schematically drawn as a sagittal section) and generating a tumor mass. Some DMG cells (light brown cells) have migrated out of the tumor mass and reached the SVZ, following a gradient of proteins including pleiotrophin (47).

A second migratory axis has been identified by Qin et al. (47) who showed that pleiotrophin, along with three required binding partners (secreted protein acidic and rich in cysteine (SPARC), SPARC-like protein 1 and heat shock protein 90B) is secreted by SVZ NPC and triggers the migration of DMG and adult GBM cells to the SVZ, through activation of the Rho/ROCK pathway (47).

Additional chemokines are known to be expressed in the SVZ-environment and could also contribute to the recruitment of GSCs (21, 46, 47). Using an array, we studied mouse SVZ conditioned media which led to the identification of several chemokines, including CXCL12 already described above. Amongst the other chemokines, we detected CXCL1, CCL5, CXCL10, and CXCL2. Furthermore, a gene expression profiling analysis was performed using real-time PCR Arrays on total RNA extract obtained from microdissected mouse SVZs. Genes were classified into high, basal and low mRNA levels. Amongst 14 genes highly expressed in the SVZ, we identified CXCL12 as well as CX3CL1 (also known as fractalkine), CCL19 and CCL12 which is the homologue of Monocyte Chemoattractant Protein-1 (MCP1/CCL2) in human (46). In a later study, chemokines were detected in condition media obtained from a human SVZ (21). This technical approach allowed the identification of eight chemokines in the conditioned media, within which Macrophage Inflammatory Protein-3 Alpha (MIP-3α/CCL20) was detected at the highest level, followed by interleukin 8 IL-8/CXCL8, MIP-1α/CCL3, Neutrophil-activating peptide 2 (NAP-2/CXCL7), and MCP-1. Interestingly, at least three chemokines detected in the conditioned media obtained from mouse SVZ were also detected in the human one, namely MCP1, Tazarotene-induced gene 2 protein (TIG2/chemerin) and IL16 (21, 46).

To the best of our knowledge, studies from our team were the only one that employed mouse and human SVZ-conditioned media to identify chemokines released by the SVZ. However, the identification of previously described chemokines was based on targeted techniques. Beside our work, numerous transcriptomic and proteomic studies of the SVZ have been performed; however, without specific focus on SVZ-secreted proteins. Indeed, these studies were based on isolated cells from the SVZ or whole SVZ extracts (61, 80–84). Recently, whole proteins forming the extracellular matrix and their associated proteins, respectively named the "matrisome" and "matrisome-associated proteins", were extracted from 8 to 12-week-old murine SVZ. In the later study, S100 proteins and Serpins were identified as highly soluble in the SVZ-matrisome. An in-depth analysis of the identified SVZ-associated soluble proteins could potentially lead to the discovery of new migratory-related soluble factors (80). In humans, the composition of the SVZ has mainly been studied during development and is based on the characterization of proteins and/or mRNA expression in specific cell-types and/or on whole SVZs (85, 86). Moreover, databases are now available to study mRNA expression in human age-related SVZ: i.e., BrainSpan Atlas of the Developing Human Brain (83, 87). These databases deserve attention as they could help identify potential SVZ-chemokines. In 2016, the comparison of the secretome of human NSC and GSC cell lines, identified 138 proteins differentially expressed (86). Although this analysis was based on cell lines, the identification of NSC-secreted proteins could help interpret and/or validate future large-scale studies based on SVZ-secreted proteins. Indeed, after reviewing the current literature, it is clear that large-scale analysis of human SVZ-chemokines or secreted proteins are still required.

To conclude, various chemokines are expressed and tightly regulated in the SVZ environment. Numerous studies on human SVZ-secreted proteins would highlight new migratory-related factors and/or confirm the one shown in murine SVZ. Interestingly, some of these secreted proteins would be responsible for specific GSCs migration toward this neurogenic niche. Delocalization of GSCs would be responsible for their maintenance even after tumor resection and their role in HGG recurrences. The identification of specific chemokines, the analyses of their role in GSCs migration capacity and the study of their targeting is therefore of interest to better understand and fight HGG recurrences.

The Subventricular Zone Offers Interesting Advantages for Glioma Stem Cells

The association of GSCs with non-tumoral cells together with soluble factors provides specific intra-tumoral microenvironments known as niches. The niche concept can be described as an environment able to maintain NSC stemness (88). This concept has been transposed to gliomas with GSC maintenance, division and differentiation in specific GBM localisations. Whereas perivascular, perinecrotic and invasive niches clearly exist in GBM, these structures are dynamic and are not always easily distinguishable one from another (89). In addition to GBM stem cells, the cellular components of these tumors include lymphocytes, macrophages, fibroblasts, pericytes, astrocytes, microglial cells, and neurons. GBM heterogeneity also occurs in different part of the tumor mass with niches not clearly distinct from each other. Niches tightly regulate GBM pathogenesis such as GBM cell survival, invasion, immune escape, and metabolic needs as well as stemness maintenance. A hypoxic environment can give rise to necrotic areas surrounded by hypoxic palisading GBM and immune cells. GBM cells can also hijack abnormal blood vessels to constitute an angiogenic niche or use blood vessels to invade surrounding brain parenchyma in the invasive niche.

This section of the review will describe the SVZ microenvironment and give an overview of the benefits that the SVZ can provide for GSCs. As going into details for each of these advantages is beyond the scope of this article, we will provide general information on the subject and briefly discuss some aspects of the SVZ environment that is beneficial for SVZ-nested GSCs.

The SVZ encompasses various cell types involved in the maintenance of endogenous NSCs and provides all soluble factors, nutrients and oxygen required for the regulation of their biological processes (90). Even the composition of the extracellular matrix of the SVZ plays a major role in the regulation of neurogenesis, cell proliferation and migration (80). Thus, this brain region is an interesting niche for GSCs as by providing an environment adapted for NSCs it also gives the same advantages to SVZ-nested GSCs. The interaction of

GSCs with the tumor microenvironment is in fact key for the maintenance of their malignancy (91). In addition to the different cell types composing the SVZ (see above), neurogenic niches encompass other cell types able to secrete soluble factors which can directly act on GSCs and regulate major biological processes involved in the development of the pathology. These cells include microglia, NPCs, and cells composing the architecture of a large vasculature network with specialized properties (91). Indeed, the BBB in the SVZ consists of a vasculature lacking astrocyte end-feet and pericyte coverage at sites. In GBM, those specialized blood vessels are altered which leads to the dysregulation of numerous factors in the brain (92, 93).

NSCs from the SVZ present an increased resistance to TMZ and radiation therapy (21, 23). This radioresistance can be explained in part by high expression of the anti-apoptotic proteins Bcl2 and Mcl1 (94). Interestingly, GSCs nested in the SVZ differ from those which remained in the tumor mass, with SVZ-nested GSCs presenting an increased resistance to irradiation (21). This increased resistance is at least partially regulated by the presence of high levels of CXCL12 in the SVZ (95). Indeed, our group has demonstrated that in addition to attracting GSCs in the SVZ, CXCL12 had a protective effect against irradiation (21). The addition of SVZ-conditioned media to human GBM cells led to a decrease in histone variant H2AX phosphorylation on Ser-139 (γH2AX), a reliable molecular marker of DNA damage repair (21). One of the mechanism involved, is the recruitment and the phosphorylation of MKP1, regulated by CXCL12, which in turn regulates DNA strand breaks repair (78). A radioprotective effect of CXCL12 is supported by a recent study by Rajendiran et al. showing that the ubiquitous overexpression of CXCL12 in a mouse model led to a significant increase in the number of multipotent progenitors and increased radioresistance by promoting quiescence (96). Piccirillo et al. studied human GSCs isolated from the tumor mass or the SVZ and found that most GSCs, isolated from different patients, were resistant to TMZ no matter their origin. GSCs were also resistant to cisplatin, an agent previously used for the treatment of GBM (23).

It is evident that multiple other soluble factors present in the SVZ could promote GSC survival. For example, CX3CL1 highly expressed in the adult SVZ (46) promotes NPC survival (97). Interestingly, CXC3L1 and its receptor are both increased in high grade gliomas, with higher CXC3CL1 being associated with shorter overall survival (OS) (98). CXCL1, also secreted by the SVZ, is overexpressed in GBM tumors, provides radioresistance and is associated with a poorer prognosis for patients affected by the disease (99).

Soluble factors secreted in the SVZ also tightly regulate the balance of NSCs between quiescence and proliferation. These extrinsic signals act through the presence of receptor at the surface of NSCs (100). Amongst receptors enriched in quiescent NSCs, there is for example cadherin 2 which has recently been suggested as a biomarker for the prognosis of GBM and as a predictive factor for the response of gliomas to TMZ (101). In addition to soluble cytokines, oxygen, and nutrients can influence the biology of NSC and thus, of GSCs. The importance of oxygen concentrations for the maintenance of stem cell normal physiology has already been reviewed (102). The level of oxygen found in the SVZ and the SGZ is higher than in the other parts of the brain including the cortex and the thalamus (103). This is interesting since higher oxygen level would help maintain NSC, as well as GSC which have reached the SVZ, in a quiescent and undifferentiated states (104). NSCs in the SVZ are in close contact with the BBB which constantly expose them to circulating molecules and nutrients (105). As already mentioned above, the BBB is often altered in GBM brains (92), which could lead to blood vessel leakage and nutrients unbalance in the SVZ and consequently influence GSC quiescence state (105).

THE SUBVENTRICULAR ZONE IN CLINICAL PRACTICE

An Independent Prognostic Factor

More than 10 years ago, a retrospective clinical study described that a GBM directly in contact with the SVZ at diagnosis was associated with invasiveness and multifocal disease in adults (106). The authors also described four patterns allowing a better characterization of the SVZ involvement: SVZ+/Cortex+ (I), SVZ+/Cortex- (II), SVZ-/Cortex+ (III), SVZ-/Cortex- (IV). A first observational study of 69 patients reported a poorer OS in patients with GBM contacting the LV in comparison to patients with GBM not bordering the LV (107). Multiple studies then confirmed that the SVZ involvement at diagnosis or at recurrence was associated with poorer OS (108–110). Recently, Mistry et al. confirmed in a meta-analysis of fifteen studies and in a retrospective study of 207 adult patients, that GBM contact with the LV was associated with lower OS and can be considered as an independent factor of survival (111, 112). In comparison, there was no decreased survival in case of SGZ involvement or corpus callosum invasion (111). Importantly, the proximity with the SVZ does not allow assessing the origin of GBM. Indeed, Han et al. reported that GSCs display similar stem gene expression in GBM with and without LV contact (113). In the same way, Mistry et al. reported that SVZ contact is not associated with molecular signatures in GBM bulk tumor (114). Finally, in the latest study by Comas et al., an analysis of GBM progression in 133 adult patients with primary GBM treated with the standard TMZ-based adjuvant radiochemotherapy showed that GBM in contact with the SVZ appears to be an independent prognostic factor for poorer progression free-survival (PFS) but not for OS. They also reported that SVZ-contacting tumors were associated with a higher rate of contralateral relapses and more aggressive recurrences which they defined as relapses occurring in patients presenting a sudden worsening of their clinical condition before it could be detected by the follow up MRIs taken every 3 months. They concluded that a direct contact of GBM tumors with the SVZ could be used as a prognostic factor (115).

A similar retrospective analysis was recently conducted in 63 children and adolescents (median age of 12.3 years) diagnosed with HGG (116). Tumors contacting the SVZ were found in 54% of the patients and were usually larger than tumors not in contact with the SVZ. Furthermore, patients with SVZ-associated tumors had a decreased survival time (HR = 1.94, 95% CI 1.03–3.64, p = 0.04). Thus, similarly to adult findings, these data suggest that in

children and adolescents, the presence of HGG attached to the SVZ is associated with a poorer prognosis (116). Targeting the SVZ could therefore be a common therapeutic target for adult GBM and pediatric HGG.

A Potential Therapeutic Target

Surgery

While more and more studies have highlighted the importance of Gross Total Resection, nay Supratotal Resection in regard to OS (117, 118), it remains unclear how large the resection should be when a GBM tumor touches the SVZ. Some surgeons are indeed reluctant to open the ventricle in order to obtain a complete resection of the tumor, as it has been associated with communicating hydrocephalus or tumor spread among the ependyma or *via* the cerebro-spinal fluid (119). However, in a retrospective study of 229 GBM adult patients, Behling et al. showed that ventricular opening was not associated with a reduced OS in a multivariate analysis and could therefore be considered to achieve gross total resection (120). Moreover, Saito et al. recently performed a retrospective study with 111 GBM adult patients and reported that a wide ventricle opening (>23.2 mm) is a strong predictive factor for longer OS (121). Information on surgical intervention for pediatric glioma tumors contacting the SVZ is lacking. Thus, retrospective and prospective studies are undeniably needed to confirm those results in children.

Irradiation

As the SVZ involvement worsens the prognosis, it seems sensible to find a way to interfere with it. In this context, many studies have considered the specific ipsilateral, nay bilateral irradiation of the SVZ, even in absence of neuroradiological clues of the presence of tumoral cells in this brain region. In 2016, Smith et al. reviewed the different studies that investigated the advantages of irradiating the SVZ to improve the OS for adult GBM patients (122). We updated their findings to include the latest publications on that topic (**Table 1**). Evers et al. published

TABLE 1 | Summary of the advantages of irradiating the subventricular zone to improve the overall survival for adult glioblastoma (GBM) patients.

Authors (year)	Study design	Number of patients (tumor subtype)	Median delivered dose	Outcomes	
Studies in favor					
				PFS	**OS**
Evers et al. (123)	Retrospective	55 (Gliomas grade III/IV)	> 43 Gy to biSVZ	15 *vs* 7.2 months (p = 0.028)	
Gupta et al. (124)	Retrospective	40 (GBM)	>53.6 Gy to cSVZ and iSVZ		Improved on multivariate analysis in group with >53.6 Gy to iSVZ
Lee et al. (125)	Retrospective	173 (GBM)	>59.4 Gy to iSVZ	Improved on univariate and multivariate analysis	
Chen et al. (126)	Retrospective	116 (GBM)	>40 Gy to iSVZ after GTR	15.1 months *vs* 10.3 months (p = 0.028)	17.5 months *vs* 15.6 months
Iuchi et al. (127)	Prospective	46 (GBM)	>50–60 Gy to SVZ		36.2 months in patients with SVZ necrosis *vs* 13.3 months in patients without SVZ necrosis
Ravind et al. (128)*	Retrospective	50 (GBM)	>50 Gy to iSVZ > 37 Gy to cSVZ		Improvement for the iSVZ: 19.83 months *vs* 6.07 months (p = 0.031) No improvement for the cSVZ
Foro et al. (129)*	Retrospective	53 (GBM)	>52.2 Gy to iSVZ, >51 Gy to cSVZ, >47.2 Gy to biSVZ		Improvement for patients receiving >51Gy in the cSVZ
Foro Arnalot et al. (130)	Retrospective	65 (GBM)	>48.8 Gy to cSVZ		Improvement
Studies not in favor					
Slotman et al. (131)	Retrospective	40 (GBM)	>43 Gy to iSVZ, cSVZ, biSVZ	No correlation with iSVZ, cSVZ, or biSVZ dose	No correlation with iSVZ, cSVZ, or biSVZ dose
Elicin et al. (132)	Retrospective	60 (GBM)	>59.2 Gy to cSVZ	7.1 months *vs* 10.37 months	
Anker et al. (133)*	Retrospective	88 (GBM)	>56.4 Gy to iSVZ, >33.4 Gy to cSVZ	No correlation with iSVZ, cSVZ, or biSVZ dose	No correlation with iSVZ, cSVZ, or biSVZ dose
Sakuramachi et al. (134)*	Retrospective	54 (Gliomas grade III/IV)	>58.2 Gy to iSVZ, >44.1 *Gy* to cSVZ	No correlation with iSVZ, cSVZ, or biSVZ dose	No correlation with iSVZ, cSVZ, or biSVZ dose
Murchison S et al. (135)	Retrospective	370 (GBM)	>59.4 Gy to iSVZ, cSVZ or biSVZ	No correlation with iSVZ, cSVZ, or biSVZ dose	No correlation with iSVZ, cSVZ, or biSVZ dose
Valiyaveettil et al. (136)	Retrospective	95 (Gliomas grade III)	>54 Gy to iSVZ	Decreased in univariate analysis, No correlation in multivariate analysis	
Valiyaveettil et al. (137)	Prospective	74 patients (GBM)	>50 Gy to iSVZ	No correlation with iSVZ, cSVZ, or biSVZ dose	No correlation with iSVZ, cSVZ, or biSVZ dose

**Some retrospective studies presented in meetings reported opposite results, in favor (128, 129) or not (133, 134) in favor for SVZ irradiation. This table is an update of Smith et al. findings and includes the latest publications on that topic (122).*

the first retrospective study revealing an improvement of PFS after bilateral irradiation of the SVZ with a median dose superior to 43 Gy (PFS: 15.0 vs 7.2 months, $p = 0.028$) for patients suffering from grade III/IV gliomas (123). In a similar way, another retrospective study of 40 patients reported a better OS if a dose equal or superior to 53.6 Gy was delivered to the ipsilateral SVZ (iSVZ) (124). Inversely, Slotman et al. used the same cut-off of 43 Gy for bilateral SVZ irradiation and did not observe any difference in OS or PFS in their retrospective study. However, and importantly, they reported less distant recurrences in case of a delivered dose greater than 43 Gy to the contralateral ventricle (131). However, the conclusions of those three studies suffer from a limited number of patients (55, 40, and 40, respectively) (123, 124, 131).

Later, using a larger cohort of 173 patients, Lee et al. retrospectively showed an increased PFS for an ipsilateral SVZ irradiation with a delivered dose superior to 59.4 Gy (125) Interestingly, Chen et al. showed that an increased iSVZ irradiation (superior or equal to 40 Gy) after GTR was associated to a better PFS and OS (126). Another retrospective study showed a poor PFS if the dose delivered to the contro-lateral SVZ (cSVZ) was superior to 59.2Gy (132). Those studies are in fact rather difficult to compare as they are retrospective studies and do not control for important variables such as (i) patient selection, (ii) irradiation dose, (iii) cut-off values, or (iv) importantly, the delineation of the SVZ. Moreover, classical prognostic factors such as MGMT or IDH status have frequently not been considered. Besides, a prospective study initially designed to test hypofractionated high-dose intensity modulated radiation therapy reported a better OS in case of radionecrosis in the SVZ (127).

In 2017, Foro Arnalot et al. reported another retrospective study of 65 patients showing an improvement in PFS but not in OS if the cSVZ received a dose superior or equal to 48.8 Gy (130). Khalifa et al. showed that a dose inferior to 20 Gy for bilateral SVZ irradiation was associated with poor prognosis (138). In 2018, Murchison et al. did not found any correlation between SVZ dose and PFS/OS in a large retrospective study of 370 GBM patients (135). Recently, in a retrospective study of 95 patients suffering from anaplastic gliomas, Valiyaveettil et al. reported no correlation between SVZ dose and PFS/OS in multivariate analysis (136). In a prospective study including 74 GBM patients, the same team did not found any correlation between SVZ dose and PFS/OS (137). Finally, in a short prospective study of 30 GBM patients, the sparing irradiation of neurogenic niches, including SVZ, did not modify PFS or OS in comparison to a matched historical control (139).

In this context, a phase II clinical trial combining standard radio- and chemotherapy to deliver irradiation to the ipsilateral (60 Gy) and the contralateral (46 Gy) SVZ is ongoing (NCT02177578). This study will provide valuable information on benefits that targeting the SVZ could offer for the treatment of GBM. It has to be said that the major constraint to investigate SVZ irradiation more deeply, or even to consider SGZ irradiation, is the fear that it might hasten neurocognitive decline as healthy NSCs would not be spared by the treatment and would suffer along with tumor cells. While some of the cited studies did not show a correlation between the delivered irradiation dose to SVZ and changes in performance status, Iuchi et al. found that high dose radiations deliver to the SVZ, leads to radionecrosis and better OS. However, it also results in progressive decline in Karnofsky performance status which measures the ability for a patient to carry out daily tasks (127). Henceforth, it is not surprising that some clinical trials focused on sparing the SVZ and the hippocampus.

In children, retrospective and prospective studies suggest an association between neurocognitive deficits and radiation dose to the hippocampus hosting the SGZ, but not the SVZ. Due to the rarity of HGG in children, the link between SVZ irradiation and survival has not yet been investigated (126, 140–143).

Targeting GSCs Nested in the SVZ

Many current researches aim at targeting GSCs. To do so in an efficient manner, it is important to know what to target. The most common pathways involved in GSC maintenance include the Wnt, the Sonic hedgehog (SHH) and the Notch pathways. As recently discussed by Sharifzad et al., targeting these pathways could help eradicating GSCs or increase chemotherapy efficiency (144). To target GSCs nested in the SVZ, it has been suggested to use perphenazine, an inhibitor of the dopamine receptor D3, in order to block the migration of GBM cells to the SVZ (145). As the activation of the dopamine receptor D3 in SVZ cells is associated with their proliferation *in vitro* (146), its blockade by perphenazine could also maintain GSC in a quiescent state in the SVZ. Bardella et al. reviewed another interesting approach which consists of interfering with the SVZ inflammatory environment as it might predispose cells to mutations and worsen cancer phenotypes (147). However, while it has been largely reported that microglia participate in GBM progression locally by adopting an anti-inflammatory state (148), their interaction and effects on SVZ-nested GSCs remain to be proven.

CONCLUSION

HGG account for a high proportion of death resulting from cancer, both in adults and children. Unfortunately, survival has not been significantly improved over the last decades. Both bench and bedside evidences strongly support the involvement of GSCs and SVZ in HGG recurrences. We and others, previously demonstrated that GSCs migrate from the tumor mass toward the SVZ, through the CXCL12/CXCR4 axis or through a pleiotrophin-driven axis. Once hosted in the SVZ, GSCs benefit from a protective environment providing increased resistance to irradiation and chemotherapy, before these cells get reactivated by a still unknown mechanism and recolonize the TM or invade other sites. In adults, the benefit/risk balance of targeting the SVZ by surgery and/or radiotherapy was investigated in clinical settings; however, the review of the current literature does not permit a clear conclusion yet. In children, it has not been evaluated and should be further investigated. Other technical approaches to target the SVZ also remain to be explored. Blocking the migration of GSCs toward the SVZ is probably not an option, given that the cells would

already have migrated out at the time of the diagnosis. Other possibilities could be to decrease or to block the recolonization of the TM. The cancer cell trap approach is another interesting and original concept that exploits the migratory potential of cancer cells in order to concentrate them toward specific locations (149). This approach has been showed to reduce the metastatic potential of human breast cancer cells implanted in female mice, through biomaterial scaffolds implanted in peritoneal fat pads (150, 151). This kind of approach should definitely be further investigated in the context of pediatric and adult HGG and DMG and could be combined with local targeted therapies.

In conclusion, there is strong evidence that the migration of GSCs toward the SVZ is implicated in HGG recurrences, both in adults and in children. The exact mechanisms supporting this process should be further investigated with the perspective of specifically targeting this particular cell population.

AUTHOR CONTRIBUTIONS

AL, MD, and CD performed the literature review, and wrote the manuscript. BR edited the manuscript. CP and NC conceived, supervised, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Evolution of Experimental Models in the Study of Glioblastoma: Toward Finding Efficient Treatments

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Glioblastoma (GBM) is the most common form of brain tumor characterized by its resistance to conventional therapies, including temozolomide, the most widely used chemotherapeutic agent in the treatment of GBM. Within the tumor, the presence of glioma stem cells (GSC) seems to be the reason for drug resistance. The discovery of GSC has boosted the search for new experimental models to study GBM, which allow the development of new GBM treatments targeting these cells. In here, we describe different strategies currently in use to study GBM. Initial GBM investigations were focused in the development of xenograft assays. Thereafter, techniques advanced to dissociate tumor cells into single-cell suspensions, which generate aggregates referred to as neurospheres, thus facilitating their selective expansion. Concomitantly, the finding of genes involved in the initiation and progression of GBM tumors, led to the generation of mice models for the GBM. The latest advances have been the use of GBM organoids or 3D-bioprinted mini-brains. 3D bio-printing mimics tissue cytoarchitecture by combining different types of cells interacting with each other and with extracellular matrix components. These *in vivo* models faithfully replicate human diseases in which the effect of new drugs can easily be tested. Based on recent data from human glioblastoma, this review critically evaluates the different experimental models used in the study of GB, including cell cultures, mouse models, brain organoids, and 3D bioprinting focusing in the advantages and disadvantages of each approach to understand the mechanisms involved in the progression and treatment response of this devastating disease.

Keywords: glioma stem cells, cell cultures of glioma cells, mouse models of glioblastoma, brain organoids, 3D bioprinting

INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive tumor of the central nervous system (CNS) (1). It represents more than 60% of all brain tumors in adults and it is associated with a bad prognosis. Also, the Central Brain Tumor Registry of the United State (CBTRUS) registered in 2019 (including dates ranged between 2012 and 2016) that GBM represents the 14.6% of all malignant

brain tumors being male population the most affected (1.5 times more than female) by this disease (2, 3). Despite the efforts of scientists and clinicians to increase the life expectancy of GBM patients, survivors do not easily exceed the 15th month (3–5) and the 5-year survival rate is as low as 5.8% (2, 3, 6). Furthermore, the median age is 65, and the most affected age ranges from 75 to 84 (3, 6). Globally, the incidence of GBM is higher in some specific areas over others, such as North America, the west and north of Europe, and Australia (7). Several risk factors have been studied as critical for GBM development such as constitutive genetic factors, ionizing radiation, or reduced susceptibility to suffer allergies and asthma. However, some inconsistencies among the different studies reveal the need of further investigations (8–11). These inconsistencies are likely to be caused by the existence of different types of GBM, which behave differently.

The World Health Organization (WHO) classified GBM in 2016 combining histopathological and molecular features (12). GBM are now classified into three subtypes based on the presence and absence of IDH mutations: GBM IDH-wild type (90% of tumors), IDH-mutant (10%), and GBM IDH-NOS in which a full IDH evaluation cannot be performed. GBM-IDH-wt include three variants: giant cell glioblastoma, gliosarcoma, and a novel and provisional variant, the epithelioid GBM characterized by large epithelioid cells and the presence of the *BRAF* V600E mutation (12, 13). Based on the expressions of genes, GBM have been classified in Classical, Mesenchymal, Proneural, and Neural (14). Classical GBM is characterized by an amplification of the chromosome 7, the loss of chromosome 10, and an increase in EGFR expression. In Mesenchymal subtype a focal deletion of *FN1 gene* is observed affecting the AKT pathway, whereas the NF-κB pathway is highly expressed. The Proneural subtype is characterized by alterations of *PDGFRA* and point mutations in IDH. In this subtype some genes such as SOX, DCX, ASCL1 are affected. The Neural type is characterized by the presence of neural markers such as *NEFL* or *GABRA1* (14). Several genetic alterations in GBM have been linked with recurrence and relapse. Thus, recurrent glioblastoma shows a higher frequency of copy number variations in several genes, particularly cell cycle genes, an enrichment in the cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B) loss, and an excessive activation of cell cycle pathway genes. Also, gen sets such as TERT promoter and IDH1 mutation or tumor protein 53 (TP53) and IDH1 mutation (15).

Given the bad prognosis associated to this type of tumors, the search for therapeutic tools that represent a real increase in the survival rate has become the main goal in GBM research. Current GBM treatment includes the complete surgical resection of the tumor mass, followed by a combination of radiotherapy and chemotherapy (16). In this context, it is reasonable to say that the most significant development in clinical management of glioblastoma over the past two decades has been the groundbreaking trial of combining radiotherapy plus temozolomide (TMZ) (17), which resulted in an increase in the 2-year survival from 8% in patients with radiotherapy alone to 20% in patients with the combined therapy. Despite this improvement, effectiveness of treatment is variable from patient to patient. Apparently, effectiveness of treatment depends on several factors such as the tumor localization and size, or the brain anatomical structures affected (18). Essentially, one of the most relevant problems surrounding GBM is its infiltration into the healthy brain tissue, which makes practically impossible to perform a complete resection using surgical tools. In addition, the posterior radiation and chemotherapy do not completely eliminate all GBM cells (19). Thus, new insights in surgical tools are being used to allow visualization of cells within the tumor and improve the tumor mass resection. These are fluorescence-guided microsurgery (20) or intraoperative MRI, and ultrasound, which have been used in the surgical resection of CNS gliomas with the goal of maximizing extent of resection to improve patient outcomes (21). Regarding chemotherapy, TMZ is still the most effective so far, however, several other chemotherapeutic agents are being used, some of them directed to modulate the activation and suppression of signaling pathways altered in GBM. Examples of these new treatments are nelfinavir, tipifarnib, tamoxifen, or enzastaurin (22). These agents have proven not to be the most effective in individualized treatments, nonetheless, considering the molecular, cellular, histological, and genetic variances found in GBM, a deep molecular characterization of the different tumors could potentially allow the design of individualized therapies using these agents.

A problem linked to the inefficacy of TMZ treatment in the long term is that some cells within the tumor have the ability to escape its action (23) as well as the complementary radiation (24). These are the glioma stem cells (GSC). These cells share many similarities with Neural Stem Cell (NSCs) present within the physiological neurogenic niche of the subventricular zone (SVZ). These similarities are principally self-renewal and differentiation capacity (25, 26) in addition to several neurogenic markers, such as CD133 (27), nestin (28, 29), CD15 (30, 31), or some transcriptional factors including Sox2, Olig2, Nanog, and c-Myc (32). The first evidences on the role of the SVZ harboring malignant GBM cells were obtained using fluorescence guided resection of GBM using the commonly used fluorescent marker 5-aminolevulinic acid (5-ALA). These resections reveal the presence of fluorescent cells not only within the tumor mass but also in the adjacent SVZ, thus suggesting the presence of malignant cells in the SVZ of GBM patients (33). Clonal analysis of the stem cell populations suggested a GBM evolution as a result of multiple, genetically diverse clonal and sub-clonal populations involving both the SVZ and the tumor mass (33, 34). The role of the SVZ as a place of origin of GBM has gained strength because of the similarities between GSC and NSC of the SVZ (35). Vasculature, hypoxia, and several growth factors that promote GSC proliferation have been deeply studied in order to clarify the role of the SVZ in the origin of GBM (36–38). The human SVZ is characterized by presenting a complex cytoarchitecture composed of layers that provide a good environment to proliferation and differentiation of NSC (39, 40). In 2018, Lee et al. showed, using single-cell sequencing, that astrocyte-like NSC in the healthy SVZ tissue of

GBM patients hide GBM driver mutations, cells that are capable of migrating from the SVZ to lead the development of high-grade malignant gliomas in distant brain regions (35).

Over the past years a vast set of methodologies have been used in the study of GBM, leading to most of our current knowledge on tumor development and prognosis (41) (**Figure 1** and **Tables 1**, **2**). However, the past 10 years have been crucial in the development on new and innovative techniques, such as the growth of GBM organoids, which are leading to novel and individualized therapies (**Figure 2** and **Table 3**) for the treatment of this disorder (99, 127). In here, we discuss some classical methodologies together with the description of the most recently developed techniques to study GBM.

CLASSICAL APPROACHES IN THE STUDY OF GLIOBLASTOMA

Cell Cultures of Glioblastoma Cells

One of the most important tools in the study of GBM has been the use of cell cultures. Cell cultures provide the maintenance of cells *in vitro* for research and clinical studies, however it is important to choose appropriately the cell line and culture because none of the currently available cell-based glioma model systems is able to reproduce the complex microenvironment of glioma cells within the brain. Thus, each *in vitro* model has its advantages and disadvantages and it is necessary to select the cell system appropriate for each experimental question (128). Some of these systems have been widely used not only in the study of GSC but also in the study of NSC giving a great deal of information about the physiology of these cells (129–132) or about the finding of small pharmacological molecules that regulate these activities (133).

Glioblastoma Cell Lines

The first studies using cell-based glioma model systems used cell lines derived from induced Wistar/Furth rats and C57BL/6 mouse tumors of the central and peripheral nervous system (42, 43). Later on, human GBM cells were also immortalized for its use in culture (44, 45). This allowed the better understanding of glioma cell biology by simplifying the studies, since glioma cell lines provided an unlimited supply of cells available without ethical concerns and the possibility of obtaining reproducible results. Most commonly used human GBM immortalized cell lines are U87MG, U252, T98G, and LN-229. These cell lines show enrichment of cancer stem cells when grown as spheres in serum containing medium (134). To date, it is the fastest way to obtain preliminary results regarding the test of new anti-tumor drugs *in vitro*. GBM cell lines are easy to manipulate and to

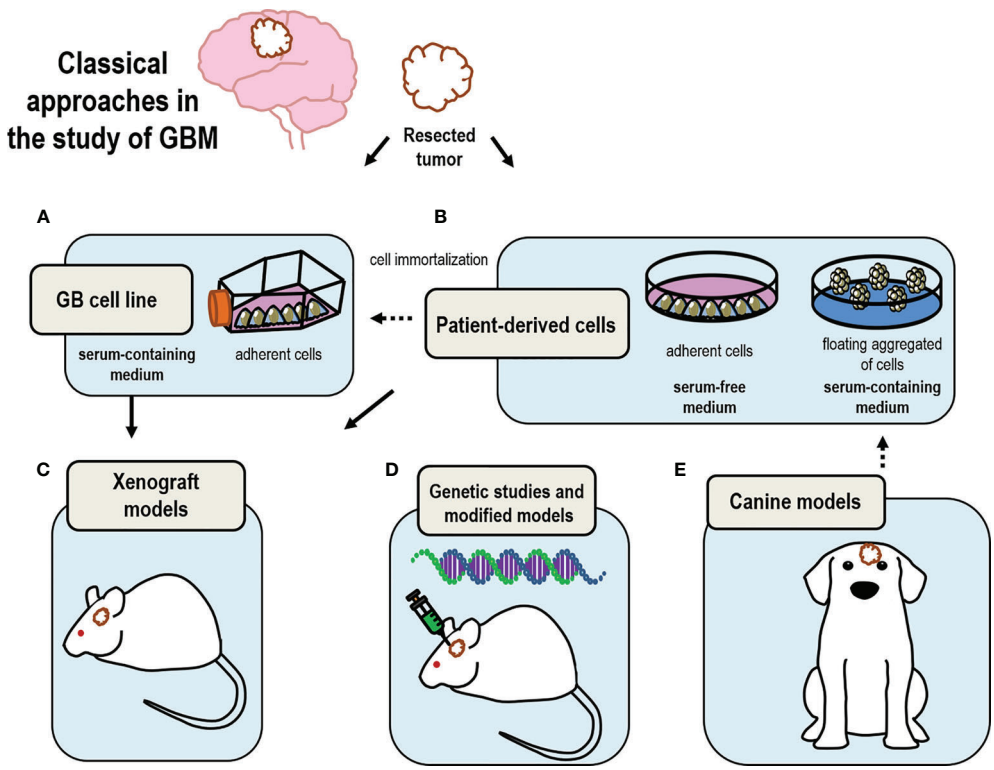

FIGURE 1 | Drawing summary of classical models currently in use to study GBM. Tumor samples resected from patients upon surgery are used to **(A)** generate immortalized cell lines that can be maintained in time; **(B)** select GSC by culturing these cells as neurospheres under floating conditions; **(C)** produce xenograft models by transplanting either cultured isolated cells or tumor tissue; **(D)** perform genomic analysis to select candidate genes to produce genetically modified mice models of GBM; **(E)** canine models are good model for the study of GBM based on their analogies with human GBM.

TABLE 1 | Advantages and disadvantages of GBM experimental models.

Method of study	Advantage	Disadvantage	References
GBM cell lines	• Good method to understand GBM biology • Simple system provided with a single type of cells • Less ethical concerns • Reproducibility • Fast method to obtain preliminary results • Easy to manipulate	• Does not allow studies about cell interactions or molecular mechanism in GBM development • Does not represent GBM microenvironment • Reduced genetic heterogeneity of cells compared with native tumor	Fields et al. (42), Sundarraj et al. (43), de Ridder et al. (44), Notarangelo et al. (45), Weeber et al. (46), Torsvik et al. (47), Allen et al. (48).
Patient-derived cells	• Respect intratumor heterogenicity (GSC), tumor initiation, invasiveness process • Genotype and phenotype characteristics resemble those of primary tumors • Give more comparable results • Can be maintained *in vitro*	• Cell cultures are limited to 20–30 passages before cells start exhibiting genomic and transcriptional changes • Quiescent GSC are deselected within the neurospheres upon the different passages • Introduce errors in terms of clonality, size, and number of neurospheres	Sottoriva et al. (49), Jiang et al. (50), Linkous et al. (51), Lee et al. (52), Brewer et al. (53), Baskaran et al. (54), Jayakrishnan et al. (55), Wang et al. (56), Chen et al. (57), Mori et al. (58), Ladiwala et al. (59), Pollard et al. (60), Fael Al-Mayhani et al. (61), Rahman et al. (62).
Xenograft model of GBM	• Allow personalized drug efficiency tests in single patients • Maintain the original tumor architecture and histological characteristic • Genetically stable	• A nude mouse is necessary to develop this model • Cells need to be cultured in spheroids forms before implantation • Does not reproduce the original niche. • It is not allow to test immunomodulatory therapies	Shu et al. (63), Lee et al. (52), Tentler et al. (64), Joo et al. (65), Patrizii et al. (66), Ashizawa et al. (67), Hutchinson et al. (68), Son et al. (69), Khaddoui et al. (70), Lynes et al. (71).
Genetically engineered and viral vector-mediated transduction models	• Modified models offer the ability to directly alter the genome of somatic cells in mouse tissues introducing or removing specific genes • Reproduce pre-clinical features • Easy approach to rapidly analyze therapeutic responses to drugs	• Genetics and histology of the modified tumor models are often not representative of the original human tumor	Furnari et al. (72), Holland et al. (73), Uhrbom et al. (74), Wei et al. (75), Baker et al. (76), Miyai et al. (77).
Canine model of GBM	• Similarities with human glioma • Good tool to perform pre-clinical studies	• Ethical issues • Difficult to detect GBM in canine models	Herranz et al. (78), Stoica et al. (79), Candolfi et al. (80), Chen et al. (81), Fernandez et al. (82).
Organotypic cultures	• Good model to study invasiveness • * Niche similarities	• * Not reproduce interactions with blood flow factors or typical hypoxic conditions	Eisemann et al. (83), Sliwa et al. (84); Marques-Torrejon et al. (85), Ravi et al. (86)
Brain organoids	• Provide a powerful tool for the *ex vivo* study of the molecular and cellular mechanisms • Maintain the architecture and organization of tissues	• Difficult approach in terms of technology • Need biopsies of patients • Complex structures to maintain	Lancaster and Knoblich (87), Clevers (88), Chen et al. (89), Sasai (90), Huch et al. (91); Hubert et al. (92), da Silva et al. (93), Ogawa et al. (94), Bian et al. (95), Krieger et al. (96), Linkous et al. (51), Hwang et al. (97), Jacob et al. (98), Zhang et al. (99), Perrin et al. (100), Vlachogiannis et al. (101), Ooft et al. (102), Ganesh et al. (103), da Hora et al. (104).

(Continued)

TABLE 1 | Continued

Method of study	Advantage	Disadvantage	References
	• Preserve important features of the original tumor heterogeneity • Useful to study GBM pathology and drug response • Good method to design personalized therapies and treatments and, also, in drug screening	• Elevated cost	
3D bioprinting	• Provide a cell network that resembles reality in a very faithful way • Replicate the architecture of tissues • Good method to test drugs effectively • Good to analyze cell signaling • Good method to test drug sensibility scanning • Good method to study invasiveness process, immunologic interactions and cellular crosstalk	• Need photo-crosslinking • Need effective biomaterials, which do not affect normal tissue development • Printing resolution still needs to be improved • Need of a bioprinter • High cost	Roseti et al. (105), Heinrich et al. (106), Ananthanarayanan et al. (107), Xiao et al. (108), Hermida et al. (109), Tang et al. (110)

maintain in cultures using serum-containing media. However, cell lines do not provide a reliable model to understand the cellular and molecular mechanism underlying the development of GBM or to evaluate therapeutic interactions because it fails to represent the native tumor microenvironment. In addition, the use of glioma cell cultures holds other issues intrinsic to immortalized cell lines. Successive cell passages select cells which have the highest proliferative potential, decreasing the genetic heterogeneity found in the parental tumor (46). It is likely that the selection imposed by the passages in *in vitro* cultures result in genetic drift, accumulation of chromosomal aberrations, and phenotypic alterations in cell lines (47, 48). Because of these drawbacks, biobanks containing annotated and validated cell lines derived from surgical samples of GBM patients that preserve GSC features are being developed. This strategy provides an open resource for the study of a large part of GBM diversity (135).

Usually, the use of cell lines is well received as an approximation or as preliminary data but it is necessary the use of other models closer to reality to obtain more relevant and representative data. However, these cultures have been fundamental to solve and understand GBM biology (**Figure 1A** and **Table 1**).

Patient-Derived Cells

Most cancers, including GBM, display intratumor heterogeneity and this could be one of the reasons why some tumors lack of satisfactory treatment (49, 50). Thus, the use of primary cell cultures derived from patients may facilitate the individualized study of GBM. Evidences show that the GSC subpopulation of GBM are very important to maintain tumor heterogeneity, as well as, the tumor initiation, maintenance, and invasion *in vivo* because of their capacity of self-renewal and differentiation (51). So far, GSC cultures have become the most accepted standard for studying GBM biology *in vitro* (52).

One of the problems found when using patient derived cultures is the use of serum since the subpopulation of GSC within the GBM is not present in cell cultures after prolonged serum exposure. GSC differentiate under these conditions, losing many of primary tumor characteristics (52). To avoid this issue, patient-derived GSC can be maintained *in vitro* under floating conditions in a serum free medium in which they form characteristic aggregates referred to as neurospheres. The starting population of cells is usually plated as a single-cell suspension in a non-adhesive substrate containing defined media supplemented with fibroblast growth factor (bFGF) and/or epidermal growth factor (EGF) (52, 53). However, it is important to consider that useful life of patient-derived glioma cell cultures is limited. After 20–30 passages cells start exhibiting genomic and transcriptional changes in metabolic and signaling pathways such as ribosomal synthesis, telomere packaging, or Wnt signaling pathways among others (54). Interestingly, the success rates of neurosphere cultures from gliomas is dependent on tumor grade and genetics, being the low-grade glioma cell cultures the most difficult to culture as neurospheres (55).

Despite its advantages, the neurosphere system presents a few drawbacks inherent to this cell culture method. Quiescent GSC may be lost with time since along the successive cell passages cells with the highest proliferative potential are selected and the subpopulation of quiescent GSC is deselected. This subpopulation is particularly important because it is believed to be responsible for chemotherapy and radiotherapy resistance (56, 57). Moreover, to ensure clonality and multipotentiality of neurospheres, cells should be seeded as single cells per well (58).

TABLE 2 | Current trends in mouse xenografts and allografts.

Reference	Implantation type	Cell type	Tumor type	Animal model	Strategy used
Tateishi et al. (111)	Orthotopic xenograft	MGG152 HT1080	Recurrent GBM Fibrosarcoma	SCID mice	NAMPT inhibitor
Ashizawa et al. (67)	Heterotopic xenograft	GB-SCC010 GB-SCC026	Primary GBM Primary GBM	NOD-SCID mice and NOG mice	STAT3 inhibitor
Szabo et al. (112)	Orthotopic xenograft	LNT-229 LN-308	GBM GBM	Nude mice	Neutralization with VEGF or PlGF antibody
Sharpe et al. (113)	Orthotopic xenograft	BT111 BT116	Primary GBM Primary GBM	Nude mice	Monoamine oxidase B-activated pro-drug
Zhang et al. (114)	Orthotopic xenograft Orthotopic allograft	LN-319 GL261	GBM GBM	NSG mice	ErbB2/HER2-Specific NK Cells
Parrish et al. (115)	Heterotopic and orthotopic xenograft	GBM12	Primary GBM	Mdr1a/b$^{-/-}$Bcrp1$^{-/-}$ knockout and wild type mice	PARP inhibitors and temozolomide
Garros-Regulez et al. (116)	Heterotopic xenograft	U251	GBM	Nude mice	mTOR inhibition with Rapamycin and Temozolomide
Karpel-Massler et al. (117)	Orthotopic xenograft	GBM164 U87MG	Primary GBM GBM	SCID mice	Bcl-xL inhibition with ABT263
Yuan et al. (118)	Orthotopic xenograft	Patient derived brain tumor initiating cells	Primary GBM	SCID mice	ABT-888 and temozolomide treatment
Chang et al. (119)	Orthotopic xenograft	LN229 U87MG	GBM GBM	Nude mice	Pyr3 treatment
Sun et al. (120)	Orthotopic xenograft	TT150630 TT150728	Primary GBM Primary GBM	B-NDG mice	Palbociclib in treatment
Bejarano et al. (121)	Heterotopic xenograft	h676 h543	Primary GBM Primary GBM	Nude mice	TRF1 Chemical Inhibitors
Guo et al. (122)	Orthotopic allograft	GL261, GL261 Red-FLuc and GL261-Luc2	GBM	C57BL mice	FTY720 treatment
Gravina et al. (123)	Heterotopic and orthotopic xenograft	U87MG	GBM	Nude mice	RES529, a TORC1/TORC2 dissociative inhibitor
Zalles et al. (124)	Orthotopic xenograft	G55	GBM	Nude mice	Neutralization with ELTD1 antibody
Jensen et al. (125)	Orthotopic xenograft	patient-derived GB brain tumor stem cells	Primary GBM	SCID mice	Afatinib and pacritinib treatment
Yang et al. (126)	Heterotopic and orthotopic allograft	GL261	GBM	C57BL/6 mice	Bip inhibition and ionizing radiation

However, this leads to a low number of cells because the concentration of paracrine and cell-to-cell signals required for cell growth and division are minimal under these conditions. On the contrary, neurospheres obtained after plating multiple cells per well show spontaneous locomotion resulting in cell aggregation and producing errors in terms of clonality, size, and number of neurospheres, thus leading to discrepancies in results and conclusions (59).

Looking for an effective manner to cultivate GSC, some reports describe a methodology to attach these cells onto a surface to expand them in serum free medium as adherent 2D cultures in the presence of growth factors EGF and bFGF (60, 61). The use of adherent 2D-cultures increases the efficiency of culture expansion avoiding the differentiation and apoptosis associated to the sphere cultures. Attached cells are more exposed to growth factors used in cultures that maintain the proliferative capacity (60). Other authors maintain that both methods, sphere and adherent cultures, are equal and useful to study GBM (62) and it is likely that a combination of both models is the best strategy for the *in vitro* test of GBM drugs, or for the study of new specific markers of GBM malignancy and progression.

Taking together all these facts prove the benefits of using this neurosphere cultures, starting with the capacity to preserve heterogeneity and also, to study the migration process and initiation of a new tumor. All these considerations are not present in cell lines models. However, the problem associated with the loss of quiescent GSC could be unfavorable in some studies. Nonetheless, this model allows the culture of cells from single individual patients, facilitating the study of inter-individual differences (**Figure 1B** and **Table 1**).

Xenograft Models of Glioblastoma

In vivo transplants of GBM in animal models have long been used to study tumor development upon the engraftment of human cells into immunodeficient mice. Transplants of patient derived xenografts, in which dissociated tumor cells or tumor tissue fragments are implanted into mouse brains have successfully been used in murine models (**Figure 1C**). Mainly, three types of mouse have been used, which are classified

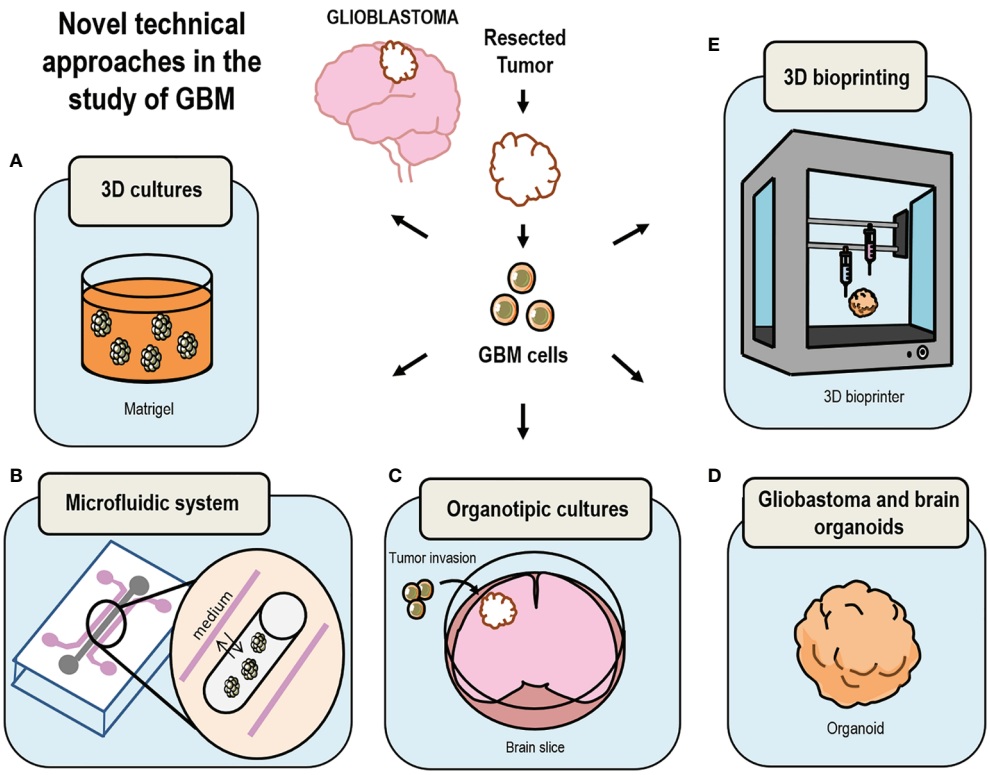

FIGURE 2 | Drawing summary of novel models currently in use to study GBM. **(A)** 3D Cultures of cells embedded in Matrigel have been key techniques in the development of brain organoids; **(B)** growth of 3D structures using microfluidic systems replicates the changing microenvironment surrounding GBM in the human brain; **(C)** GBM cells can be grafted *in vitro* in human brain slices grown as organotypic cultures replicating the natural environment of a GBM tumor; **(D)** culture GBM organoids by either using glioma cells or by inserting GSC into brain organoids; **(E)** bio-print GBM 3D-spheroids using extracellular matrix materials and other cell types.

according to their immune response: i) nude mice which are unable to produce T cells; ii) non-obese diabetic severe combined immunodeficiency (NOD-SCID) and SCID-beige mice, which lack T and B cells; and iii) NOD-SCID IL2R-γ null (NSG or NOG) mice, which lack T, B, or NK cell activity (136). GBM implantation is usually done in the subcutaneous flank location (heterotopic implantation) facilitating the visual observation of tumor development, which allows testing the efficacy of anti-tumor drugs by analyzing the tumor dimensions. However, an important limitation to heterotopic models is that the established microenvironment has an important role in GBM tumors. For this reason, xenografts based on the implantation of GBM in the brain (orthotopic implantation) are more extensively used nowadays because it provides a CNS microenvironment, and preserves the integrity of tumor-initiating cells (63). We have summarized in **Table 2** series of studies involving PDX models, showing how orthotopic or heterotopic xenografts have been used in different immunodeficient mouse strains to perform preclinical studies on the efficacy of novel drug treatments.

Invasive xenografts have been established from surgical specimens that were first maintained as tissue spheroids in short-term culture to obtain cell lines (137, 138). Many human and mouse cell lines have been used in PDX models (111, 112, 139, 140). The patient-derived cells are engrafted into immunocompromised mice to maintain the histopathologic, genomic, and phenotypic characteristic of the primary tumor across early passages (64–66). Lee et al. used GSC lines to initiate tumors in mice and they found a high percentage of engraftments compared with patient derived tumor xenografts. This type of tumor induction was much more potent and maintained the heterogeneity typical of GBM (35, 67). While NSC implantation into nude mice does not result in tumor formation, GSC implantation leads to the generation of tumors in a healthy brain, preserving tumor heterogeneity. This strategy opens the possibility to development of personalized drugs facilitating individual drug-screening and the search for resistance mechanisms. Notwithstanding, several drugs showing favorable results in pre-clinical studies using PDX have failed posteriorly in clinical trials (68). Indeed, this is not completely surprising since orthotropic implantation in PDX models does not reproduce the conditions of the niche of origin. The human stroma and microenvironment are not similar to those in the mouse, limiting the study to tumor biology and therapy resistance (69). Moreover, since these mouse models lack inflammatory responsive cells or an intact immune system it does not allow testing for immunomodulatory therapies. This limitation is currently critical since immune therapeutics have recently been very successful in treating a diverse group of

TABLE 3 | Current preclinical trials using organoids for the study of GBM.

Reference	GBM organoid from	Contribution to GBM study	Potential clinical use	Advantage	Disadvantage
Hubert et al. (92)	Patient-derived GBSC	Microenvironmental impact and cancer stem cell biology	Tumor sensitivity to radio-chemotherapy	Preserve phenotypic heterogenicity and tumorigenic capacity	No vessels No immune cells Slow growth No interaction with no-tumor cell
Ogawa et al. (94)	hESC-derived cerebral organoid genetically modified by introducing $HRas^{G12V}$ through CRISPR/Cas9	Model for tumor formation and transplantation	Platform to test human cancer phenotypes and personalized therapy	Interactions between tumor and non- tumor cell	No vessels No immune cells Slow growth
Bian et al. (95)	hESC/iPSC-derived cerebral organoid genetically modified by introducing several mutation combinations through CRISPR/Cas9	GBM invasiveness and evaluation of drug response	Personalized therapy	Interactions between tumor and non- tumor cell	No vessels No immune cells Slow growth
Da Silva et al. (93)	Co-culture of human GBM spheroids with mouse ESC	Identification of anti-GBM invasion strategies	Drug screening. Tumor sensitivity to radio-chemotherapy	Interactions between tumor and non- tumor cell	No vessels No immune cells
Linkous et al. (51)	Co-culture of patient derived GBM stem cells with hESC-derived cerebral organoid	GBM biology in the human brain environment.	Drug screening Tumor sensitivity to radio-chemotherapy	Interactions between tumor and non- tumor cell	No vessels No immune cells
Krieger et al. (96)	GBM cells co-cultured with hESC-organoid cells	Invasion and transcriptional heterogeneity	Drug screening Tumor sensitivity to radio-chemotherapy	Interactions between tumor and non- tumor cell	No vessels No immune cells
Jacob et al. (98)	Patient-derived resected glioblastoma tumor tissue	GBM cells heterogeneity, tumor microenvironment and GBM infiltration	Useful to test therapy response including immunotherapy Construction of a living organism bank Biobank	Fast growth Vessels and immune cells	No interaction with non-tumor cell
Hwang et al. (97)	Patient derived- IPSC with c-met mutation	Reproduction a genomic network described in the most aggressive primary human GBM	Therapy response for GBM with c-met mutation	Good model to study progression and aggressiveness	No vessels No immune cells Slow growth No interaction with non-tumor cell

cancerous lesions and there has been an explosion in the study of immune therapeutics for cancer treatment over the past few years (70, 71).

Among the advantages of these xenograft models three can be highlighted: i) they allow personalized drug efficiency tests in single patients, ii) the original tumor architecture and histological characteristic are preserved and iii) they are genetically stable (**Tables 1** and **2**). However, there are important caveats that need to be addressed in xenograft models: i) immunodeficient rodents may not respond to certain drugs, ii) the surrounding microenvironment of mouse origin may interfere with drug response, and iii) they do not allow the test of immune therapies, thus limiting the type of drugs to be tested.

Genetically Engineered and Viral Vector-Mediated Transduction Mouse Models

As mentioned before, several genetic alterations have been found after the analysis of large numbers of uncultured GBM tissue samples removed from patients, leading to the discovery of several genes commonly mutated in GBM. Some of these mutations are already present in common cancer genes, such as EGFR, BRAF, RAS, PIK3CA, PIK3R1, PTEN (141, 142), particularly, EGFR variant III (EGFRvIII) is the most common active EGFR mutant in GBM. The presence of this mutant correlates with a poor patient prognosis due to its ability to extend downstream signaling (143). Accordingly, kinases like v-Src, which regulate the activity of these type of receptors have also been involved proliferation and migration of glioma cells (144). Additionally, a very clinically relevant discovery was the finding of the IDH1 mutation appears in a high percentage of secondary GBM and a small percentage of primary GBM (142). While PTEN loss, and EGFR amplification, are associated with primary glioblastoma, IDH1 mutation is common in secondary glioblastoma and show a higher survival rate.

The study of genetic alterations has led to the development of genetically engineered mouse models of GBM (77). Transgenic mouse models and knockouts as well as vector-mediated genetic approaches accurately reproduce pre-clinical features of GBM including the accumulation of genetic and epigenetic alterations in tumor suppressor genes or to the activation of oncogenic pathways, which lead to the progression of tumors (72, 142, 145). There are several examples employed in the development of mouse glioma modelling of aberrant expression of relevant downstream signaling pathways, resulting from i) expressing v-src kinase under the control of glial fibrillary acidic protein

(GFAP) gene regulatory elements (77, 146), ii) aberrantly activating the p21-RAS signaling pathway mimicking the effect of EFGRvIII mutation (147, 148) to iii) overexpression of $IDH1^{R132H}$ in the SVZ of the adult mouse brain (149).

In addition transduction of genes with viral vectors have been used to efficiently reproduced GBM development: i) retroviruses engineered to express relevant gain-of-function genes that result from overexpression of the viral oncogene v-sis, the cellular counterpart of which is c-sis or PDGF-B (73, 74); ii) adenovirus containing the EGFRvIII mutant into mice harboring activated RAS, which led to the efficient formation of glioblastoma (75); or iii) lentivirus expressing oncogenes such as HRAS or AKT (150, 151).

Models used in the study of GBM are the knockout of *p53* tumor suppressor gene harboring a conditional allele of the tumor suppressor *Nf1* (152), a model that displays an upregulation of Ras signaling; the Cdkn2a knockout mice combined with *Kras* and *Akt* upregulation by viral induction (74); the combination of both p53 and Cdkn2a knockout; the introduction of EGFRvIII or *Pten* loss in the glioma-prone mouse strain RasB8 mice with activated HRAS; the combined conditional knockout of tumor suppressors *p53, Nf1* and *Pten* genes (151).

Genetically engineered or viral vector-mediated models offer the ability to directly modify the genome of somatic cells in mouse tissues (by targeting the genomic alterations driving tumor behavior) for a rapid generation of complex mouse tumor models, that harbor specific genetic alterations providing the chance to potentially study specific drugs interfering with the function of such genes (76, 77) (**Figure 1D** and **Table 1**).

Canine Models

The use of canine models to understand GBM biology represents a good option to simulate human conditions. The incidence of brain tumors in dogs is high and its characteristics are similar to those found in human (78, 79). Also, the comparison of GBM histopathological features in dog *versus* those in murine models leads to the conclusion of dog models as a good tool to perform preclinical studies (80, 81). The advantages of using the canine models of glioma are based on the similarities with human gliomas including the presence of several neural precursor markers such as nestin (82) and the capacity to form spheres (78). In contrast, an intrinsic problem to its use is the difficulty of detecting canine brain tumors and the ethical issues involved and the need to comply with the 3R (refinement, replacement, and reduction) for animal use in experimentation (**Figure 1E** and **Table 1**).

NOVEL TECHNICAL APPROACHES IN THE STUDY OF GLIOBLASTOMA

As it can be inferred from the studies discussed above, *in vitro* cell culture systems for the study of GBM do not exactly reproduce the real conditions surrounding brain tumors and the use of animal models may not be the best approach either to reproduce the particular niche in which GBM cells reside inside a human brain. Thus, a refinement of the *in vitro* techniques was required to produce humanized GBM models based on the three-dimensional (3D) culture of GBM cells in a system that reproduced the microenvironment of human brain tumors (**Figure 2**).

2D vs 3D Co-Cultures

Studies on GBM require the culture of patient derived GBM reproducing *in vitro* the conditions that establish interactions between different types of cells and between the cells with the extracellular matrix. GBM include a combination of fibroblasts, endothelial cells, and stem cells that release signaling ligands that determinate the characteristic. One of the challenges in cell culture for GBM has been the search for coating elements that reproduce these conditions as opposed to the effect of plastic. In this context a collection of hydrogels have been produced from ECM derived polymers that replicate the GBM microenvironment such hyaluronic acid, chitosan, chondroitin sulfate polysaccharides, alginates, and collagen/gelatin proteins (153). Matrigel (a mix of mouse collagen, laminin, and ECM-associated growth factors) is a hydrogel widely used in cultures of GBM, which allows cells to grow interacting on multiple sides (154). However, these conditions do not faithfully reflect the situation *in vivo* since proper tissue architecture and cell-cell contacts may be lost in such 2D systems as well as the contacts with the extracellular matrix.

Thus, recent works aimed to produce 3D GBM structures by culturing GBM cells onto hydrogel coated 3D scaffolds in which GSC or pieces of patient derived GBM are cultured reproducing a more real tumor environment (**Figure 2A**). These systems reproduce cell growth environment of GBM cells in combination with more than one type of cell, soluble signaling factors as well as the extracellular matrix signaling. Matrigel-coated 3D polystyrene scaffolds have already been successfully used to test drug efficacy (155), morphological structures in human tumors (156) and invasion (reviewed in Caragher et al., 2019) (154). The limitations of these scaffolds are the substrate stiffness, the selection across passages of cells that attach more loosely to the scaffold, which in turn are more invasive and apparently show expression patterns more similar to GSC. Also, the mouse origin of Matrigel makes it different from human brain ECM and more humanized Matrigel is being developed (154) together with other new promising biomaterials (157).

Microfluidic Technology in Glioblastoma

GBM tumors are structures surrounded by a constantly changing microenvironment, which leads its development, however, the particular invariable conditions of the medium in 3D cultures do not reproduce this important attribute. Microfluidic technologies solve the problem of GBM cells growing in static medium (**Figure 2B**). These systems allow liquid media to be continually delivered to growing cells (158, 159). GBM primary cells can be grown in a scaffold of hydrogel tubes with circulating medium. Cells are pumped into the tubes in a solution containing brain ECM elements (160). The latest advances in

this field include several devices that allow the maintenance of tissue for 3–7 days facilitating drug tests. As an example Olubajo et al. fabricated a device using glass in a photolithographic process, getting a high percentage (61.1%) of tissue viability compared with fresh tissue at the beginning of the experiment (68.9%) (159). This approach constitutes a new way to study GBM progression since it replicates microenvironmental and extracellular conditions prevailing in the brain and facilitates the measurement of biological phenomena with high resolution and in a high-throughput manner. In this context, Ayuso et al. used a new microfluidic model in GBM to understand GBM aggressiveness related to blood vessel obstruction. They studied the area surrounding the necrotic part of the tumor usually known as pseudopalisades finding a new method to study nutrient and oxygen behavior in tumor progression and in the migratory cell response (161).

All of these findings and promising methods have several benefits such as the reproduction of the environmental conditions typical of GBM, the generation of specific targeted drug tests, and the possibility of keeping the tissue for longer periods of time. On the contrary, it entails economic and time cost and the search for the adequate materials to deliver the microfluids depending on the type of study.

Organotypic Cultures

A new but not so novel approach in the study of GBM is the use of organotypic cultures (**Figure 2C** and **Table 1**). These systems widely used in other types of studies allow the transplantation of GBM cells into brain slices that are kept alive for several weeks. This type of organotypic cultures have already been used for the study of tumor cell invasion the role of microglia in tumor growth and the niche factors governing tumor growth (83–85). In addition, microinjection of patient-derived tumor cells into cultured sections the use of human slices allows the study of GBM progression in its natural environment (86). This method enables GBM cells to be grown surrounded by cells like those in the niche of origin and therefore it is a good system to test microenvironmental interaction, however, it does not reproduce the interactions with blood flow factors or the hypoxic conditions.

Glioblastoma and Brain Organoids

Brain organoids are a promising new technology that has offered new perspective for disease modelling, including cancer, in human tissues (87, 162, 163). These brain like structures, so called "mini-brains" provide a powerful tool for the *ex vivo* study of the molecular and cellular mechanisms of human brain disorders as they can accurately represent human organ histology and physiology (87–89). Brain organoids involve the generation of 3D tissues from pluripotent stem cells, such as induced pluripotent stem cells and embryonic stem cells, or adult-tissue-resident cells, that, in a controlled environment, slowly grow and differentiate (**Figure 2D** and **Table 1**). This architecture arises from the great self-organizing ability of these cells to form whole tissues (90, 91). Brain organoids from different regions are constructed resembling their *in vivo* counterpart and recapitulating at least some functions found *in vivo*. Forebrain organoids exhibit the multi-layer progenitor zone organization that recapitulates human cortical development, including a prominent SVZ layer with radial glial cells-exclusive expression of defined molecular markers. Besides, these organoids present a diverse collection of functional neuronal and other cell types found in developing human brains. Midbrain and hypothalamic organoids from human pluripotent stem cells have also been developed showing specific neuronal markers found *in vivo* (164).

Based on this technology, several laboratories have attempted to developed GBM models using different approaches:

As a first approach GBM specimens were embedded in Matrigel and cultured in serum free conditions in the presence of growth factors EGF and bFGF. This pioneering study proved the suitability of this method to GBM studies (92). This organoid model of GBM preserved important features of the original tumor such as phenotypic heterogeneity among stem cells as well as a hypoxic gradient that regulated stem cell mitotic activity. Also, they successfully demonstrated its high tumorigenic capacity after implantation in mouse brain. As an alternative approach, da Silva et al. co-cultured human GBM spheroids with early-stage brain organoids forming a hybrid organoid with spontaneous infiltration of tumor cells into the organoid demonstrating and invasive tumor phenotype (93).

A recent study has genetically engineered brain organoids to generate a GBM model. Thus, Ogawa et al. generated a GBM model organoid by entering the HRasG12V oncogene into human brain organoids modifying the fourth exon of TP53 locus through CRISPR/Cas9. This mutated cell which profile resembles the aggressive mesenchymal subtype of GBM, proliferate and invade the normal organoid speedily. Besides, in this work they also demonstrated that primary human-patient-derived glioblastoma cell lines can be transplanted into human cerebral organoids to induce tumors (94). In a related study, Bian et al. introduce different oncogenic mutations found in GBM into human cerebral organoids to study GBM pathology and evaluate drug response (95).

Linkous et al. developed an organoid model by using patient-derived glioma stem cells and human embryonic stem cell. This model called GLICO showed that GSC move into the human brain organoid invading and proliferating within the host tissue and forming tumors that closely phenocopy patient GBMs (51). Additionally, Krieger et al., co-cultured GBM cells with hESC-organoid cells and showed that tumor cells within organoids extend a network of long microtubes, recapitulating the *in vivo* behavior of GBM. They also demonstrated that transcriptional changes implicated in the invasion process are coherent across patient samples, indicating that GBM cells reactively upregulate genes required for their dispersion (96). In a different approach, Hwang et al. generated a neuronal organoid model mimicking GBM using induced pluripotent stem cells from a patient with c-met mutation, a mutation in receptor for hepatocyte growth factor involved in the progression and aggressiveness of GBM (97).

Finally, a recent study by Jacob et al. generated and created a live biobank of GBM organoids, called GBO, from fresh tumor

without single-cell dissociation that mimic inter- and intra-tumoral heterogeneity and key aspects of their corresponding original tumors. These GBOs can be successfully transplanted into the adult mouse brain with an aggressive and fast infiltration profile and preserving original mutation expression (98). The field of GBM organoids has rapidly developed years, we have summarized in **Table 3** how this type of studies have progressed along the past four years from the first study by Hubert et al. (92) to the more recent advances.

In general, this revolutionary and developing technology reviewed by Zhang et al. (99) and summarize in **Table 3**, provided its limitations such as the lack of vasculature, immune cells or blood-brain barrier functions (100) has many advantages in GBM studies because it allows to: i) analyze the interactions between tumor and non-tumor cells (94, 95), ii) functionally analyze the consequences of genome aberrations within the same genetic background (94, 95), iii) study the interactions between tumor cells and their microenvironment, iv) test the susceptibility of individuals to different combinations of driver mutations (95, 97), and v) design personalized therapies and treatments (94, 97, 98). Related to the potential use of organoids to evaluate GBM treatment response, there are some ongoing promising trials in other cancers that demonstrated their powerful and utility. The use of this approach is especially important in cancer treatment due to the inherent resistance of cancer cells and the different response to the treatment among patients. Vlachogiannis et al. demonstrated that cultured cancer-derived organoids from patients with gastrointestinal metastatic cancers treated with a broad set of anticancer agents could retrospectively predict response with an 88% positive predictive value and 100% negative predictive value using a generalized cell viability assay (101). Several clinical studies showed the feasibility of testing patient-derived tumor organoids for evaluation of sensitivity to chemotherapy (102, 103) and radiation. Ooft et al., demonstrated that patient-derived tumor organoids predicted response of the biopsied lesion in more than 80% of metastatic colorectal cancer patients treated with irinotecan-based therapies without misclassifying patients who would have benefited from treatment (102). Therefore, organoid models are not only very useful for studies of essential tumor biology, but also they are suitable for preclinical investigations, such us drug screening and analysis of antitumor effects accompanied by a rapid and safety test in the same system (104).

3D Bioprinting in Glioblastoma

Three dimensional biological constructions (3D bioprinting) represent a new and promising method of study not only in GBM but also in other types of diseases (**Figure 2E**). Layers of biomaterials are deposited generating an extracellular matrix, which contains live cells of different types organized into a cell network resembling the real tumor in a very faithful way (105). Particularly, most studies on 3D bioprinting of GBM have been used to study the role of glioblastoma-associated macrophages (GAMs), which are key cells in tumor progression, angiogenesis and also in invasiveness (165–167). The advantage of 3D bioprinting resides in the possibility to replicate the architecture of tissues being crucial to test drugs effectively and also, fulfilling the principle of 3R of animal experimentation (106).

This technology allows the creation of a "mini-brain" in the form of 3D bioprinting model and although it is not a real representation owing to the lack of stem cells, the model includes a combination of different types of cells that are able to interact with each other. The technique is based on bioprinting a brain model using mouse macrophages which is then filled with mouse glioblastoma cells (GL261). The resulting model is adequate to test chemotherapeutic agents as well as macrophage modulating drugs (106). Other authors such as Hermida et al. used another bioprinting method to produce multilineage GBM models (109) and reveal that it is an advantageous method to test drugs and to perform cell signaling analysis using fluorescence-bound protein kinase reporters. Other authors use biomaterials such as hyaluronic acid (HA)-based hydrogels and also, synthetic polymers such as polyethylene-glycol (107, 108).

Furthermore, Tang et al. analyzed 3D printed GBM macrophages in combination with GSC alone or in combination with astrocytes and NPC. They identified molecular characteristics of GSC and evidenced the use of this type of model as an important approach for drug sensibility scanning, the study of invasion, immunologic interactions, and cellular crosstalk (110).

Advances in 3D bioprinting have represented a new vision in the discovery of effective drugs for this type of pathologies, but there are also disadvantages that must be taken into consideration. One is the materials required to print, which may not be physiological molecules. Also, it is necessary to improve the current resolution of printing in order to introduce vasculature (106). Comparing with other methods raised above, the 3D bioprinting is the most adequate when it comes to analyzing the interactions between cells and also to understand the microenvironment created within the tumor (**Figure 2E** and **Table 1**).

FUTURE DIRECTIONS

Despite the greatest advances made within the field, GBM is still a highly malignant tumor, resistant to the currently available treatments. Research in the field of GBM should not only be guided towards understanding the behavior of the tumors, but also towards the finding of a new and effective medication. Therefore, the development of experimental models in the study of GBM should be focused on those that facilitate the discovery of new and more potent therapeutic options. In this context, attention needs to be paid to therapies directed to exploiting the potential of the immune system (70, 71). Future experimental models in the study of GBM would need to allow the study of the crosstalk between GBM and the components of the immune system in order to facilitate the development of immune based therapies. In addition, techniques such as GBM organoids that allow the understanding of the individual behavior of each tumor as well as the screening of the available pharmacological options for each individual tumor would be those preferably developed in the short future.

CONCLUSIONS

As time has passed since the first discoveries and advances in the study of GBM using cultures of cells (patient-derived cells and GBM cells lines), and mouse models (xenograft, genetically engineered and viral vector-mediated transduction models) new techniques are now defining the future of GBM studies, which will probably be characterized by the use of individual organoids combined with single cell sequencing of genetic alterations to understand the processes involved in GBM origin and development. Also, the combination of different techniques, such as organotypic cultures and organoids, with 3D bioprinting could lead to an improvement in the study of cell interactions in GBM. Using all these latest scientific advances, targeted therapies can be tested and designed specifically for each patient resulting in a better prognosis and shedding light into the mechanisms controlling this devastating disease.

AUTHOR CONTRIBUTIONS

CC, PN-A, and NG-D contributed to the conception and design of the review. RG-O wrote the first draft of the review and created **Figures 1** and **2**. NG-D organized the table information. SD-G, JA-M, LC, CV, RP-D, CC, PN-A, and NG-D wrote sections of the review. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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The Role of Microglia in Glioblastoma

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Glioblastoma (GB), the most aggressive malignant glioma, is made up of a large percentage of glioma-associated microglia/macrophages (GAM), suggesting that immune cells play an important role in the pathophysiology of GB. Under physiological conditions, microglia, the phagocytes of the central nervous system (CNS), are involved in various processes such as neurogenesis or axonal growth, and the progression of different conditions such as Alzheimer's disease. Through immunohistochemical studies, markers that enhance GB invasiveness have been shown to be expressed in the peritumoral area of the brain, such as Transforming Growth Factor α (TGF-α), Stromal Sell-Derived Factor 1 (SDF1/CXCL12), Sphingosine-1-Phosphate (S1P) and Neurotrophic Factor Derived from the Glial cell line (GDNF), contributing to the increase in tumor mass. Similarly, it has also been described 17 biomarkers that are present in hypoxic periarteriolar HSC niches in bone marrow and in hypoxic periarteriolar GSC niches in glioblastoma. Interestingly, microglia plays an important role in the microenvironment that supports GB progression, being one of the most important focal points in the study of therapeutic targets for the development of new drugs. In this review, we describe the altered signaling pathways in microglia in the context of GB. We also show how microglia interact with glioblastoma cells and the epigenetic mechanisms involved. Regarding the interactions between microglia and neurogenic niches, some authors indicate that glioblastoma stem cells (GSC) are similar to neural stem cells (NSC), common stem cells in the subventricular zone (SVZ), suggesting that this could be the origin of GB. Understanding the similarities between SVZ and the tumor microenvironment could be important to clarify some mechanisms involved in GB malignancy and to support the discovering of new therapeutic targets for the development of more effective glioblastoma treatments.

Keywords: glioblastoma, microglia, signaling pathways, therapeutic target, epigenetic

INTRODUCTION

Among primary brain tumors, glioblastoma (GB) has been described as the most aggressive and is generally associated with a poor prognosis (1). GB is commonly treated with a combination of elements, starting with surgery and followed by radio- and chemo-therapy (2). However, the life expectancy of patients is reduced to approximately 15 months, and they face a high likelihood of the cancer recurring (3). New approaches for the treatment of newly diagnosed and recurrent GB such as Tumor Treating Fields (TTF), have shown a prolonged survival in these patients up to 20 months (4). In addition, GB therapies based on engineering Chimeric Antigen Receptors (CARs) have emerged as an immunotherapeutic approach with high specificity for target tumorigenic cells, but with some adverse effects that must be well defined, in order to design effective control strategies (5).

GB is classified as a grade IV glioma due to its patterns of histological necrosis and vascular changes (6). GB is composed of different types of cells, including glioblastoma stem cells (GSCs) that are responsible for tumor malignancy and expansion (7). Other types of cells that are also present in the tumor mass include NK cells, plasma cells, B cells, gamma delta (γδ) T cells, regulatory T cells (Treg), Follicular helper T (Tfh) cells, Th1, Th17, Th2, naïve CD8+ T cells, EMRA CD8+ T cells, effector memory CD8+ T cells, central memory CD8+ T cells, plasmacytoid dendritic cells, granulocytes, dendritic cells, monocytic cells, macrophages type 2 and type 1, which are the most common cells in GB (8–11). Hira et al. demonstrated that GSC niches are located close to tunica adventitia of a small subset of arterioles in hypoxic areas in GB. Thus, the hypoxic condition of GSH niches promotes the conservation of stem cells (12).

Microglia cells are the resident macrophages in the central nervous system (CNS) and could respond to tumorigenesis signaling by producing chemokines and cytokines that favor tumor progression (1, 13, 14). Glioma-associated microglia/macrophages (GAMs) are abundant in the tumor mass and favor tumor progression (15–17). In the tumor, microglia cells can polarize into two different phenotypes, the typical M1 and M2 phenotype (18). The M1 phenotype is functionally distinguished by its ability to eliminate microorganisms or tumor cells, and to secrete proinflammatory cytokines, such as IL-23, IL-12, IL-6, IL-1β, tumor necrosis factor α (TNF-α), with production of reactive oxygen species (ROS), and a low expression of IL-10 favoring the polarization of T helper cells to Th1 lymphocytes (19, 20); while M2 phenotype is characterized by a low expression of MHC-II, IL-12, IL-23 and a high expression of arginase 1 (Arg1) and anti-inflammatory cytokines, such as TGF-β and IL-10. Thus, M2 phenotype is associated with prolonged neural survival, restriction of brain damage, and prevention of destructive immune responses (21, 22). It has been shown that human GB has a heterogeneous population of M1/M2 macrophages, and M1:M2 ratio is associated with a better prognosis in IDH1 R132H wild-type GB (23). Using automated quantitative immunofluorescence Sørensen et al. found that M2-like TAMs (Tumor associated macrophages) show worse progression in high-grade gliomas and these favor a pro-tumorigenic microenvironment (24). This negative correlation was corroborated by Caponegro et al. (25) and Zhou et al. (26).

Nowadays researchers are focused on discovering the underlying mechanisms of this awful disease, understanding its biology, and researching therapeutic targets to alleviate the symptoms associated with GB, one of which could involve microglial cells' interactions within the tumor origin and the epigenetics associated therewith.

NEURAL STEM CELLS, MICROGLIA, AND GLIOBLASTOMA STEM CELLS: INTERACTIONS IN THE NEUROGENIC NICHE

Neurogenesis is the action through which the neurons are generated out of neural stem cells (NSCs). This process occurs during the embryonic stage and during adulthood where neurogenesis is relegated to two principal regions in the mammalian brain. These specific neurogenic sites are the dentate gyrus of the hippocampus (DG) and the subventricular zone (SVZ) (27, 28). However, other regions have also been described as neurogenic niches, such as the hypothalamus or the striatum in some species (29–31). The SVZ in the lateral ventricles is a neuroepithelium that contains the specific conditions to form and maintain NSCs. NSCs could be differentiated into neurons or glial cells, such as astrocytes, oligodendrocytes, and neurons (**Figure 1A**), and share some specific characteristics with astrocytes (32). NSCs, also called type B cells, embed apical processes into the cerebrospinal fluid, and at the opposite side, embed their basal processes into blood vessels, creating a unique site to drive cell fate according to environmental signaling (33). NSCs are closely related to microglial cells within the SVZ as they are the primary macrophages of the CNS (34). In fact, microglia within the SVZ show a specific morphology, differential expression of some types of receptors, as well as some differences in expression of typical microglial markers such as Iba1, which is underexpressed (35, 36). Furthermore, some studies revealed that microglia release several factors that stimulate migration (37), promote the generation of neuroblasts (38), and enhance not only neurogenesis but also oligodendrogenesis (39). In fact, microglial cells are related to synaptic connectivity, programmed cell death, and regulation of neuronal activity (40–42). All things considered; microglia are a crucial component for determination of NSC fate.

For many years, researchers focused their attention on the SVZ as a potential contributor to GB development. That is because 50%–60% of GB is related to the SVZ and is also associated with the short life expectancy typical of glioblastoma patients (43, 44). This relationship is likely to cause a multifocal diagnosis, as well as an NSC transformation to a new form of cancer cell called glioblastoma stem cells (GSCs) (45). In 2018, Lee et al. described the relationship between GB and its SVZ origin, directing their attention to GSC characteristics using

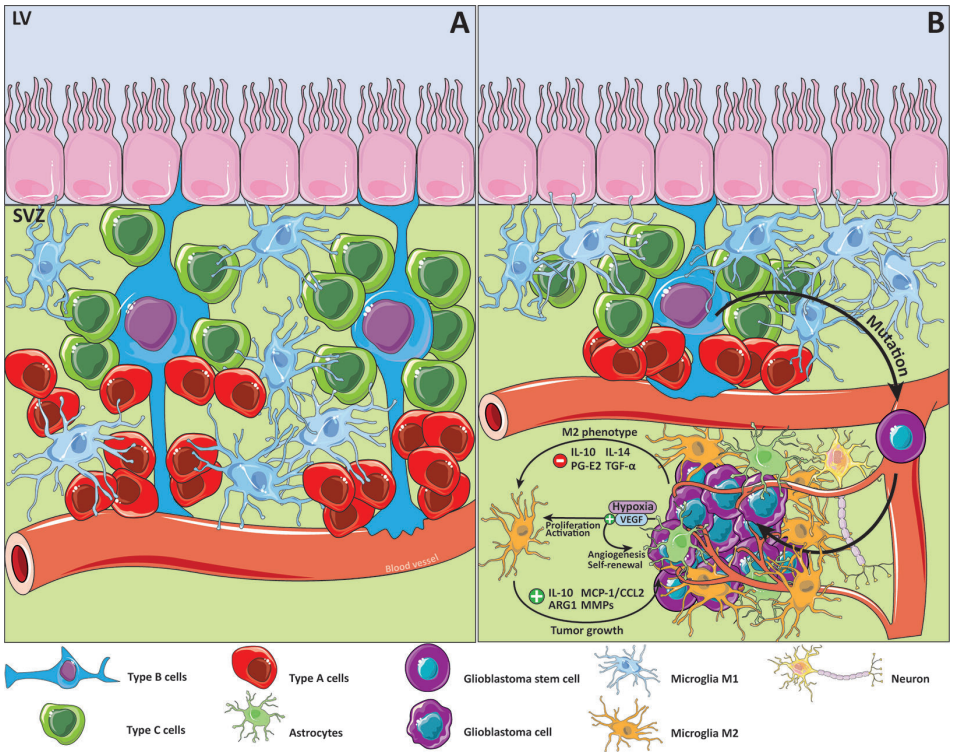

FIGURE 1 | Glioblastoma and the subventricular zone. Details of the subventricular zone (SVZ), microglia cells and its relationship with glioblastoma (GB). Type B cells, knowing as the resident neural stem cells (NSC) within the SVZ are postulated as an origin of glioblastoma stem cells (GSC) because of the accumulation of some mutations. Signaling pathways are modified in response of the changing cancer niche soluble factors that promote M2 microglial cells phenotype. **(A)** SVZ niche in physiological conditions. **(B)** SVZ niche in the GB context.

single-cell sequencing to show that some mutations could transform healthy cells into cancer cells (46) (**Figure 1B**). GSCs show similarities with NSCs, such as the capacity to form cell aggregates called neurospheres (47–49), and the expression of several markers such as nestin, Sox2, Musashi-1, or BMI1 (50, 51). It has been postulated that GSCs are responsible for resistance to medical treatments and chemotherapeutic agents such as temozolomide (TMZ) (52–55). GSCs are self-renewing and are important for other components in the tumor origin, such as the microglial cells. Glioma tissues suppress the secretion of some factors, such as TGF-α, IL-10, prostaglandin E2 and IL-14, that promote M2-like microglial phenotype polarization (56), which is implicated in some immune response downregulation processes (57). In tumor masses, M2 microglia are associated with protumorigenic activities that are capable of stimulating tumor growth through several cytokines and chemokines like IL-10, monocyte chemotactic protein-1 (MCP-1/CCL2), some metalloproteinases (MMPs), and ARG1 (13, 42, 58, 59). These factors could affect cell behavior by enhancing the crosstalk between microglia and astrocytes.

Another point associated with the tumor origin is related to the hypoxic environment. In this respect, the Vascular Endothelial Growth Factor (VEGF) is important because it induces the proliferation and activation of microglia and the neural precursor cells are involved in its secretion (60–62). Regarding GB, hypoxia induces angiogenesis and promotes GSCs self-renewal *via* VEGF secretion (63, 64).

Therefore, researchers are now focused on identifying alterations in the signaling pathways and looking for new therapeutic targets to treat GB, focusing on microglia and their relationship with the neurogenic niche.

ALTERED SIGNALING PATHWAYS IN MICROGLIA IN GLIOBLASTOMA

Various alterations have been described in GB signaling pathways that involved microglia (1). Walentynowicz et al. characterized the functional response and transcriptional activity in human and mouse microglial cultures with fresh human cell glioma–conditioned substrate. They found activated pathways related to immune evasion and TGF-β signaling (65). Brennan et al. performed a protein analysis in surgical glioma specimens to identify differential patterns of coordinated switch on between glioma-relevant signal transduction pathways, which revealed three patterns of protein expression and activation: Epidermal Growth Factor Receptor (EGFR) expression related to receptor mutation and amplification; stimulation of the platelet-derived growth factor (PDGF) pathway that is mediated by ligands; or loss of

Neurofibromatosis type I NF1 gene expression (66). In addition other researches have shown that the polarized M2 microglia induces the transcription of PDGF Receptor Beta in glioma cells and stimulates their motility capacity (67).

Furthermore, several alterations have also been described in signaling pathway of CCL2 chemokine receptor CCR2 and its major (CCL2/CCR2) GB (68). CCL2 is over-expressed in GB. Interestingly, the secretion level of this chemokine correlates with tumor grade. Glioma cells initially secrete low levels of CCL2 to chemotactically attract microglia cells, which increase CCL2 generation in the tumor environment. The amplified secretion of CCL2 by microglial cells recruits even more microglial cells into the tumor, stimulating the progression and development of the glioma (69).

Relevant findings from Hira et al. show that Mesenchymal Stem Cells (MSCs), expressing SDF-1α and OPN, capture CXCR4-CD44 positive GSCs into GSC niches and protect them from chemotherapy and irradiation (12).

Another altered signaling pathway associated with a negative regulation of T-cells, promoted by microglia, is the Programmed cell Death protein 1 (PD-1), due to the overexpression of ligand Programmed cell Death-Ligand 1 (PD-L1), in GB cells. The PD1 pathway alteration increases the possibility of PD-1/PD-L1 binding in microglia, which is associated with an increased invasion of GB cells into the brain tissue (70).

In addition, blocking the myeloid checkpoint of Signal regulatory protein alpha (SIRPα)/CD47 has shown to be efficient improving tumor phagocytosis and thus decreasing tumor burden (71, 72). SIRP-α in microglia exerts action in the neuronal CD47 to repress microglial stimulation (73). SIRP-α has a receptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic region (74) that is phosphorylated after CD47–SIRP-α interaction, promoting the binding and activation of SHP-1 and SHP-2 [(Src 2 (SH2) -like domain possessing protein tyrosine phosphatases (PTP)],which inhibits phagocytosis by preventing myosin IIA deposition at the phagocytic synapse (75, 76). Hence, the documented overexpression of CD47 in GB tumor cells (71) favors the immunosuppressive characteristics of microglia in the tumor microenvironment.

EPIGENETIC MECHANISMS IN MICROGLIA IN THE CONTEXT OF GLIOBLASTOMA

The phenotype of microglia is characterized by its own expressed gene pattern. This transcriptional signature is modified when cells are stimulated by a signal, or under pathological conditions such as GB. In this context, under homeostatic conditions, microglia have a transcriptional spectrum of expression with a main signature consisting of *P2RY13*, *TMEM119*, *CX3CR1*, *P2RY12*,*CSF1R*, *MARCKS*, and *SELPLG* genes and a diminished expression of MHC class II and lipid metabolism genes (2, 3). In the context of GB, microglia present higher expression of proinflammatory and metabolic genes, including *SPP1*, *HLA-DR*, *TREM2*, *APOE*, *CD163*, *GPR56*, and several type I interferon genes, which is substantially different from the genetic expression in homeostatic microglia (4). These changes in expression are modulated by epigenetic mechanisms that regulate the accessibility of genetic loci to transcriptional machinery, gene expression levels, and chromatin architecture without altering the sequences in the DNA (5). This can be demonstrated by treatment with Valproic acid, which inhibits class I HDAC catalysis, promotes proteasomal hydrolysis of HDAC2 and primary adult human microglia, and decreases phagocytosis and levels of PU.1 and CD45, indicating that the regulation of the phagocytic activity of the microglia is carried out by epigenetic mechanisms (6). These HDAC inhibitors (HDACi) have a proapoptotic effect on cancer cells, which involves the interruption of the mitochondrial membrane potential and the increasing of acetylation in the protein histone H3 (7).

Global hypomethylation has been reported in 80% of GB (8), showing intratumoral DNA methylation heterogeneity (9). DNA methylation is closely related to the response to temozolomide (TMZ) treatment, with O6-methylguanine-DNA methyltransferase (MGMT) being the only predictive biomarker for a patient's response to first-line chemotherapy with TMZ (10). Hypermethylated CpG in the promoter of the connexin 30 (*Cx30*) gene have also been establish in grade III and IV GB, but not in grade I and II gliomas. This hypermethylated region is related to Sp1 and Ap2 expression factor recognition sites and it is correlated with progressive downregulation of *Cx30* mRNA and with the degree of GB (11).

MiR-138 has been found to effectively inhibit cell division in GB *in vitro* and tumorigenicity *in vivo* by arresting a transcription factor EZH2–mediated signaling loop (12). Inhibition of EZH2 in GB decreases the transcription of M2 profile and increases the expression of M1 related proteins in microglia cells (13). We still have a long way to understand the role that epigenetic modifications play in microglia in GB, but research effort is focused on bringing light to this issue (**Figure 2**).

A detailed view of gene expression of microglia under homeostatic conditions versus GB, supports the understanding of the dysregulation processes in this disease, and could help to find new GB therapeutic targets.

MICROGLIA AS A THERAPEUTIC TARGET FOR GLIOBLASTOMA

Tumor-associated microglia have been shown to be a key therapeutic target in GB (14) since microglia cells decline in animal experimental models reduces tumor growth (15). Thus, therapies based on microglia as a target could complement the treatments currently used against this disease. Among the molecules that block microglial/macrophages' infiltration of GSC-derived tumors, the integrin inhibitor arginine-glycine-aspartic acid (RGD) peptides have been shown to interfere with GSC-secreted periostin, thereby these peptides could suppress tumor growth and augment survival of GB-bearing animals (16).

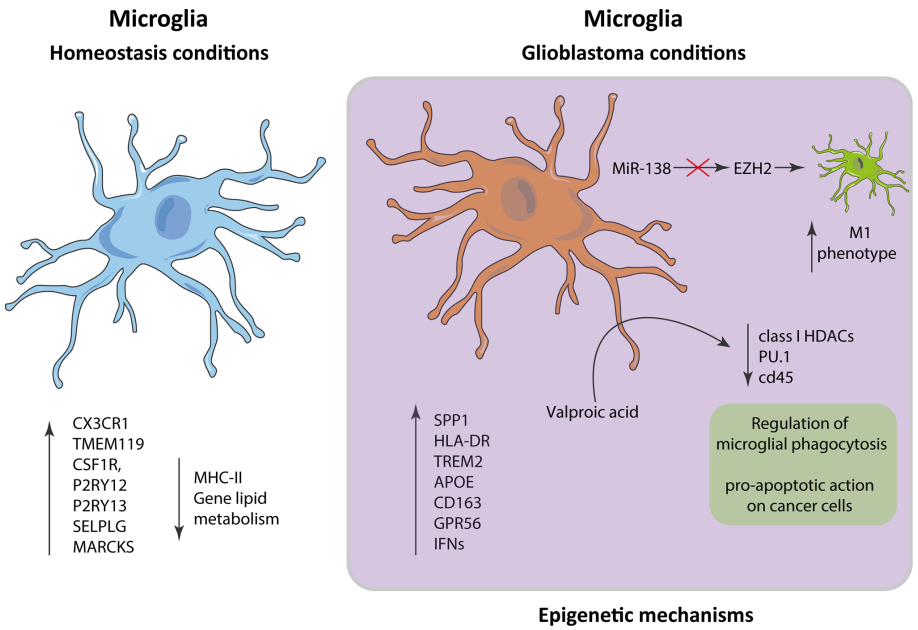

FIGURE 2 | Alterations in microglia in the context of glioblastoma. The gene expression patterns in microglia in homeostatic conditions vs glioblastoma differ significantly, presenting in the latter an inflammatory pattern characterized by an increase in the expression of *SPP1*, *HLA-DR*, *TREM2*, *APOE*, *CD163*, *GPR56*, and interferons.

Another strategy employed promotes the antitumor activities of GAMs. For example, in an experimental model of GSC tumors derived from humans implanted in non-obese mice with combined diabetic/severe immunodeficiency (NOD-SCID), it was shown that systemic administration of amphotericin B (AmpB) significantly reduces tumor growth and increases the chances of survival. In animals treated with AmpB, a greater tumor penetration of M1 macrophages and microglial cells was found (77), showing a significant positive regulation of iNOS, and resulting in a higher production of cytotoxic nitric oxide (NO). In these same experiments, positive effects of AmpB it was also found in immunocompetent C57BL/6 mice against very aggressive tumors from stem-enriched CD133 + GL261 glioma cells (17).

Additionally, mTORC plays a key role in the integration of c-MET and PDGFRα signal transduction that are co-activated with EGFR in the context of GB, and it has been shown that inhibition of mTOR activity in rat microglial cells can promote its antitumor properties while restricting its protumorigenic characteristics. Therefore, mTOR inhibitors have the potential to attack both glioblastoma and the protumor functions of GAMs (78–81).

Intracranial injection of a viral recombinant adeno-associated vector (rAAV2) expressing IL-12, induce an increased level of IL-12 in tumor-bearing animals, contributing to microglial penetration in the tumor and reactivation of GAMs' protective effects. This immunological reactivation of GAMs significantly decreases tumor growth and prolongs animal life (19).

An oncolytic virotherapy using Herpes simplex virus type 1 (HSV-1) has been authorized by the FDA for cancer therapy after the optimum completion of clinical trials (20). In GB, the antitumor efficacy of oncolytic HSV-1 (oHSV-1) is determined, in part, by the amount of microglia/macrophages that phagocytize viruses with the ability to express reporter genes. Thus, viral replication was inhibited, forming an unpermissive OV barrier, and avoiding the spread of oHSV-1 in the glioma mass. The decrease in viral replication, in microglial cells, was related to the suppression of some viral genes by phosphorylation of STAT1/3, responsible for suppressing oHSV-1 replication in microglia/macrophages (21). Together, these strategies employ microglia as a promising therapeutic target in treating glioblastoma.

Microglia are executors of the innate immune response and are specialized in sensing and eliminating abnormal cells, however these cells can change their phenotype and become tumor-promoting cells due to the influence of tumor signals. As part of the tumor mass, tumor-associated macrophages (TAM) are interesting therapeutic target based on data that have shown that the antiphagocytic protein CD47 is increased on the surface of cancer cells, allowing them to evade the innate immune system To avoid the interaction of CD47 with SIRP-α, it is used an anti-CD47 monoclonal antibody (mAb). In microglia cells, anti-CD47 could prevent the expression of their protumorigenic phenotype and turn them into a potential weapon, to arrest GB progression (72).

Stupp et al. In a study with 695 patients with glioblastoma who have completed their initial radio-chemotherapy, the combination of tumor treatment fields (TTFields) with maintenance chemotherapy using alkylating agent TMZ demonstrated a statistically significant improvement with a median overall survival of 20.9 months in this group vs. 16 months in the temozolomide-only group (HR, 0.63, 95% CI, 0.53–0.76, P <0.001) (4).

Pang et al. demonstrated the ability of macrophages as cell carriers of drugs. Culture of RAW264.7 cells in presence of LPS and IFN-γ, shown that these molecules bind to Toll-like receptor 4 and the IFN-γ receptor respectively, activating and promoting the exocytosis of the drug loaded by these cells. Thus, they propose the use of patient-derived M1-type macrophages loaded *in vitro* with the drug of interest, and then transferring them back to the patient to treat GB (82).

CONCLUSION

Microglia and TAMs comprise up to 30% of cells in the brain tumor environment (56, 83–88). Microglia cells in the CNS are keys regulators of homeostasis, but their function in immunological surveillance of glioma cells remains little known. Tumor cells, through the expression of different surface and secreted molecules, modulate the phagocytic activity of microglia by altering various signaling pathways and epigenetic mechanisms. Therefore, the modulation and reeducation of the set of microglia constitute a promising antitumor strategy against glioblastoma.

AUTHOR CONTRIBUTIONS

NG-D and EN conceptualized the study. IS-G and CF contributed to the methodology. LG and IS-G conducted the formal analysis. RN conducted the investigation. EN provided the resources. NG-D and EN was in charge of the data curation. RN, CF, NG-D, and EN wrote and prepared the original draft. RN, AS, CF, LG, EP, IS-G, NG-D, and EN wrote, reviewed, and edited the manuscript. IS-G and NG-D created the cartoon in **Figures 1** and **2**. All authors contributed to the article and approved the submitted version.

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Human Cerebrospinal Fluid Modulates Pathways Promoting Glioblastoma Malignancy

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Glioblastoma (GBM) is the most common and devastating primary cancer of the central nervous system in adults. High grade gliomas are able to modify and respond to the brain microenvironment. When GBM tumors infiltrate the Subventricular zone (SVZ) they have a more aggressive clinical presentation than SVZ-distal tumors. We suggest that cerebrospinal fluid (CSF) contact contributes to enhance GBM malignant characteristics in these tumors. We evaluated the impact of human CSF on GBM, performing a transcriptome analysis on human primary GBM cells exposed to CSF to measure changes in gene expression profile and their clinical relevance on disease outcome. In addition we evaluated the proliferation and migration changes of CSF-exposed GBM cells *in vitro* and *in vivo*. CSF induced transcriptomic changes in pathways promoting cell malignancy, such as apoptosis, survival, cell motility, angiogenesis, inflammation, and glucose metabolism. A genetic signature extracted from the identified transcriptional changes in response to CSF proved to be predictive of GBM patient survival using the TCGA database. Furthermore, CSF induced an increase in viability, proliferation rate, and self-renewing capacity, as well as the migratory capabilities of GBM cells *in vitro*. *In vivo*, GBM cells co-injected with human CSF generated larger and more proliferative tumors compared to controls. Taken together, these results provide direct evidence that CSF is a key player in determining tumor growth and invasion through the activation of complex gene expression patterns characteristic of a malignant phenotype. These findings have diagnostic and therapeutic implications for GBM patients. The changes induced by CSF contact might play a role in the increased malignancy of SVZ-proximal GBM.

Keywords: glioblastoma, cerebrospinal fluid, cancer progression, tumor stem cells, brain tumor, subventricular zone

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INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive type of primary brain cancer in adults, accounting for 54% of new gliomas and 45% of primary malignant tumors (1). In the United States alone, 15,000 people die and 26,000 new cases are detected annually (2) with an estimated economic burden exceeding US$300 million per year (3). Survival expectancy of a patient suffering from GBM averages 14 months, despite the most advanced therapeutic strategies combining surgery, chemotherapy, and radiation (4, 5). Invariably, GBM will

eventually reoccur in almost 100% of cases, due to the highly invasive nature of the tumor that makes complete resection impossible and the presence of a subpopulation of cells called brain tumor initiating cells (BTICs) (6). These cells exhibit neural stem cell (NSC) properties, such as self-renewal and the ability to differentiate into defined progenies (7, 8). BTICs are also more resistant to chemo- and radio- therapy, and if not completely removed during surgical resection, have the capacity to generate new tumors (9).

Tumor location greatly influences the prognosis of GBM patients. More than half of all patients with GBM have tumors that touch the lateral ventricle or even reach into an important brain neurogenic region known as the subventricular zone (SVZ) (10–15). These patients have significantly worse outcomes in terms of median overall survival, time to progression, and recurrence (3, 12).

The cause for worse outcome for patients suffering from SVZ-infiltrating GBMs is not known. Although it is tempting to speculate that the neurogenic characteristics of the SVZ are the underlying causes of this clinically observed phenomenon. The SVZ is the largest neurogenic niche in adults and is highly regulated by the flow of cerebrospinal fluid (CSF). The CSF milieu has the properties to sustain the neurogenic niche environment, regulating neural stem cells proliferation, differentiation, and migration (13, 16–18). Given the neurogenic potential that CSF holds on NSCs and the similarities between NSCs and BTICs, we explored the possibility that CSF might similarly affect GBM proliferation, differentiation, and migration.

The role of CSF in modulating the aggressiveness of GBM, or other gliomas for that matter, is largely understudied in the field of neuro-oncology. In this work we have taken on the hypothesis that CSF contact enhances GBM malignant characteristics and we have resolved to study the effects of CSF exposure on human GBM-derived BTICs. Our study evaluated CSF impact on GBM gene expression profile as well as cell proliferation and migration *in vitro* and *in vivo*. We observed that CSF is an important contributor to tumor growth and invasion through the activation of gene expression patterns characteristic of a malignant phenotype.

MATERIALS AND METHODS

Primary-Cultured BTICs and Human CSF Collection

Under Mayo Clinic institutionally-approved protocol we established primary BTIC cultures from tumor tissue from patients undergoing surgical resection for newly diagnosed GBM without prior treatment as described previously by our group (4, 19). Clinical data for primary BTICs and CSF samples used in this study are described in detail in **Supplementary Table 1**.

Primary-cultured BTICs derivation and culture protocols were performed as previously described (20, 21). Briefly, intra-operative brain tissue was chemically and mechanically dissociated in Accutase® and cell number and viability were determined by trypan blue exclusion. Cells were maintained in media composed by DMEM/F12 (Invitrogen) supplemented with 2% GEM21 Neuroplex (Invitrogen), 1% antibiotic/antimycotic (Invitrogen), 20 ng/mL human EGF (Peprotech) and 20 ng/mL FGF (Peprotech). Cells were maintained in suspension to evaluate their neurosphere formation and pluripotency *in vitro*. Tumor initiation potential was determined *in vivo* by orthotopic implantation in immunosuppressed mice (J:Nu, Jackson Labs).

For CSF collection and processing, samples were obtained intra-operatively upon opening of the dura mater. CSF samples were spun at 200 × g for 5 min at 4°C, filtered by 0.45 μm to eliminate cells and debris, and immediately aliquoted and stored at −80°C until use.

For CSF stimulation, BTICs were cultured on laminin-coated plates. Twenty-four hours prior to experimentation, cells were maintained in base media (without EGF and FGF) to avoid confounding effects. Protein concentration in CSF was measured and CSF was utilized at a 1:100 dilution in base media, unless otherwise stated. CSF samples were matched based on gender and age, specifically cCSF73 was matched to ncCSF12 or ncCSF1276 and cCSF-37 to ncCSF25. Cells were maintained under CSF stimulation in incubation conditions for 24 h or as required for analysis.

RNA Expression Microarray

Cells were grown in 25 cm^2 flasks until 80% confluency was reached, then treated with cancer or non-cancer CSF (CSF was pooled from 3 cases each). Cells were incubated in CSF or control conditions for 24 hrs. Upon incubation, cells were centrifuged and RNA was extracted. Samples were assessed for RNA quality and quantity using a 2100 Bioanalyzer (Agilent) with a RNA 6000 Nano Chip (Agilent) and diluted to a final concentration of 50 ng/μl. All samples used in this study had RIN values >9.2 μg of RNA were submitted for microarray using the Illumina Human HT-12 v4 chip. The scanned images of the microarrays (.dat files) were processed using Illumina's GenomeStudio software. The probe level intensities were quantile normalized across samples with background subtraction. Probes with detection p-values > 0.05 in all samples were excluded. All samples had >40% probes expressed and passed this step of QC. In addition, all samples passed the Illumina's internal QC threshold of signal intensity ratios between 5′ vs. 3′ probes targeting house-keeping genes. The bimodal distributions of the normalized and log2 transformed probe intensities were plotted to determine the threshold of expression as 6.6 (data not shown). Genes with average expression values in all experimental groups below the threshold of expression were filtered out. The principle component analyses (PCA) as well as the unsupervised hierarchical clustering analyses of all samples using the remaining 21,023 probes identified two outlier samples which were consequently removed for the following analysis of variance (ANOVA) and weighted gene co-expression network analysis (WGCNA). Source of variance analyses identified CSF treatment, cell line, and CSF treatment/patient interactions as main contributors to variance, and were included in the ANOVA model. Pair-wise group comparisons were also performed to obtain fold change and p-values between any two groups.

Weighted Gene Co-expression Network Analysis and Pathway Analysis

WGCNA (22) was performed to identify modules of highly correlated genes. Briefly, the gene co-expressions were calculated using Pearson Correlated Coefficient to the power of 6 ($\beta = 6$) optimized for discoveries of scale-free topology. A signed hybrid Topological Overlap Measures (TOM) matrix were calculated to reduce sporadical correlations between gene-pairs. In addition, modules whose eigengenes highly correlated with treatment groups were tested for enrichment of known functional gene sets using Broad's Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb).

Gene ontology (GO) and pathway analysis were performed on differential gene expression (DEG) results using Metacore (Clarivate Analytics) and Ingenuity Pathway Analysis (IPA, Qiagen). DEGs were defined as genes with a Fold change (FC) higher than 1.2 or lower than −1.2 and adjusted p-value < 0.05. Canonical pathways were assessed for significant enrichment and directionality of effect utilizing a z score $> \pm 2$ and p-value < 0.05 (right-tailed Fisher's exact-test) on IPA. Network Analysis was also performed on Metacore, using Analyze Networks (AN) algorithm (default settings) to generate a list of biological sub-networks highly enriched and unique for the uploaded data. In this workflow the networks are prioritized based on the number of fragments of canonical pathways on the network, ranked by p-value, G-score and interpreted in GO terms. Transcriptional regulators of DEG genes were also identified with MetaCore Interactome. Transcription factors (TF) are ranked according to their Z-score (the level of connectivity of the TF to the DEG list). Larger Z-scores represent higher levels of connectivity between the transcription factor and the DEG list.

Molecular and Clinical Data Collection and Patients Survival Analysis

Pre-processed and normalized gene expression data was retrieved from GlioVis portal (http://gliovis.bioinfo.cnio.es/) (mRNA expression from the Affimetrix HT Human Genome U133 array). Unsupervised hierarchical clustering of both genes and samples was performed using Partek Genomics Suite (https://www.partek.com/partek-genomics-suite/, St. Louis, Missouri). The quantile normalized and log2 transformed expression values were used for unsupervised hierarchical clustering of both samples and probes. Pearson Correlation Coefficient was used as the distance metric between samples and genes. The distance between two clusters is defined as the average of distances between all pairs of objects using the average linkage method. The expression values of each gene across samples were standardized to mean of 0 and scaled to standard deviation of 1. Clinical data collection for overall survival and disease/progression free was obtained from the GBM provisional cohort in the cBioPortal for Cancer genomics (https://www.cbioportal.org).

Cell Viability and Migration Assay

Cell culture growth and viability was evaluated by alamarBlue (Invitrogen). GBM cells were seeded at 2.000 cells/well in a laminin-coated 96-well plate ($n > 4$ per condition) and incubated overnight to allow for attachment in base media. A 10 μL aliquot of alamarBlue reagent was added to the wells containing 100 μL of base growth media with or without treatments, and cells were incubated for at least 4 h at 37°C. Fluorescence was measured using a plate reader (Ex 540–570 nm, Em 580–610 nm) at 24, 48, 72 h after CSF stimulation.

Cell migration response to CSF stimulation was evaluated by transwell and gradient migration assays. For transwell migration, we utilized a modified Boyden chamber. Fifty thousand cells were seeded in culture inserts with an 8 μm pored permeable membrane in 3 replicates per condition, to allow migration to the bottom compartment. CSF was applied to the bottom compartment. Chambers were maintained for 24 h in incubator conditions. Non-migrated cells were removed from the upper compartment and migrated cells were stained with DAPI, and counted at 10X magnification from nine different fields by an independent observer.

For gradient migration assay, cells were plated on a glass bottom multiwell plate (ibidi™) coated with poly-L-ornithine solution (0.01%, Sigma-Aldrich) and laminin. A 2×10^5 cells/ml suspension was plated per chamber and allowed to adhere overnight in base media. CSF was applied on one side of the chamber to establish a CSF gradient. Time-lapse of cell migration was recorded using an inverted microscope with environmental chamber. 10x images were acquired every 10 min for 24 h and processed with Zeiss ZEN Blue software. At least 3 time-lapse videos were collected per conditions. Cells were tracked (at least 30 cells/video) using ImagePro Software (Media Cybernetics).

In vitro Extreme Limiting Dilution Assay

The assay was performed as previously described (23). Briefly, cells were seeded at 1, 5, 10, or 50 cells/well in a laminin-coated 96-well plate and incubated overnight to allow for attachment in base media. The following day, attached cells were counted and treated with CSF. Sphere formation was monitored over a period of 14 days and each well was quantified and scored thereafter. Colonies measuring 250 μm or above were counted as positive. A semilogarithmic plot was generated of the fraction of negative cultures also referred to as "non-responding" (i.e., wells lacking spheres) as a function of the dose of cells placed in each culture. Results were analyzed using the online software tool at the following website from Walter and Eliza Hall Institute of Medical Research: http://bioinf.wehi.edu.au/software/elda/.

Cell Proliferation

Nuclear Ki67 expression was evaluated to determine cells in active proliferative phases. Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, and blocked for 1 h in PBS containing 0.1% Triton X-100 (Sigma-Aldrich) with 10% goat serum prior to overnight incubation with anti-Ki67 (RM-9106-s1; Thermo Fischer Scientific, 1:500). Alexa 594-labeled secondary antibody (Invitrogen, 1:500) was used for visualization, and DAPI was used to counterstain cell nuclei. Slides were visualized and recorded with an inverted fluorescence microscope and the number of Ki67+/DAPI cells was counted in at least 8 randomly selected fields at 20x per slide. For mouse tissue, after fixation in 4% paraformaldehyde and

paraffin embedding, brains were sliced at 5 μm, deparaffinazed and treated for antigen retrieval in citrate buffer for 30 min, followed by primary antibody staining as described above.

Cyclin D1 expression was measured by real-time quantitative PCR, after 24 h exposure to CSF. Total RNA was isolated using TRIzol (Invitrogen) and the RNeasy mini kit (Qiagen) following the manufacturer's instructions. Reverse transcription was performed using Superscript III (Invitrogen) and real time quantitative PCR was performed on a Quant Studio 3 (Applied Biosystems) with Power SYBR Green PCR Master Mix. Relative quantification of mRNA expression was calculated by the ΔΔCT method after adjusting the levels to the corresponding internal GAPDH control for each sample. Primers sequences were as follows: human GAPDH sense: 5′-AGGTCGGTGTGAACGGATTTG-3′, antisense 5′-TGTAGACCTGTAGTTGAGGTCA-3′; human Cyclin D1 sense 5′-CAATGACCCCGCACGATTTC-3′, antisense 5′-CATGGAGGGCGGATTGGAA-3′.

Orthotopic Tumor Implantation

All animal procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines. Athymic nude mice (J:Nu, Jackson Laboratories) were housed three to five per cage and maintained on *ad libitum* access to food and water with a 12 h light/dark cycle. All animals were used for study between 8 and 15 weeks of age.

Glioblastoma cells GBM1A (previously known as GBM 0913) originally established by Vescovi et al. (7) and previously characterized by our collaborators were used for the *in vivo* studies (7, 24–26). To identify GBM cells in our *in vivo* mouse experiments, we transduced the cells with lentiviral vectors coding for Green Fluorescence protein/Luciferase (GFP-Luc) proteins (RediFect^{TM} Red-FLuc-GFP, Perkin Elmer cat: CLS960003). One week post-transduction, cells were sorted by flow cytometry to select GFP-expressing cells. Cells were washed in PBS and resuspended in PBS before injection to remove any trace of growing media.

We performed intracranial injection as described previously by our group (18, 19, 27). Mice were anesthetized using isoflurane and immobilized in a stereotaxic apparatus. 1×10^5 GBM1A GFP-Luc cells were resuspended in 3 μl of CSF (or PBS for controls) plus 2 μl 0.2% Pura Matrix (Corning) and injected intracranially into the right hemisphere (coordinates from bregma in mm: Y: 0.86; X: 2; and Z: −3). Injection was performed with an automatic system at a 0.5 μl/min rate and injection needle was left in place for an extra minute to reduce backflow. Injection needle was then retrieved and skin incision was closed with surgical glue. Isoflurane was discontinued, and the animal placed on a heating pad. All mice completely recovered within 5 min. Buprenorphine (10 mg/kg) was used as pre- and post-operative analgesic.

Mice were randomized into 3 experimental groups of 8 animals each: (1) control GBM1A coinjected with PBS and Pura Matrix, (2) GBM1A coinjected with cancer CSF and Pura Matrix, and (3) GBM1A coinjected with non-cancer CSF and Pura Matrix. Tumor formation and growth were followed by bioluminescence (BLI) every week. Mice were euthanized 4 weeks after injection. Brains were fixed using transcardiac perfusion, and post-fixed overnight at 4°C in 4% paraformaldehyde.

Tumor volume was calculated performing a morphometric analysis of brain sections that presented tumor. The morphometric volume (MFV) determination was done using the Cavalieri principle (28), which allows an accurate estimation of the volume of a structure independently of its shape and size, estimating the surface area (A) of a number (n) of parallel sections spaced at a constant distance (t), using the following equation: $est\ (V) = t\ ^*\ (A1 + A2 + A3 + ...\ An)$. Three 5 μm brain slides per tumor sectioned at 50 μm intervals were used for this calculation.

Bioluminescence Imaging

In vivo bioluminescence images of tumor-implanted mice were obtained using the IVIS Spectrum System (Perkin Elmer) that has a cooled CCD camera to capture images of animals and tissues in a light-tight box. D-luciferin (XenoLight D-Luciferin—K+ Salt Bioluminescent Substrate 15 mg/ml, Perkin Elmer) was injected intraperitoneally at a dose of 10 mg/kg and allowed to distribute for 5 min. Mice were then anesthetized using isoflurane and imaged in prone position. Imaging times ranged from 5 s to 5 min, depending on the total tumor burden as a function of light emission from tumor cells. Region of interest analysis was performed using Living Image Software.The mean ± SD light emission over the time was plotted for each brain tumor engrafted mice. Bioluminescent signal was normalized and analyzed as the absolute total flux (photons/steradian/cm^2). Mice were imaged at 1, 2, and 3 weeks after tumor engrafting.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8. Multiple comparisons analysis was determined by a one-way ANOVA, followed by Tukey *post-hoc* analysis. Results represent the mean ± SEM of four replicates in three independent experiments unless stated otherwise. Statistical significance is represented by $*p<0.05$, $**p<0.01$, $***p< 0.001$.

RESULTS

Pathways Analysis Indicates That CSF Modulates Aggressive Features of GBM

In order to establish the extend of the effects of CSF contact on GBM, we performed a transcriptome analysis in CSF-treated GBM cells. To the best of our knowledge, there is not data to date on CSF induced transcriptomic changes in GBM cells. To identify common properties of CSF derived from brain cancer compared with non-cancer patients, we have pooled in this study CSF derived from 3 brain cancer patients (high grade gliomas) and CSF from 3 control patients (hydrocephalus) (**Figure 1A**).

Of the 47,323 probes initially detected, only 21,023 probes remained after filtering and these were used for differential expression analysis (**Figure 1B**). Consensus module Eigengene clustering confirmed that cancer CSF and non-cancer CSF exposure induces the segregation of GBM samples into intrinsically different subsets, with the control group naturally

A

Sample	Age	Sex	Diagnosis
GBM276	53	F	GBM - Mesenchymal
GBM612	56	M	GBM - Proneural
CSF-1211	70	M	GBM - cCSF
CSF-1221	45	M	GBM - cCSF
CSF-1267	43	M	GBM - cCSF
CSF-1174	52	F	NPH - ncCSF
CSF-1245	78	M	NPH - ncCSF
CSF-1276	67	F	NPH - ncCSF

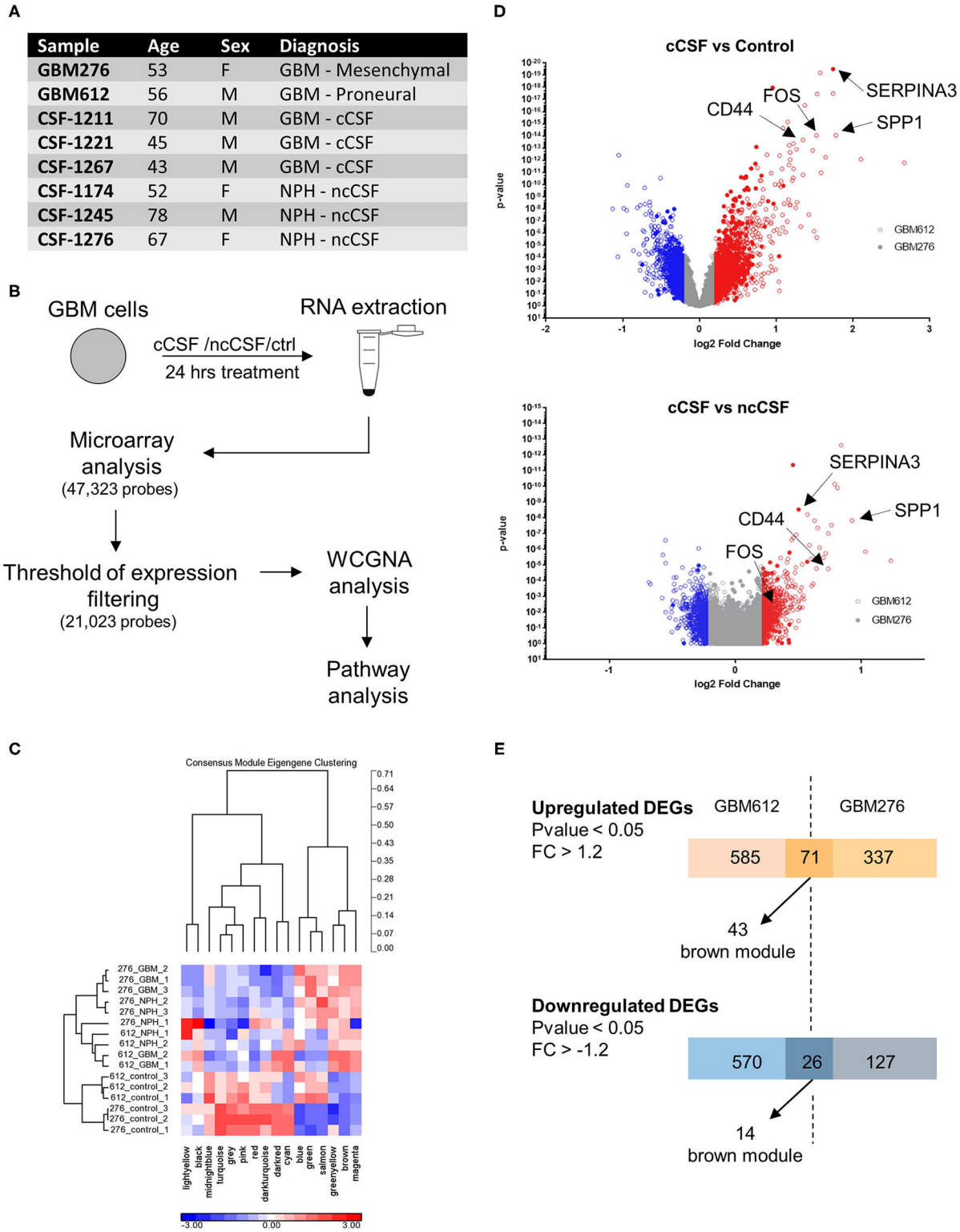
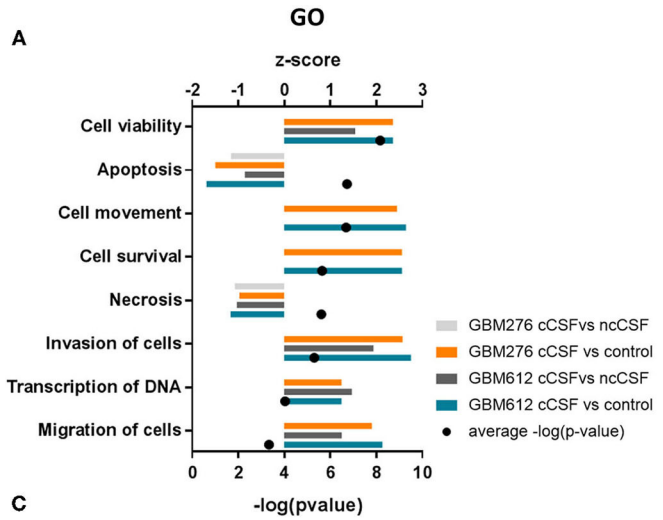
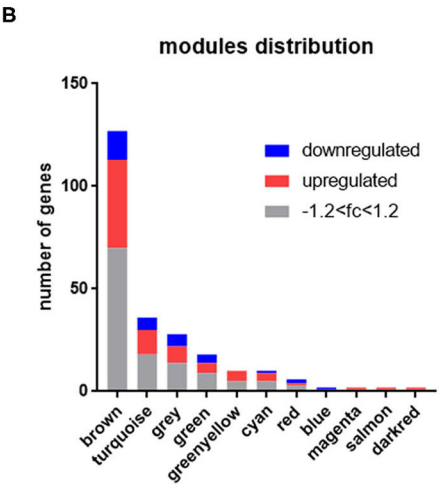


FIGURE 1 | RNA expression microarray WGCNA analysis. **(A)** Demographic data of samples utilized for RNA expression analysis. **(B)** Schematic of the experiment. Cells were cultured for 24 h in the presence of cCSF or ncCSF then processed for RNA extraction and microarray analysis. **(C)** With a Weighted Gene Co-expression Network Analysis (WCGNA) we identify 16 modules of highly correlated genes, of which 10 reached significancy. **(D)** Volcano plot showing the expression levels of the transcripts derived from the 2 cell lines analyzed. GBM276 is represented by diamonds, GBM612 is represented by circles. **(E)** Venn diagrams showing unique and common DEGs between the 2 BTICs cells used for analysis (GBM612 and GBM276). GBM, Glioblastoma; cCSF, cancer derived CSF; ncCSF, non-cancer derived CSF; NPH, normal pressure Hydrocephalus; M, male; F, female.





C

BROWN MODULE TOP GENES		GBM276				GBM612			
		cCSF vs Ctrl		cCSF vs ncCSF		cCSF vs Ctrl		cCSF vs ncCSF	
	GENE	FC	p-value	FC	p-value	FC	p-value	FC	p-value
top up GBM276	SERPINA3	3.344	3.1E-20	1.417	2.9E-09	1.254	1.4E-05	1.225	1.7E-04
	AGT	1.803	2.8E-07	1.238	1.5E-02	-1.359	2.6E-03	1.073	4.9E-01
	WARS	1.752	1.2E-05	1.250	3.6E-02	1.935	6.1E-06	1.318	3.4E-02
	PTP4A3	1.663	2.0E-10	1.115	2.8E-02	1.477	1.5E-07	1.214	2.4E-03
	ALDH1A3	1.658	1.9E-12	1.123	3.9E-03	-1.111	1.6E-02	1.014	7.5E-01
top up GBM612	SPP1	1.172	1.5E-02	1.031	6.1E-01	3.430	8.7E-15	1.902	1.4E-08
	CEBPD	1.327	5.4E-07	1.097	3.3E-02	3.347	3.1E-18	1.755	1.2E-10
	FOS	1.374	1.3E-04	1.045	5.2E-01	3.128	5.6E-13	1.253	1.3E-02
	S100A3	1.193	8.1E-06	1.117	1.5E-03	2.976	6.4E-20	1.791	2.3E-13
	CCL2	1.262	1.3E-01	1.052	7.4E-01	2.883	2.2E-06	1.208	3.1E-01
top down GBM276	STC1	-1.349	4.9E-05	-1.040	5.1E-01	1.475	7.0E-06	1.070	3.7E-01
	EPHB1	-1.439	2.5E-05	-1.160	4.1E-02	-1.983	8.8E-09	-1.335	2.3E-03
	MYC	-1.461	7.7E-07	-1.011	8.5E-01	-1.279	6.8E-04	-1.031	6.6E-01
	DLL3	-1.469	3.9E-08	-1.106	4.4E-02	-1.634	4.0E-09	-1.147	2.6E-02
	NINJ1	-1.487	1.2E-06	-1.106	1.1E-01	-1.417	3.3E-05	-1.115	1.5E-01
top down GBM612	HEY2	1.415	9.5E-07	1.232	5.4E-04	-1.706	5.0E-09	-1.132	6.3E-02
	SERPINE2	1.085	1.3E-01	1.016	7.6E-01	-1.917	1.1E-10	-1.489	2.2E-06
	PDLIM3	-1.103	2.8E-01	1.039	6.7E-01	-1.932	1.1E-06	-1.495	1.2E-03
	EPHB1	-1.439	2.5E-05	-1.160	4.1E-02	-1.983	8.8E-09	-1.335	2.3E-03
	LEFTY2	-1.054	5.1E-01	-1.064	4.4E-01	-2.188	9.9E-09	-1.410	1.8E-03

D

Most Relevant Networks					
No	Network name	Processes	p-Value	zScore	gScore
1	TNF-R1, Tissue factor, SPP1, RelA (p65 NF-kB subunit), FAK1	response to lipid (69.8%), response to oxygen-containing compound (79.1%), positive regulation of cell migration (51.2%), positive regulation of cell motility (51.2%), positive regulation of cellular component movement (51.2%)	2.53e-09	14.07	67.82
2	Ephrin-B receptor 1, NRCAM, ALDOC, RPL22, Podocalyxin-like 1	locomotion (56.0%), chemotaxis (42.0%), taxis (42.0%), cell migration (46.0%), cell chemotaxis (28.0%)	9.70e-15	20.66	21.91
3	VPS37B, TESC, B4GT5, Factor I, SP1	sulfur compound biosynthetic process (24.0%), carbohydrate metabolic process (32.0%), sulfur compound metabolic process (28.0%), N-acetylglucosamine metabolic process (12.0%), regulation of behavior (18.0%)	1.34e-12	17.91	17.91

FIGURE 2 | Pathway Analysis of DEGs. **(A)** Top gene ontology processes for DEGs common to both lines tested. Bars represent z-scores for each pairwise comparison, dots represent –log(*p*-value) averages of the 4 pairwise comparisons. **(B)** Distribution of DEGS in WGCNA modules, with the brown module containing most of the significant DEGs defined by *p*-value <0.05. Red indicates upregulation of fold change +1.2, blue indicates downregulation of fold change −1.2. **(C)** Top 5 up or down-regulated DEGs in brown module for GBM276 and GBM612 with relative fold change (FC) and *p*-value in each pairwise comparison. Common DEGs are represented in red. **(D)** Most relevant networks for common DEGs in the brown module using Metacore algorithm Analyze Networks. FC, fold change; FDR, false discovery rate.

being the most distant cluster (**Figure 1C**). Pair-wise group comparisons were also performed to obtain fold change and *p*-values between any two groups. We then analyzed the expression fold change of the GBM-CSF treated samples compared to controls (using a threshold of fold change ±1.2 and *p*-value < 0.05), and we found 1,252 differentially expressed genes (DEGs) in the GBM612 sample (of which 656 upregulated, 596 downregulated) and 561 DEGS in the GBM276 sample (of which 408 upreguated, 153 downregulated) (**Figure 1D**), among these, 97 DEGs were shared between the 2 different GBM primary cultures (71 upregulated, 26 downregulated) (**Figure 1E**). Most importantly, when we performed a gene ontology (GO) analysis on the common DEGs for pathway enrichment we observed processes involved in regulation of cell invasion, apoptosis, survival, and transcription of DNA (average $p < 0.001$) (**Figure 2A**), all processes that support the clinical observations of SVZ-proximal GBM being more malignant than the distal counterpart.

WGCNA Analysis Reveals the Presence of a Biologically Significant Module for GBM Progression

Global analysis of the entire dataset by WGCNA highlighted the relevance of one module (brown module, 828 genes) containing the highest number of differentially expressed transcripts common to both cell lines (**Figures 1E**, **2B**). WGCNA modules group genes based on similar expression profiles that are highly connected and these genes often also share similar biological functions. The brown module contained 127 common DEGs, of which 43 upregulated and 14 downregulated by cancer CSF exposure in both cell lines. The top 5 upregulated genes in the brown module for GBM276 and GBM 612 were SERPINA3, AGT, WARS, PTP4A3, ALDH1A3, and SPP1, CEBPD, FOS, S100A3, CCL2 respectively. The top 5 downregulated genes in the brown module for GBM276 and GBM 612 were respectively NINJ1, DLL3, MYC, EPHB1, STC1, and LEFTY2, EPHB1, PDLIM3, SERPINE2, HEY2 (**Figure 2C** and **Supplementary Figure 1A**). The enrichment analysis of the entire brown module in Metacore revealed GO and process networks terms involved in angiogenesis, cell adhesion and integrin signaling, cell proliferation and response to inflammatory stimuli, again suggesting the role of CSF in promoting GBM aggressive characteristics (**Figure 2A** and **Supplementary Figures 1B,C**) The unique genes from the top 15 pathways in the brown module are listed in **Supplementary Table 2**. Using the Analyze network algorithm in Metacore, we have identified the most connected items in the brown module collected in 3 main networks as shown in **Figure 2D**. These biological networks again confirmed the positive regulation of cell migration by CSF, as well as metabolic and biosynthetic processes which are also affected during tumorigenesis. Upstream regulator analysis using MetaCore showed key transcription factors whose targets are overrepresented in the differentially expressed gene lists, that are involved in the regulation of the pathways mentioned above, such as MYC, STAT3, and FOS, which were also found upregulated in our dataset (**Supplementary Table 3**).

The results of this analysis summarize the complexity of functions regulated by the CSF in the contest of GBM pathophysiology.

CSF-Induced Gene Expression Signature Determines Patients' Clusters With Different Survival Patterns

In order to establish the clinical relevance of the gene signature induced by CSF in GBM cells *in vitro*, we have attempted to identify patient subgroups in the TCGA database that carry this particular gene expression profile. We have selected DEGs from **Figure 1D** (cCSF vs. control analysis) with a *p*-value < 0.05 and a FC > 2 in either cell line analyzed and generated a list of 35 upregulated genes to represent the CSF-induced gene signature in GBM. Gene expression measurements for the entire dataset in the TCGA GBM provisional cohort were retrieved from GlioVis portal (http://gliovis.bioinfo.cnio.es/). We then performed independent hierarchical clustering of both genes and patient samples and we generated 11 patient clusters with different transcriptomic profiles (**Figure 3A**). We next determined whether these clusters in the TCGA data were associated with differences in survival outcomes. Kaplan-Meier (KM) analyses revealed the existence of a cluster (Cluster 8) with significant longer overall survival (OS) and disease/progression free survival (DFS) (**Figure 3B**). Cluster 8 patients show an overall reduced expression of the 35 up-regulated signature genes, which all in concert contributed to the clinical outcome differences (**Supplementary Figure 2**). The genes with the most significantly low expression (compared to the TCGA cohort) were EMP ($p < 0.0001$), NNMT ($p < 0.0001$), CCL2 ($p = 0.002$), GBP1 ($p = 0.0004$), RCAN1 ($p = 0.0044$), SERPINA3 ($p = 0.0075$), ZFP36 ($p = 0.018$), CEBPD ($p = 0.0423$), and TNC ($p = 0.0462$).

CSF Derived From GBM Patients Induces an Increase in BTIC Proliferation

To validate the results obtained from the pathway analysis, we next evaluated whether CSF is capable of modulating growth, migration, and stem cell features of GBM-derived BTICs. We have assessed proliferation and viability of 2 different BTICs lines by Alamar blue assay upon exposure to human CSF samples (**Figure 4A**). Both treated cell lines showed significantly different viability in response to the two types of CSF, with the cancer CSF (cCSF) inducing a greater viability rate when compared to non-cancer CSF (ncCSF) and control ($p < 0.01$). This response was proved to be dose-dependent (data not shown). Interestingly, GBM1A and GBM965 showed maximum response at different concentrations, with the GBM1A line being the most susceptible to respond to treatment (**Supplementary Figure 3A**). No evident morphological differences were observed in GBM cells after exposure to CSF of either origin (**Supplementary Figure 3B**). Single pairs of cCSF and ncCSF, matched on age and gender, were used for these arrays. When comparing CSF samples from several patients, we consistently observed very mild effects

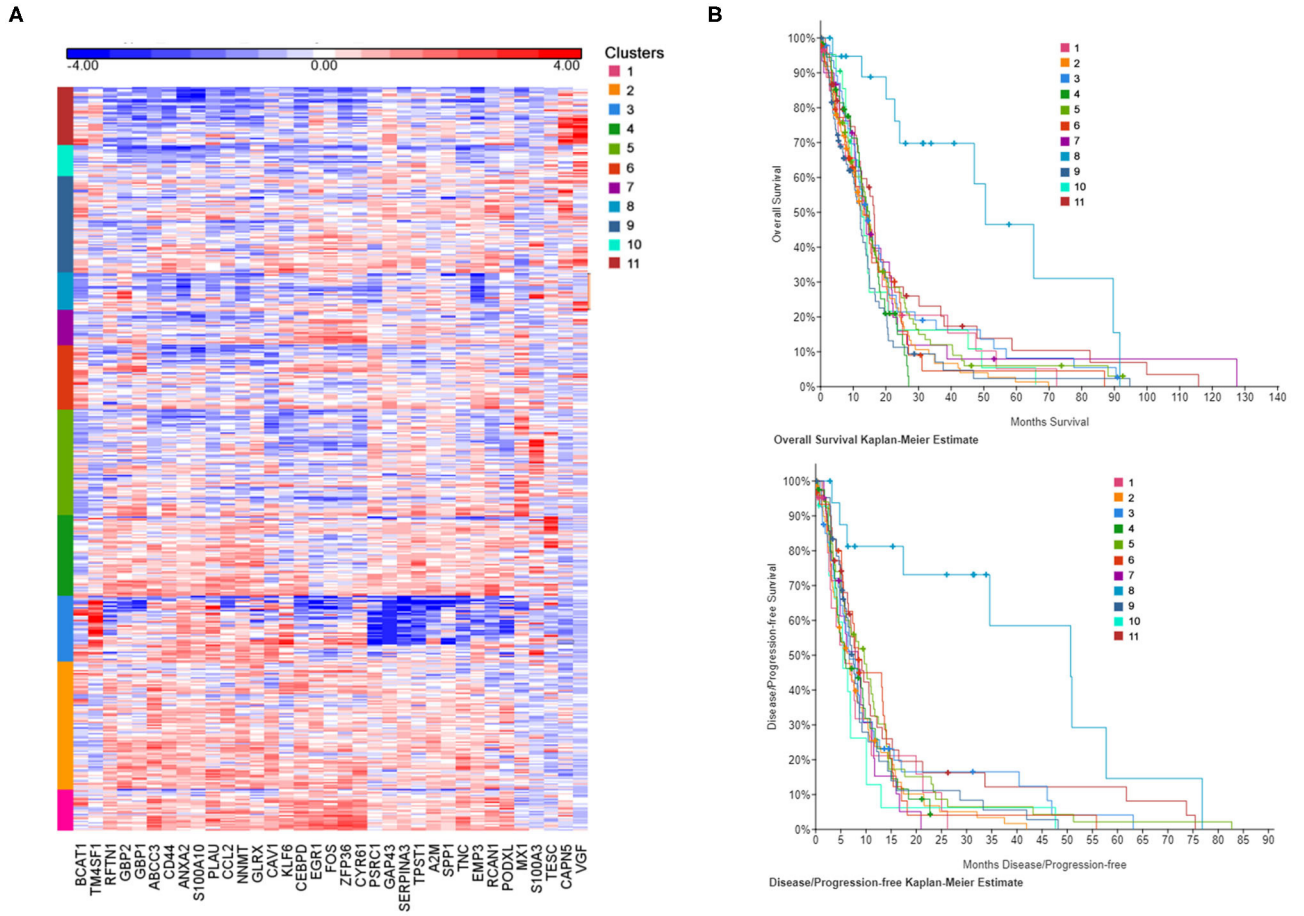


FIGURE 3 | CSF-induced gene signature influence survival in GBM patients. **(A)** The heat map represents each expression component. Each row represents a patient sample and each column a gene selected for a CSF-induced signature in GBM *in vitro*. For each patient, red indicates upregulation, blue indicates downregulation. Clusters (1–11) are color coded and represent gene-specific expression patterns. **(B)** Overall survival and disease/progression free Kaplan-Meier plots for the clusters derived by CSF-induced gene expression show a distinct cluster (8) with significant better clinical features.

in response to ncCSF in GBM lines, in contrast to cCSF (**Supplementary Figure 3C**).

The increase in proliferation rate induced by cancer CSF was also substantiated by immunohistochemical staining for Ki67, which labels actively proliferating cells. We observed a significantly higher percentage of cells Ki67 positive compared to the other treatments ($p < 0.01$, **Figures 4B,C**). We also measured a significant increase in mRNA levels of Cyclin D1, an important cell cycle regulator, required to overcome the restriction point in the G1 stage of the cell cycle in cCSF-treated BTICs compared to controls ($p < 0.01$, **Supplementary Figure 3D**).

Furthermore, a significant increase in self-renewing capacity was observed in both cell lines following cancer CSF exposure using ELDA, as cCSF treated BTICs produced more readily and bigger colonies than in any other condition (cCSF vs. control $p = 0.0008$, cCSF vs. ncCSF $p = 0.0425$, ncCSF vs. control $p =$ n.s.) (**Figure 4D**). Taken together, our results demonstrate that CSF obtained from GBM patients promotes tumor cell proliferation and self-renewal capacity in GBM cells *in vitro*.

CSF Derived From GBM Patients Induces an Increase in BTIC Migration

Here we evaluated the cell migration response of BTICs to human CSF by transwell assay and video-microscopy on a 2D surface during 24 h as described previously (17, 29). GBM-derived CSF treated cells showed an increase in their transwell migratory capacity compared to the other treatments ($p < 0.05$, **Figure 4E**). We also followed cell migration in response to a CSF gradient, in a µSlide IV (IBIDI) for 24 h using timelapse microscopy. This assay confirmed that cCSF enhances migration in GBM cells. Cell migration distance was highest in cCSF-treated BTICs ($p < 0.05$, **Figure 4F** and **Supplementary Figure 4A**). Speed was also significantly increased by CSF compared to control ($p < 0.0001$, **Supplementary Figures 4B,C**). CSF from both sources appeared to have a repellent effect on GBM cells inducing migration in the opposite direction of the gradient (**Supplementary Figures 4D,E**). These results demonstrate that CSF can enhance the migratory capabilities of GBM-derived BTICs *in vitro*.

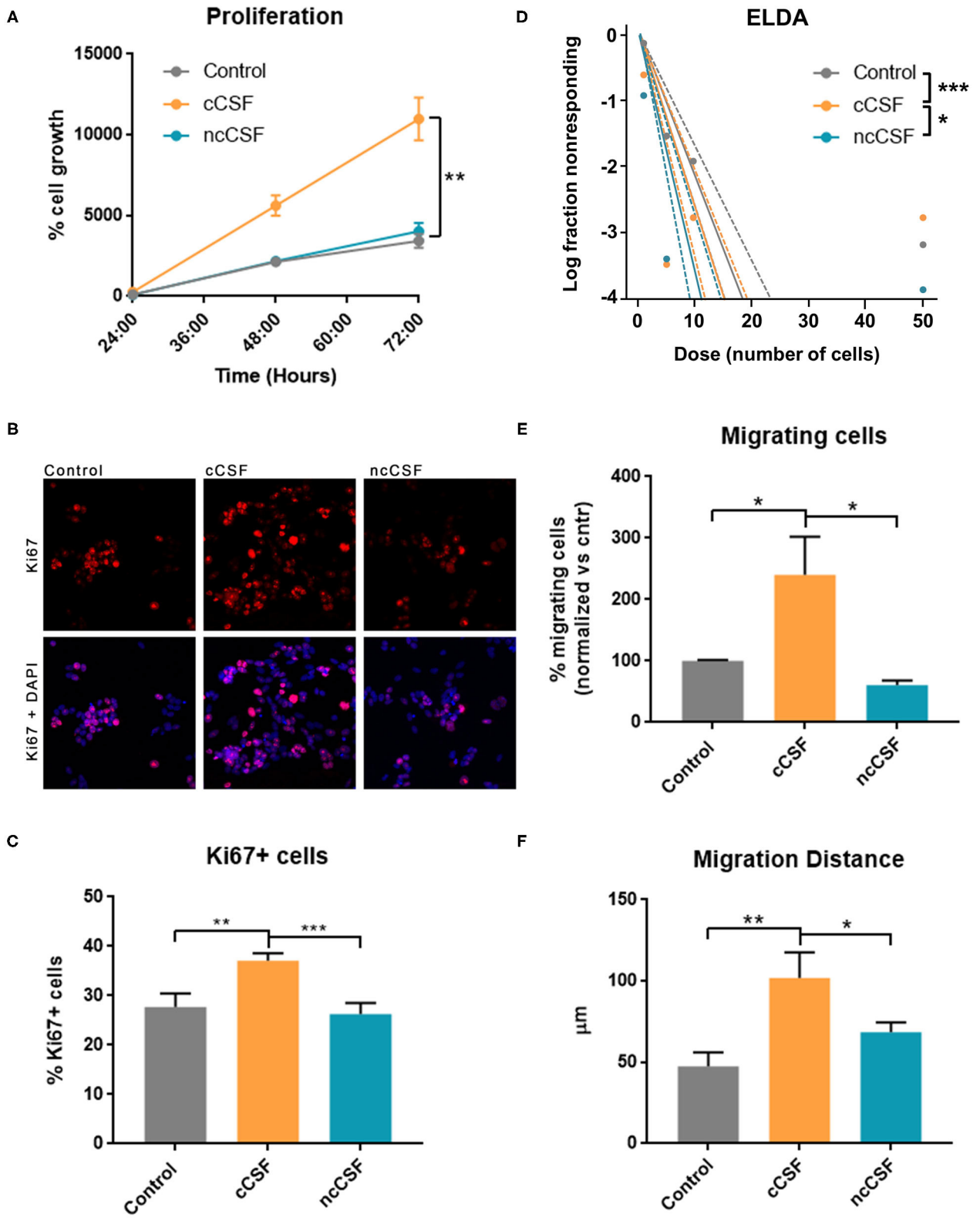


FIGURE 4 | CSF derived from GBM patients induces an increase in BTIC proliferation and migration *in vitro*. GBM cells were treated for 24 h with either glioma derived CSF (cCSF) or non-cancer (ncCSF). After incubation time cells were processed for analysis. **(A)** Alamar blue assay tracked cells proliferation over time (graph shows growth between 24 and 72 h post-treatment) revealing an increase proliferation rate in the cCSF treatment group (CSF pairs: cCSF73/ncCSF12)

(Continued)

FIGURE 4 | (B) Immunohistochemistry evaluation for proliferation marker Ki67 shows a predominant presence of positive cells in the cCSF treated groups, confirmed by quantification in **(C)** (cCSF on GBM1A: 151.38% increase vs. ncCSF, $p = 0.0002$; 130.33% increase vs. untreated, $p = 0.0004$; cCSF on GBM965: 189.28% increase vs. ncCSF, $p = 0.01$; 104.70% increase vs. untreated, $p =$ n.s.) (CSF pairs: cCSF73/ncCSF1276). **(D)** Extreme Limited Dilution Assay (ELDA) determines cells' self-renewal by measuring their ability to form colonies when seeded at a very sparse density. When cells were incubated with cCSF, their propensity to generate colonies was higher compared to the other treatments. *p*-Values: Control vs. ncCSF $p = 0.175$, Control vs. cCSF $p = 0.000882$, cCSF vs. ncCSF $p = 0.0425$ (CSF pairs: cCSF73/ncCSF12). **(E)** Transwell migration assay allows to quantify the number of cell that migrate through a porous membrane. Cells treated with cCSF demonstrated a higher tendency to migrate when compared to untreated and ncCSF. (250% increase cCSF vs. ncCSF, $p = 0.0429$; 157.84% increase cCSF vs. control, $p =$ n.s.) (CSF pairs: cCSF37/ncCSF25). **(F)** GBM cell migration in a 2D gradient, cells exposed to cCSF migrated longer distances. (215.24% increase cCSF vs. control $p = 0.004$; 149.04% increase cCSF vs. ncCSF $p = 0.04$, ncCSF vs. control $p =$ n.s.) (CSF pairs: cCSF73/ncCSF12). Scale bars = mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Graphs are representative of gender/age matched pairs of CSF samples.

CSF Promotes GBM Growth *in vivo*

The effects of CSF, from cancer or non-cancer source, on GBM have never been reported *in vivo*. In order to determine whether the *in vitro* effects of CSF on BTIC proliferation and migration persist when cells are in the brain microenvironment, we tested using a human orthotopic GBM model in mice (30). To evaluate the effect of CSF *in vivo*, we injected GBM cells encapsulated in a CSF-containing hydrogel (Pura Matrix). PuraMatrix is a non-toxic, biodegradable, and biocompatible hydrogel composed by 16 amino acids (RADA16; AcN-RADARADARADARADA-CONH2). This hydrogel mimics several properties of the natural extracellular matrix in which cells can easily proliferate, differentiate, and migrate (31, 32) and, in our case, allowed the creation of a CSF-enriched microenvironment surrounding the implanted BTICs. The use of a hydrogel allowed the CSF to remain in contact with the GBM cells. Importantly, xenographs were generated at a location distal to the SVZ or the subgranular zone. This location allowed us to avoid any interference of the mouse neurogenic niche proximity on our readouts (**Figure 5A**). A single pair of age/gender matched CSF samples was used for this study (cCSF73/ncCSF1276). Bioluminescence imaging, collected throughout the experiment, demonstrated an increased tumor growth in the group co-injected with cCSF compared to vehicle and CSF from control patients (**Supplementary Figure 5A**). Upon euthanasia, we observed differences in tumor volume between our groups as identified by H&E staining (**Figure 5B**) and immunohistochemistry against eGFP (**Supplementary Figure 5B**). Brain histology showed cCSF co-injected tumors were significantly larger compared to the other 2 groups (cCSF 345.45% larger than PBS, $p = 0.0169$, cCSF 493.12% larger than ncCSF, $p = 0.0089$, ncCSF vs. PBS $p =$ n.s). Interestingly, female mice developed larger tumors than their male counterparts (**Figure 5C**). In addition, immunohistochemistry against Ki67 revealed again a higher proliferative rate in cCSF-coinjected mice (cCSF vs. control $p < 0.001$, cCSF vs. ncCSF $p < 0.0001$, ncCSF vs. control $p =$ n.s.) (**Figure 5D**). No differences were observed between genders in Ki67 positivity. Our results demonstrate that cCSF alone exacerbates the progression of GBM tumors *in vivo*.

DISCUSSION

In this work we first demonstrated by transcriptomic analysis that CSF induces changes in pathways regulating apoptosis, survival, cell mobility, angiogenesis, response to inflammatory stimuli and metabolism. Based on these results, we identified a brain cancer CSF-induced gene signature linked to survival outcome in GBM patients in the TCGA database. We then further validated our findings *in vitro* and *in vivo* confirming that CSF exposure to GBM-derived BTICs affects their malignant phenotype. Our functional experiments showed that CSF modulates the most critical features of aggressive behavior in cancer cells: proliferation, invasiveness and tumor initiation.

As briefly mentioned earlier, clinical outcome for GBM tumors is strongly influenced by their location in the brain (3, 12). It is thought that tumors arising close to the SVZ (SVZ+) in the lateral ventricles (LV), have a much more aggressive profile than SVZ-distant tumors (SVZ–) (11). SVZ+ GBMs recur at distant locations of the brain with a higher incidence than SVZ-distal tumors and within a shorter period of time (14, 33), this could be related to an increased in cell migration due to the closeness to the CSF as seen in our results. Moreover, studies evaluating survival suggest that patients with SVZ+ tumors tend to have a decreased overall survival and a worse prognosis, which could be related to an increase in cell proliferation and migration combined, due to CSF contact. A study conducted by Chaichana K, et al. in 2008, in which 26 out of 69 patients presented with contrast-enhancing lesions in close contact to the LV, presented a lower median overall survival when compared to patients with SVZ– GBM, 8 vs. 11 months respectively (12). These results were further confirmed by other studies, demonstrating that regardless of tumor size, patient's characteristics and extent of resection, contact to the SVZ represents an independent factor for survival (33, 34).

Altogether, these characteristics support the theory that SVZ+ and SVZ– GBMs carry a different biological behavior, concluding that SVZ+ GBM has a much more aggressive profile with a worse overall prognosis for patients. The prognostic difference could theoretically stem from intrinsic characteristics of the tumor, or from its SVZ-contacting or SVZ-distal location. At this time, there is no conclusive evidence that links the clinical features of SVZ+ GBM to a defined molecular subtype or other intrinsic tumor characteristic (35). Although some have shown a differential distribution of GBM subtype in preferential brain regions (36), a recent work found no differences in molecular signature in tumor bulk tissue from SVZ+ or SVZ– tumors (37). This furthermore suggests that SVZ proximity does not lead tumors to evolve toward a certain molecular subtype or another, nor selects for survival of an intrinsically more aggressive cellular subtype. All of this evidence supports the conclusion that discrepancies in clinical outcomes are not due

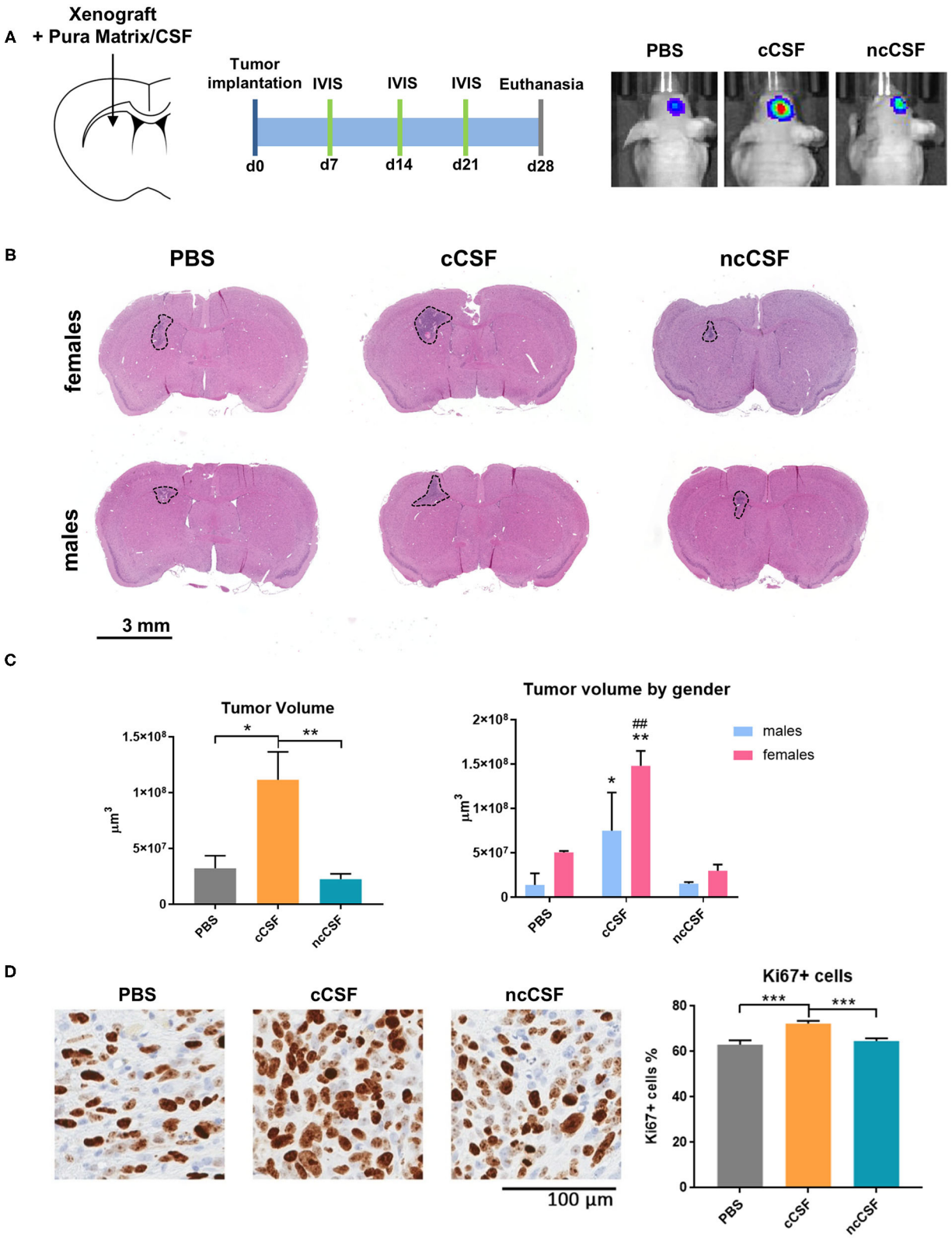


FIGURE 5 | CSF promotes GBM growth *in vivo*. **(A)** Schematic representation of the *in vivo* study design, showing injection site and timeline of the experiment. **(B)** Implanted tumor size. Mice receiving GBM cells co-injected with cCSF harbored larger tumors compared to the other groups. Interestingly, female mice developed bigger tumors than males. **(C)** Quantification of the tumor volumes overall and segregated by female and male mice in the different treatment groups. (*$p < 0.05$, **$p < 0.01$ for cCSF vs. PBS; ##$p < 0.05$ for cCSF vs. ncCSF). **(D)** Immunohistochemistry staining for Ki67 in xenograft. A higher percentage of KI67+ cells were observed in the cCSF coinjected cohort. Scale bars = mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

to intrinsic characteristics of the tumor as a whole, but rather to the environment of its location (38), i.e., the resident SVZ cell population (NPCs) (39) and, importantly, as supported by our results, the CSF and its components, creating a permissive tumor-supportive environment in which any GBM subtype thrives. Studies using directed biopsies in SVZ+ GBM patients and possibly single cell analysis, although often technically very challenging, would be necessary in the future to further dissect the local changes induced by the brain environment.

We have shown here that pathways pivotal for cancer development are indeed modulated by exposure to CSF, particularly when derived from cancer patients (which has obvious implications in the context of tumor recurrence), as regulation of genes involved in cell proliferation, migration, angiogenesis, metabolism and inflammatory responses was observed in our microarray analysis. Most of these pathways converge upstream on the activation of common master regulator genes, such a STAT3, MYC, and FOS which have been proven consistently to be involved in tumorigenesis (40), although have never been studied in the context of or linked to SVZ proximity or CSF exposure.

One of the most significant pathways identified by our analysis involves the upregulation of STAT3 and SERPINA3, which could both be induced by the presence of cytokines of the IL-6 family in the CSF (41). These cytokines are involved in a variety of biological activities such as inflammation, remodeling of extracellular matrix and modulation of cell growth and differentiation via the induction of important regulator elements of these processes, such as C/EBPD, VEGF, Cyclin D1, Matrix metallopeptidase 1 (MMP1) and TIMP metallopeptidase inhibitor 1 (TIMP-1). SERPINA3, not only can be induced by cytokines through STAT3 (42), but itself could be mediating and/or enhancing the same processes as an extracellular soluble protein present in the CSF (43, 44). Converging on the same pathways, we have identified the upregulation of CD44, which may be mediated by the binding of one of its ligands SPP1 (the expression of which is also induced by CSF in our study) and/or MIF (both cytokines present in the CSF), with the coincidental phosphorylation of ERK1/2, c-Jun and c-Fos. This would lead to the induction of a plethora of processes involved in oncogenesis, including proliferation, migration and angiogenesis, all driven by the interaction of GBM cells with CSF. It is possible that the presence of other cytokines in the CSF, highlighting here a crucial role for the inflammatory response, can sustain the overexpression of SERPINA3, among other genes, and together concert the buildup of a tumor supportive environment. Although hypothesizing the identity of such possible modulator candidates is fascinating, it goes beyond the scope of this study; here we have not conducted a systematic analysis of CSF molecules, and we particularly wanted to focus on the effects elicited by CSF, and the downstream targets of this interaction, on GBM cells and GBM cells behavior. The leads generated by this study are presently investigated in our laboratory, we have recently proven in details the role of SERPINA3, one of the top DEGs identified by this analysis, in enhancing GBM tumorigenesis (44).

Consistent with the fact that the SVZ is the largest neurogenic niche in direct contact with the CSF, and with the above clinical features of GBM, our work suggests a pivotal role for CSF in GBM malignancy and progression. As we have shown here, the gene expression changes induced by CSF in GBM cells have important repercussion for the clinical outcome of these patients: the 35 CSF-induced signature genes that we used in our analysis, showed an average lower expression in patients with a significantly better survival. This suggests that modulation of targets of CSF components could contribute significantly to disease outcome. It is crucial for future studies to investigate the nature of the CSF components responsible for the changes observed in GBM cells that we have reported here, and identify the upstream regulators of such tumorigenic effects. Such CSF components could be soluble proteins or ligands present in suspended cells or exosomes. Previous reports have found both populations of shed tumor cells as well as immune cell sets in the CSF of GBM patients. However, it has been described that the lymphocyte population of the CSF in malignant brain tumors has a far fewer percentage of T cells than non-tumoral neurosurgical disorders, implicating depressed cell-mediated immunity of the cells in the CSF (45). Additionally, recent research has identified an exosome-contained protein, LGALS9, in GBM patient CSF that binds to the TIM3 receptor of dendritic cells, inhibiting antigen recognition and presentation. This ultimately leads to failure of the cytotoxic T-cell-mediated anti-tumor immune response (46). Therefore, because the immune cells within the CSF of GBM patients are already immunosuppressed and are unable to respond to tumor antigens, we believe that the response of GBM cells would likely be very similar to the unfiltered CSF as to the filtered CSF used in our study.

An interesting and quite surprising observation in our *in vivo* study was the increased tumor size in female mice compared to males. This is also somewhat in contrast with the clinical observation that GBM is more prevalent and more severe in male patients than females (47). Due to the fact that no significant differences were observed in the proliferation marker Ki67, our results may reveal an ulterior role for either CSF and/or recipient gender in tumor implantation survival rather than solely proliferation. On the other hand, the factors influencing this phenomenon could be various, and not controlled for in this study, among which the original gender of both GBM cells and CSF donors and the gender of the xenographs recipients, and their participation and contingent role in GBM progression should all be further elucidated.

The relatively small number of samples in our study is a limitation due the heterogeneity of GBM and the impact that age, gender, and disease stage have on CSF composition. Future studies with a wider cohort of cases are needed to corroborate our findings and investigate the specific contribution of sex, age, tumor location, and molecular status in the interplay between CSF and GBM progression.

To summarize here we have proven that:

1) CSF alone enhances proliferation and tumor initiating properties of GBM cells;

2) CSF promotes GBM cells migration and invasiveness, regardless of LV proximity;
3) CSF affects GBM cells behavior, *in vitro* and *in vivo* and it alters GBM gene expression to induce a malignant signature with relevant clinical implications.

That CSF modulates GBM is extremely important in explaining what we observe in clinical settings of increased aggressiveness of those tumors close to the SVZ. Components of the CSF, once identified, should be the target of the next class of drugs used to treat this disease.

DATA AVAILABILITY STATEMENT

The data presented in this study are deposited in the GEO repository, accession number GSE161528.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Mayo Clinic Internal Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Mayo Clinic Institutional Animal Use and Care Committee.

AUTHOR CONTRIBUTIONS

AC, KC, AQ-H, YA, and HG-C: conceptualization. AC, NZ, JP, ML-V, PS-M, KC, AQ-H, and HG-C: methodology. AC, NZ, JP, ML-V, PS-M, EN, YA, and HG-C: data analysis. AQ-H, YA, and HG-C: supervision. AQ-H and HG-C: funding acquisition. All authors have reviewed, edited, read, and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.624145/full#supplementary-material

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REVIEW
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Aberrations of Genomic Imprinting in Glioblastoma Formation

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In human glioblastoma (GBM), the presence of a small population of cells with stem cell characteristics, the glioma stem cells (GSCs), has been described. These cells have GBM potential and are responsible for the origin of the tumors. However, whether GSCs originate from normal neural stem cells (NSCs) as a consequence of genetic and epigenetic changes and/or dedifferentiation from somatic cells remains to be investigated. Genomic imprinting is an epigenetic marking process that causes genes to be expressed depending on their parental origin. The dysregulation of the imprinting pattern or the loss of genomic imprinting (LOI) have been described in different tumors including GBM, being one of the earliest and most common events that occurs in human cancers. Here we have gathered the current knowledge of the role of imprinted genes in normal NSCs function and how the imprinting process is altered in human GBM. We also review the changes at particular imprinted loci that might be involved in the development of the tumor. Understanding the mechanistic similarities in the regulation of genomic imprinting between normal NSCs and GBM cells will be helpful to identify molecular players that might be involved in the development of human GBM.

Keywords: genomic imprinting, glioblastoma, neural stem cells, methylation, subventricular zone

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GENOMIC IMPRINTING AND GENE DOSAGE CONTROL

Genomic imprinting is an epigenetic process in which a small group of genes, called imprinted genes, are expressed depending on their parental origin (1–3). Whereas non-imprinted genes express both copies contained on homolog chromosomes, in imprinted genes either the maternal or paternal copy is expressed thus bypassing mendelian inheritance laws (4, 5) (**Figure 1A**). Therefore, parental genomes are not functionally equivalent due to genomic imprinting, implying that both genomes are required for normal mammalian development (6, 7). To date, around 200 imprinted genes have been described in mice (8) and more than 150 in humans (9, 10). Although imprinted genes represent <1% of total genes in the mammalian genome, they play important roles in different biological processes such as embryonic and placenta growth, fetal development and adult metabolism (11–13).

Most imprinted genes are grouped in clusters (3, 14) and it has been postulated that a *cis* regulatory DNA element could regulate the expression of all genes contained in the same cluster (4). Indeed, imprinted gene expression is known to be co-ordinately controlled by epigenetic mechanisms, being DNA methylation the most important one occurring in specific genomic regions enriched in cytosine and guanine dinucleotides (CpG) (15). These regions, known as

imprinting control regions (ICRs), are differentially methylated (DMRs) exhibiting a specific parental methylation pattern (2, 14) (**Figure 1A**). Importantly, deletion of these sequences implies a loss of imprinting (LOI), which results in alterations of the expression of imprinted genes in the cluster (14, 16).

The establishment of imprints takes place in the germline through a multistep mechanism termed imprinting life cycle, which ensures monoallelic expression of imprinted genes (17) (**Figure 1B**). During embryogenesis, the primordial germ cells (PGCs), which will give rise to the gametes, have the methylation patterns characteristic of somatic cells. However, in the genital ridges, the imprints are erased during gamete formation to allow re-establishment of new parental-specific marks at the ICRs (4, 8). This process takes place during development at different times in males and females (18). Paternal-specific methylation occurs prenatally in pro-spermatogonia before meiosis, whereas maternal-specific ICR methylation takes place postnatally in growing oocytes (19) (**Figure 1B**). After establishment of imprints, methylation patterns of each chromosome must be kept in somatic cells, thus imprints are protected against the extensive genome demethylation that occurs after fertilization (17), and then transmitted to every somatic cell (10) (**Figure 1B**).

During development and adult life, genomic imprinting can be modified leading to tissue or cell type specific imprint patterns (2). Indeed, loss of imprinting (LOI) has consequences in physiological processes and is the cause of some human imprinting syndromes such as Angelman, Prader-Willi or Beckwith-Wiedemann, which course with severe neurological defects (3, 9, 20). Moreover, disruption of imprinting can cause a predisposition to tumor formation, and LOI in several genes is considered to be the most common and early event in human cancers such as colorectal or esophageal cancer, meningiomas, gliomas, and chronic myeloid leukemia among others (13, 21–23).

IMPRINTED GENES AND NSCs

In the mammalian brain, two regions generate new neurons throughout adulthood: the subventricular zone (SVZ) in the walls of the lateral ventricles, and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (24, 25). The process of neurogenesis in these adult neurogenic niches is continually sustained by the activity of NSCs, which are characterized by their ability to balance self-renewal with multipotential differentiation into astrocytes, oligodendrocytes and neurons (26). Activated and quiescent NSCs (also known as type B1 cells) coexist in the adult SVZ (27) and once activated, slowly dividing NSCs give rise to fast cycling cells called transit-amplifying progenitors (TAP or type C cells). Mash1-positive type C cells in turn generate immature neurons or neuroblasts (type A cells) that migrate tangentially through the rostral migratory stream (RMS) toward the olfactory bulb (OB). These chains of polysialylated neural cell adhesion molecule (PSA-NCAM) positive neuroblasts reach the core of the OB, where they integrate and differentiate into inhibitory interneurons, playing an important role in rodent olfaction (28). Although less frequently, subventricular NSCs are also capable of producing some oligodendroblasts that migrate to the corpus callosum and striatum, where they differentiate into myelinating and non-myelinating oligodendrocytes (29, 30). The human SVZ is also considered as an important pool of neuronal and glial progenitor cells, and this pool has been implicated in injury, neurodegeneration and cancer (31).

In the SVZ, type B1 cells have many features of astrocytes and retain expression of NESTIN or GLAST (astrocyte-specific glutamate aspartate transporters), markers that are also expressed in radial glia cells (RGCs), the NSCs in the developing brain (32, 33). The majority of NSCs in the adult SVZ originate from these RGC cells between embryonic days (E) 13.5 and 15.5 and remain largely quiescent until they become reactivated postnatally (34, 35).

Recent studies on the developing brain and postnatal neurogenic niches raise many intriguing questions concerning the role of genomic imprinting and gene dosage in gliogenesis and neurogenesis, including how imprinted genes operate in concert with signaling cues to contribute to these processes (36). For example, during cortical neurogenesis, radial glia cells express high levels of the paternally-expressed zinc finger protein *Zac1,* which leads to the expression of other imprinted genes such as the maternally-expressed cyclin-dependent kinase inhibitor *Cdkn1c*, known to promote NSC cell cycle arrest and proglial differentiation (37). Interestingly, *Cdkn1c* has been shown to also promote NSCs quiescence in the adult hippocampus, and long-term deletion of the gene leads to NSC exhaustion and impaired neurogenesis in aged mice (35). Moreover, in the embryonic mouse neocortex, the proliferative capacity of cortical progenitors is repressed by paternal expression of *Necdin*, which suppresses neural progenitor proliferation by antagonizing the polycomb protein BMI1 function (38).

Genomic imprinting can be selectively lost or "*switched off*" in particular cell types or at specific developmental points to activate an allele that is usually repressed by imprinting (36). For example, in the adult SVZ, the insulin-like growth factor 2 (*Igf2*) gene, canonically expressed from the paternally-inherited allele, is biallelically expressed in the choroid plexus and secreted into the cerebrospinal fluid to regulate NSC proliferation (39, 40). IGF2 is also biallelically expressed in the postnatal human and mouse choroid plexus epithelium and leptomeninges, acting as a paracrine factor that regulates NSC homeostasis (39, 41). In contrast, in the SGZ, *Igf2* is expressed in NSCs in an imprinted manner, suggesting that the regulatory decision to imprint or not is an important mechanism of transcriptional dosage control in adult neurogenesis (39). Another example of LOI in the SVZ is the paternally-expressed gene *Delta-like homolog 1* (*Dlk1*), an atypical Notch ligand located on mouse chromosome 12 (human chromosome 14) that plays a relevant dual function to regulate postnatal neurogenesis (42). *Dlk1* is a single gene that encodes for both a secreted factor (expressed by niche astrocytes) and a bound receptor (expressed by NSCs). *Dlk1*, which is a canonically imprinted gene elsewhere in the brain, shows a selective absence of imprinting in these cell types, and biallelic expression of *Dlk1* is required for stem cell maintenance in the SVZ and final neurogenesis in the olfactory bulb (42). In conclusion, genomic imprinting might be reversible and context-dependent and is likely to be essential to control neural stem cell potential

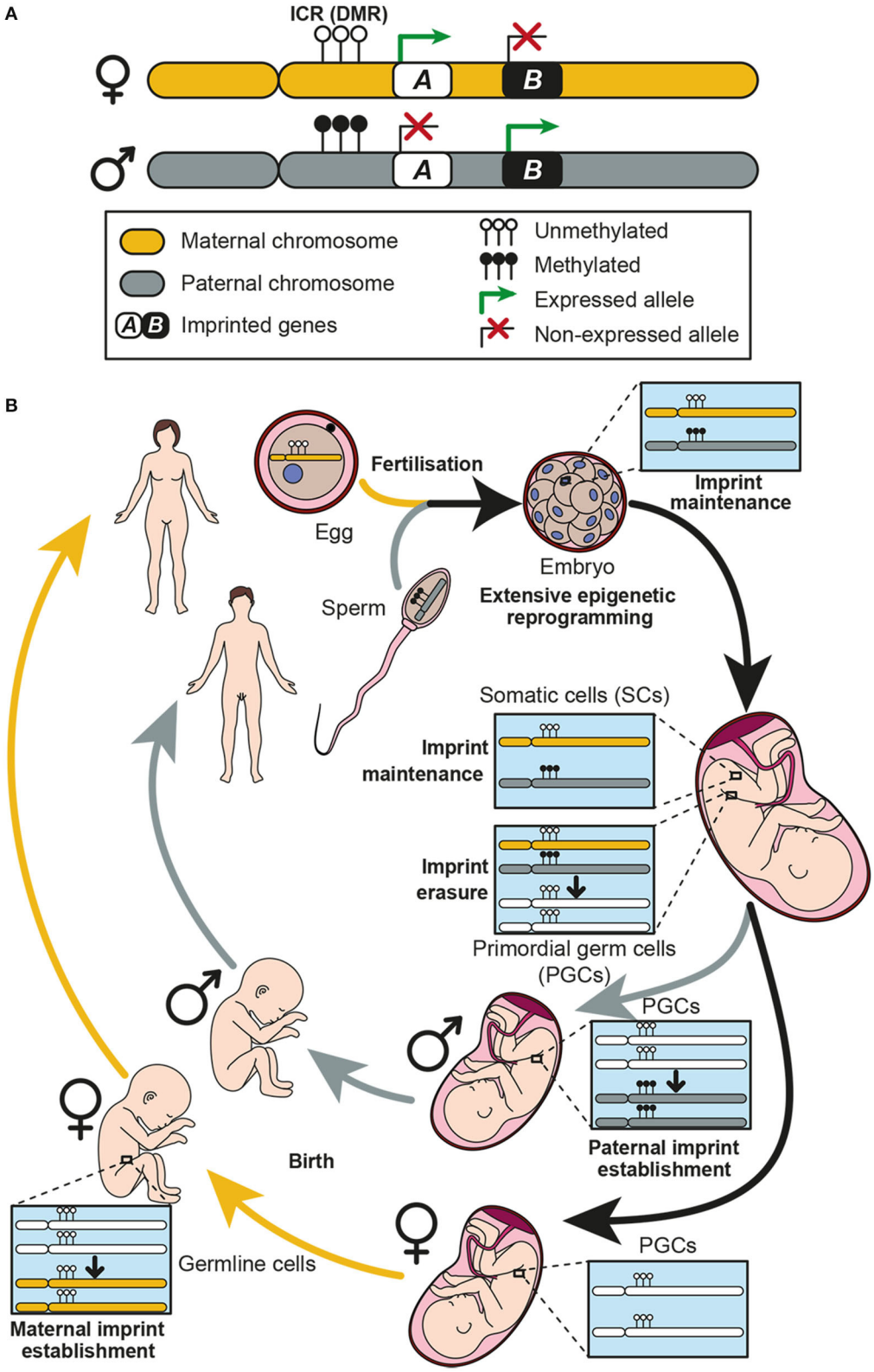

FIGURE 1 | Genomic imprinting and the establishment of imprints in the germline. **(A)** Two homolog chromosomes are represented, each one inherited from one progenitor: maternal chromosome in yellow and paternal chromosome in gray. An imprinting cluster containing two imprinted genes (genes *A* and *B*) is represented. Gene *A* is maternally expressed, while gene *B* is paternally expressed. Expression of both genes is controlled by methylation at the imprinting control region (ICR)

(Continued)

FIGURE 1 | which is a differentially methylated region (DMR) between the two chromosomes. **(B)** Genomic imprinting life cycle is represented. When fertilization occurs, the zygote receives a maternal and a paternal copy of the genome, each one imprinted accordingly. Methylation patterns of each chromosome must be kept in somatic cells, thus imprints are protected against the extensive genome demethylation that occurs after fertilization. Imprints are maintained along the individual life in somatic cells, while they are erased in primordial germ cells (PGCs) during development. Afterwards, a new imprint is established in the germline according to the individual chromosomal sex. These imprints are established during development in males and postnatally in females.

and for normal development and tissue regeneration in the adult brain.

GENOMIC IMPRINTING IN HUMAN GLIOBLASTOMA

In the central nervous system (CNS), as in many other tissues, diverse types of tumors may emerge throughout life. Gliomas arise from glial cells and are the most frequent primary tumors in the brain (43). According to the criteria established by the World Health Organisation (WHO) in 2016, gliomas are classified as grades I to IV based on histology and clinical criteria (44). Grade I tumors are generally benign and frequently curable, whereas malignant glioma are subdivided from the least aggressive grade II to grade IV, which is more proliferative, more necrosis-prone and angiogenic and has a poorer prognosis (45–47). GBM is the most aggressive and frequent grade IV type glioma and despite its low incidence (3.21 cases per 100,000 people), up to 46% of primary malignant brain tumors are GBM (43, 48). Patients diagnosed with GBM survive on the average 15 months and the 5-year-survival rate is only 5.6% (48, 49).

Due to its frequency and lethality, several studies have been carried out in order to characterize different human GBM subtypes based on genome and transcriptome changes. For example, the epidermal growth factor receptor (EGFR) is altered in almost 50% of GBM and represents one of the most promising therapeutic targets (50). Other mutations affecting TP53, PTEN, RB1, ERBB2, PIK3R1 or PIK3CA pathways have been identified in different GBM patients (51). Another recurrent mutation is the one occurring in the isocitrate dehydrogenase 1 gene (IDH1). This mutation is much more frequent in LGG and secondary GBM (GBM arising from LGG) than in primary GBM, and it is associated with an increased survival (52, 53). Interestingly, IDH1 mutations are associated with the existence of a glioma-CpG island hypermethylation phenotype (G-CIMP tumors), which also correlates with a significantly improved outcome (54). Thus, the study of epigenetics of GBM and the consequence of its mutations is also relevant. Among all epigenetic phenomena, genomic imprinting could be particularly important in GBM since several imprinted genes function as cellular mitogens or tumor suppressors, and misexpression of some of these imprinted genes has been postulated in human GBM (55). For example, repression of the tumor suppressor *CDKN1C* ($p57^{KIP2}$), a maternally expressed gene, or overexpression of an oncogene, such as the paternally-expressed imprinted gene *IGF2,* increases the chance of developing the malignant process (21–23, 56). Precisely, upregulation of *IGF2* as a result of a LOI has been associated with several cancers due to over-proliferation effects (57, 58). Also, the maternally expressed *H19* is overexpressed in GBM samples compared to healthy brains, and its role as an oncogenic lncRNA through inhibition of *β–catenin* expression is clearly recognized (59). Low expression of the maternally expressed gene *MEG3* significantly correlates with short survival in GBM patients, and *in vitro* restoration of *MEG3* impairs tumorigenic abilities of GBM cells (60). Moreover, epigenetic silencing of the paternally expressed gene *PEG3* was confirmed in GBM (61). *Contactin 3* (*CNTN3*), another imprinted gene, has been postulated as a biomarker that predicts overall survival in GBM patients (62). Similarly, expression of the paternally expressed gene *DLK1* is higher in GBM cells than in normal brain thus increasing their proliferation and migration capabilities (63). Therefore, an important role of genomic imprinting in human GBM is starting to also be elucidated.

In order to corroborate the potential relevance of genomic imprinting in human GBM, we searched for imprinted genes expression in different tumor and non-tumor samples using the GlioVis database (64). Eight datasets were chosen, five of them containing RNAseq data: Bao (65), CGGA (66), Gill (67), TCGA_GBM (68), and TCGA_GBMLGG (69); and the other three containing microarray data: Rembrandt (70), Gravendeel (71) and Kamoun (72). Expression analysis was executed comparing non-tumor (NT), low grade glioma (LGG, grade II-III gliomas), and GBM (grade IV gliomas) human samples. Using these datasets, analysis of the expression of 81 imprinted genes was performed in three different comparisons: GBM and NT samples (GBM vs. NT), LGG and NT samples (LGG vs. NT) and GBM and LGG samples (GBM vs. LGG). Different numbers of datasets were used in each case: five datasets for GBM vs. NT comparison (Gill, TCGA_GBM, Rembrandt, Gravendeel and Kamoun); three datasets for LGG vs. NT comparison (Rembrandt, Gravendeel and Kamoun); and six datasets for GBM vs. LGG (Bao, CGGA, TCGA_GBMLGG, Rembrandt, Gravendeel and Kamoun). The results show that a high number of imprinted genes alter their expression levels in all comparisons. For example, 53.8% of imprinted genes resulted differentially expressed in GBM compared to NT samples (**Figure 2A**), 46.5% in LGG compared to NT samples (**Figure 2B**) and 60.9% in GBM compared to LGG samples (**Figure 2C**). These data support the hypothesis of genomic imprinting having a relevant role in glioma development and progression. Additionally, we have performed a similar analysis comparing the expression of imprinted genes in IDHwt and IDHmut LGG samples using TCGA_GBMLGG database. This study shows that 69.1% of imprinted genes are differentially expressed in IDHwt and IDHmut samples (**Figure 2C**), suggesting that imprinted genes could also be important for patient prognosis.

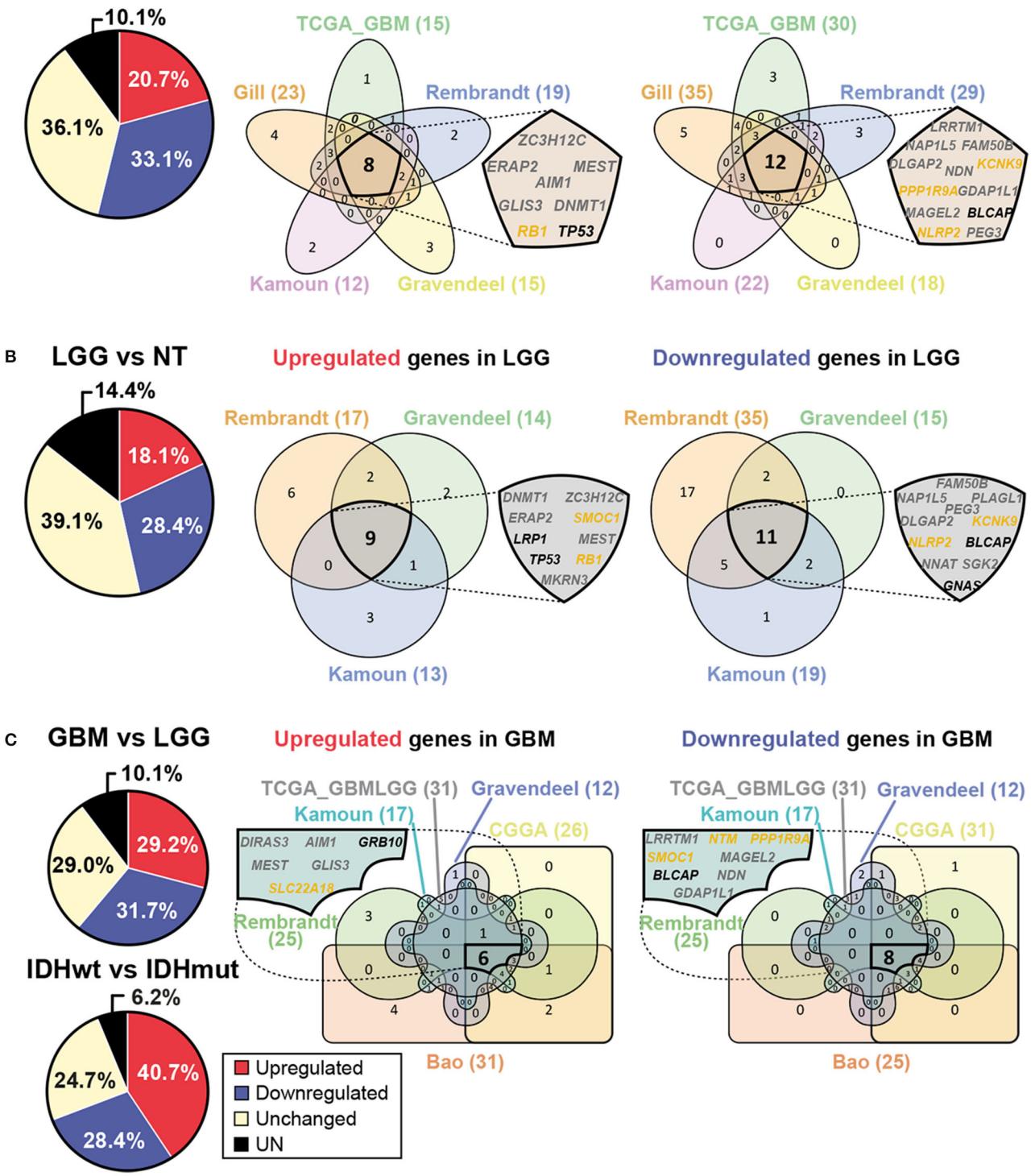

FIGURE 2 | Expression of imprinted genes is altered in human GBM. **(A)** Pie chart representing average percentages of upregulated (red) and downregulated (blue) genes in GBM when compared with non-tumor (NT) samples and obtained with different human GlioVis datasets. Non-significant gene expression is also included (yellow). The average percentages of genes which data are not available are shown in black (UN, left panel). Venn diagrams represent imprinted genes which are upregulated or downregulated in GBM when compared with NT samples. Each dataset used is represented (right panel). Intersection of all sets shows genes which expression pattern is coincident in every analyzed dataset. Maternally expressed genes are indicated in yellow whereas paternally expressed genes are indicated in gray. Genes with unknown specific-parental expression are in black. **(B)** Pie chart representing average percentages of imprinted genes which are upregulated

(Continued)

FIGURE 2 | (red) or downregulated (blue) in low grade glioma (LGG) compared to NT samples (left panel). Venn diagrams representing imprinted genes which are differentially expressed between LGG and NT samples (right panel). Intersection of all sets represents genes which expression pattern is coincident in every analyzed dataset. **(C)** Pie charts representing average percentages of imprinted genes which are upregulated (red) or downregulated (blue) in GBM compared to LGG samples and in IDHwt compared to IDHmut LGG samples. Venn diagrams representing imprinted genes which are differentially expressed between GBM and LGG samples (right panel). Intersection of all sets represents genes which expression pattern is coincident in every analyzed dataset. GlioVis datasets used are Bao, CGGA, Gill, TCGA_GBM, TCGA_GBMLGG, Rembrandt, Gravendeel and Kamoun.

Expression patterns in every dataset were analyzed using Venn diagrams (73) and three lists of imprinted genes were obtained from the analysis, one per comparison, each of them containing upregulated and downregulated genes in different samples (**Figure 2**). A list of 20 differentially expressed genes between GBM and NT samples was obtained from the analysis, 8 of them upregulated (*ZC3H12C, ERAP2, MEST, AIM1, GLIS3, DNMT1, RB1,* and *TP53*) and the other 12 downregulated (*LRRTM1, NAP1L5, FAM50B, DLGAP2, NDN, KCNK9, PPP1R9A, GDAP1L1, MAGEL2, BLCAP, NLRP2,* and *PEG3*) in GBM (**Figure 2A**). Another list of 20 genes with different expression levels was obtained when comparing LGG and NT samples, 9 of them upregulated (*DNMT1, ZC3H12C, ERAP2, SMOC1, LRP1, MEST, TP53, RB1* y *MKRN3*) and the other 11 genes downregulated (*FAM50B, NAP1L5, PLAGL1, PEG3, DLGAP2, KCNK9, NLRP2, BLCAP, NNAT, SGK2* y *GNAS*) in LGG (**Figure 2B**). A third list of 14 differentially expressed genes was obtained when comparing both types of tumor samples (GBM and LGG), being 6 of them upregulated (*DIRAS3, AIM1, GRB10, MEST, GLIS3* y *SLC22A18*) and the other 8 downregulated (*LRRTM1, NTM, PPP1R9A, SMOC1, MAGEL2, BLCAP, NDN* and *GDAP1L1*) in GBM (**Figure 2C**). This analysis reveals potential candidates for future research on the role of concrete imprinted genes and gene dosage control in GBM formation.

ABERRATIONS OF GENOMIC IMPRINTING IN NSCs AND GBM FORMATION

Due to its similarities with astrocytes, GBM is considered an astrocytoma (45, 46). However, the cell of origin of GBM is not completely understood. Several studies have described the presence of a cell population with stem cell characteristics within the tumors, the glioma stem cells (GSCs), which have GBM potential and are responsible for the origin of the tumors (74–77). These cells can give rise to new tumors by themselves and are thought to be responsible for the resistance to treatment and the high risk of recurrence in this kind of tumor (78). GSCs express stem cell markers, sharing some features with NSCs, such as the expression of some surface antigens and the activation of some signaling pathways (79). In addition, both cell types exhibit a similar proliferation rate, a similar transcriptome and are closely associated to blood vessels (80, 81). Although some authors have demonstrated that differentiated cell types can be reprogrammed and form GBM when bearing some specific-gene mutations (82–84), NSCs have also been proposed to be the cell of origin of GSCs (76, 85, 86). Indeed, some authors have described that susceptibility to malignant transformation of NSCs decreases with the increase of lineage restriction in the brain, suggesting a GBM hierarchy in which NSCs are the most common cell-of-origin and differentiated cell types are less susceptible to tumorigenesis (87).

As we mentioned before, imprinted genes are defined by their monoallelic expression with implications in development and placentation, but also in metabolism of the adult organism (11, 12). These characteristics make these genes extremely susceptible to mutations. LOI most likely precedes tumor formation and several studies suggest this to occur originally in stem cell populations, leading to their transformation (23, 57). This theory posits that epigenetic modifications such as LOI take place in stem cells and this is supported by the presence of non-malignant cells around the tumor with LOI events (23). Indeed, an increase of the stem cell pools due to LOI (for example with high levels of *IGF2*) could favor the accumulation of mutations, creating a suitable context for transformation (21, 57). Thus, genomic imprinting seems to play an important role in converting stem cells into cancer stem cells, although very little is known about how aberrations of genomic imprinting might participate specifically in the malignant transformation of NSCs. It has been recently described that the imprinted lncRNA *MEG3* acts as a tumor-suppressor gene in GSCs, inhibiting cell growth, migration and colony-forming abilities of GSCs *in vitro* (60). Moreover, the imprinted gene *DLK1,* essential for the maintenance of NSCs in the murine adult SVZ (42, 88), increases its expression in human glioma and promotes proliferation of GBM cell lines (60, 63). Nonetheless, the molecular mechanisms governing the tumor suppressing or promoting activities of these genes and other imprinted genes in GBM remain elusive.

In order to further elucidate the potential regulation of genomic imprinting during malignant transformation of NSCs, we performed an analysis of single-cell RNA sequencing data, which had been previously generated from 28 human GBM samples (88), and compared it with non-malignant oligodendrocytes and adult human NSCs (89). Of the 222 imprinted genes analyzed, 92 showed significant expression in oligodendrocytes and 68 were expressed in human NSCs. Interestingly, more than 70% of these genes were altered in GBM when compared to non-malignant oligodendrocytes (**Figure 3A**), whereas only 16% of genes were altered when compared to human NSCs (**Figure 3A**). This suggests that the transcriptomes of NSCs are more closely related to those of tumor cells than to non-malignant cells.

It has been described that malignant cells in GBM exist in four main cellular states that recapitulate distinct neural cell types within the tumors: oligodendrocyte-progenitor-like (OPC-like), astrocyte-like (AC-like), mesenchymal-like (MES-like)

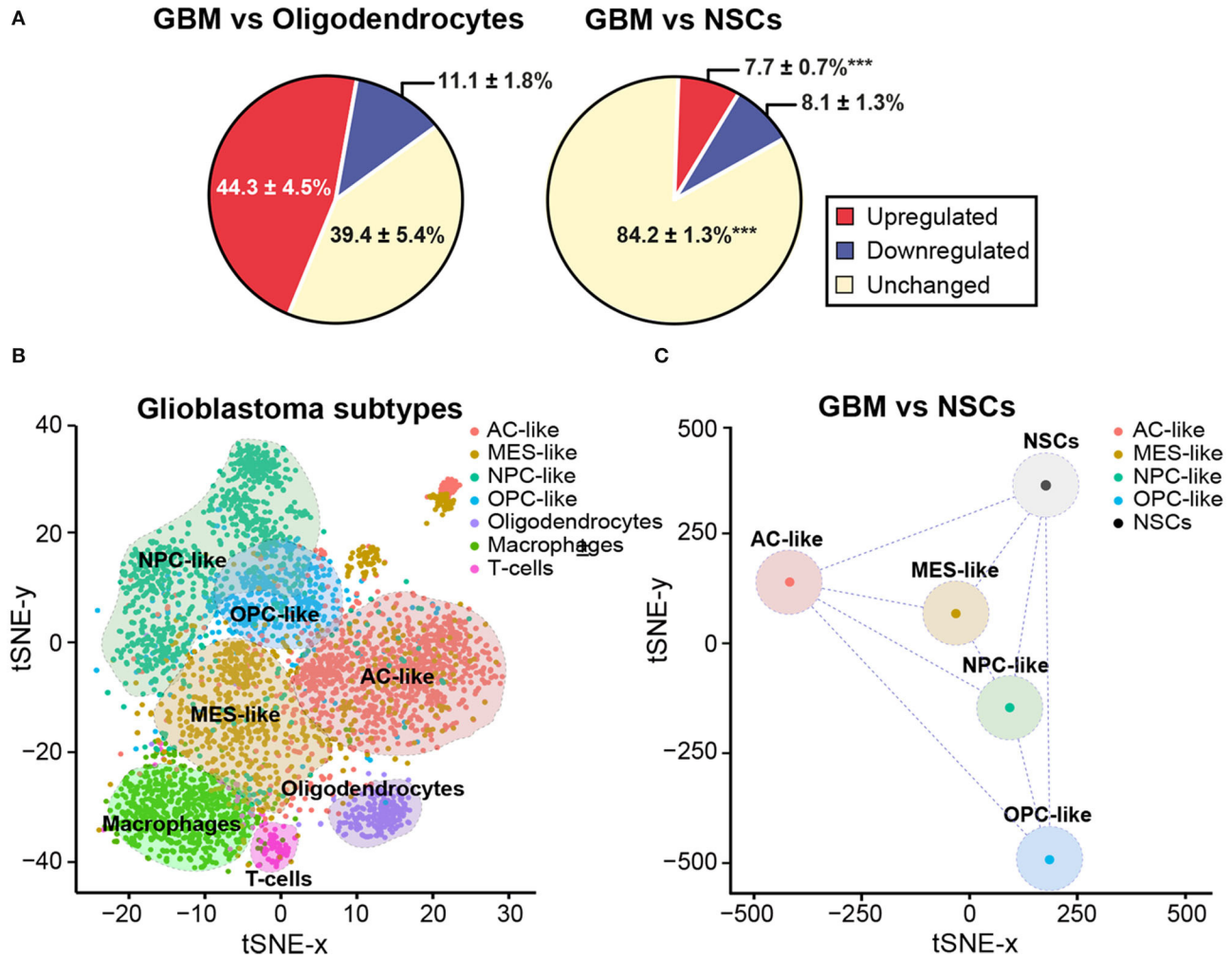

FIGURE 3 | NSCs share imprinting gene expression profile with GBM cells. **(A)** Pie chart representing average percentages of upregulated (red) and downregulated (blue) imprinted genes in GBM when compared with oligodendrocytes (left panel) or NSCs (right panel). Percentage of imprinted genes that do not change their expression levels is also included (yellow). A statistical analysis was performed to determine the changes in the percentage of downregulated, upregulated or unchanged genes in NSCs and oligodendrocytes when compared to GBM. Mean percentage and s.e.m are indicated. *P*-values: $^{***}p < 0.001$. **(B)** tSNE dimensional reduction plot of single-cell RNAseq data from Neftel et al. (88) (downloaded from GSE131928) showing that the four GBM cell state subtypes and the three non-malignant cell types form discrete clusters based on the expression of 222 imprinted genes. AC-like (astrocytic-like), MES-like (mesenchymal-like), NPC-like (neural progenitor-like) and OPC-like (oligodendroglial progenitor-like). Non-tumoural cells: oligodendrocytes, macrophages and T-cells. Assignment of cell state names to individual GBM cells was based on the reanalysis of the two-dimensional hierarchical representation of cellular states from Neftel et al. (88). From each of the four quadrants, cells that displayed relative meta-module scores > 1 were selected and named according to their corresponding cellular state, as defined in the figure. In total, 2,528 GBM cells and 1,014 non-malignant cells were used to generate the tSNE plot. **(C)** The tSNE dimensional reduction plot of GBM cells was repeated after converting the scRNAseq data of tumoural states into pseudo-bulk RNAseq data and incorporating to the input expression matrix the four biological replicates of the bulk RNAseq datasets for NSCs from Donega et al. (89) (downloaded from GSE130752).

and neural-progenitor-like (NPC-like) states (88). Importantly, plasticity between states and the potential for a single cell to generate all four states have been shown. Based on the same single-cell RNAseq datasets, we performed a tSNE dimensional reduction analysis taking into account only the molecular profiles of the 222 imprinted genes present in the expression matrix. On top of the tSNE plot, cells were color-coded according to their assignment as each of the four tumoral states (88). Based on the expression of imprinted genes only, cells appeared as visually distinctive groups that nicely matched either their cell states in case of GBM cells, or their cell type in case of non-malignant cells (**Figure 3B**). Non-malignant cells, which highly expressed previously described markers of specific cell types such as oligodendrocytes, macrophages or T cells (88), formed three discrete groups at the bottom of the plot clearly separated from GBM cells (**Figure 3B**). Aiming to compare the single-cell transcriptomes of GBM cells and bulk RNA-seq datasets previously generated of NSCs (89), we averaged the single-cell datasets to convert them into comparable pseudo-bulk datasets and repeated the dimensional reduction analysis.

Interestingly, the resulting plot indicated that the distance in the two-dimensional plane was not higher between NSCs and GBM states than among the four tumoral subtypes (**Figure 3C**). Our analysis overall indicates that imprinted gene expression programs might have biological significance in tumor identity, thus being of potential value for diagnosis and GBM treatment.

CONCLUDING REMARKS

Genomic imprinting is an epigenetic phenomenon consisting in the expression of imprinted genes only by one allele depending on its parental origin. This process is susceptible to alterations that not only can cause some human syndromes but are also involved in cancer development. Indeed, some imprinted genes act as oncogenes or tumor suppressor genes and have been involved in malignant transformation. In GBM, which is the most frequent and malignant primary brain tumor in humans, the misexpression of some concrete imprinted genes has been previously described. In this review, we show the results of an expression data analysis performed in GBM and non-tumor samples, confirming that an extensive alteration in the expression of imprinted genes does exist in GBM. Although the cell-of-origin of GBM has not been completely elucidated yet, NSCs seem to be good candidates as they share multiple features with GBM cells. There is emerging evidence pointing out that NSCs could undergo malignant transformation and give rise to GBM, and that genomic imprinting could be important in this process. In contrast to other non-malignant cells, adult NSCs from the human SVZ cannot be distinguished from GBM cells based on imprinted gene expression data, supporting the hypothesis that NSCs are the cells-of-origin of GBM. Taken together, all these data reveal genomic imprinting as an important epigenetic mechanism in GBM origin and development, and thus make aberrations of imprinting a potentially valuable tool for both diagnosis and cancer treatment. However, the causal relationship between aberrations of imprinting and GBM formation has not been resolved yet and needs to be studied further in the future.

AUTHOR CONTRIBUTIONS

AL-U and EJ-V performed the GlioVis analysis. AJ-P performed the RNAseq data analysis. SF initiated, designed, and wrote the manuscript. All authors contributed to data analysis, discussion, and writing of the paper.

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Glioblastoma Proximity to the Lateral Ventricle Alters Neurogenic Cell Populations of the Subventricular Zone

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Despite current strategies combining surgery, radiation, and chemotherapy, glioblastoma (GBM) is the most common and aggressive malignant primary brain tumor in adults. Tumor location plays a key role in the prognosis of patients, with GBM tumors located in close proximity to the lateral ventricles (LVs) resulting in worse survival expectancy and higher incidence of distal recurrence. Though the reason for worse prognosis in these patients remains unknown, it may be due to proximity to the subventricular zone (SVZ) neurogenic niche contained within the lateral wall of the LVs. We present a novel rodent model to analyze the bidirectional signaling between GBM tumors and cells contained within the SVZ. Patient-derived GBM cells expressing GFP and luciferase were engrafted at locations proximal, intermediate, and distal to the LVs in immunosuppressed mice. Mice were either sacrificed after 4 weeks for immunohistochemical analysis of the tumor and SVZ or maintained for survival analysis. Analysis of the GFP+ tumor bulk revealed that GBM tumors proximal to the LV show increased levels of proliferation and tumor growth than LV-distal counterparts and is accompanied by decreased median survival. Conversely, numbers of innate proliferative cells, neural stem cells (NSCs), migratory cells and progenitors contained within the SVZ are decreased as a result of GBM proximity to the LV. These results indicate that our rodent model is able to accurately recapitulate several of the clinical aspects of LV-associated GBM, including increased tumor growth and decreased median survival. Additionally, we have found the neurogenic and cell division process of the SVZ in these adult mice is negatively influenced according to the presence and proximity of the tumor mass. This model will be invaluable for further investigation into the bidirectional signaling between GBM and the neurogenic cell populations of the SVZ.

Keywords: glioblastoma, subventricular zone (SVZ), lateral ventricle, neural stem cell (NSC), cancer stem cell (CSC), neurogenic niche

INTRODUCTION

Glioblastoma (GBM) is the most frequent and aggressive type of malignant primary brain tumor in adults (1, 2). Patients suffering from GBM have a median survival of approximately 15 months despite advanced therapeutic strategies of combinatorial surgery, chemotherapy, and radiation (3, 4). Interestingly, tumor progression for GBM patients is greatly affected by tumor location. Lateral ventricles (LVs) infiltrating tumors account for over 50% of all GBM patients (5). These LV-contacting tumors result in higher incidence of distant recurrence, as well as larger tumor volume and worse survival expectancy in patients (6–9). Furthermore, GBM patients who receive radiotherapy that includes the ventricular wall ipsilateral to the tumor show increased survival when compared to patients where the ipsilateral ventricular wall is avoided (10), indicating the involvement of LV-derived factors in GBM progression.

The reason for worse patient outcome in these cases is unknown, though could be due in part to contact with the subventricular zone (SVZ) present in the lateral wall of the LV. The SVZ is the largest stem cell niche in the mammalian adult brain, including humans (11–14). In rodents, neural stem cells (NSCs) of the SVZ form new neurons and glia throughout life, differentiating to neuroblasts or glial progenitors that then migrate to their site of terminal differentiation (15–18). Studies have shown a high amount of similarity in the biology of NSCs and stem-like GBM cells, including shared pathways of self-renewal, differentiation, and cell migration (19, 20). Additionally, several studies have identified NSCs of the SVZ as a potential cell-of-origin for GBM, pointing to the potential involvement of NSCs in GBM progression (21–25).

Shared mechanisms between NSCs and GBM support the idea that stem cell-supportive factors contained within the SVZ support the proliferation and stemness of LV-proximal tumors. This may include a bidirectional crosstalk between NSCs/progenitors and GBM cells that leads to changes in SVZ biology and patient outcome. Previous work has shown that cell types within the SVZ, including NSCs and their progeny, are altered in response to GBM in rodents (26). Here, we develop a novel rodent model of LV-proximal GBM and examine the reciprocal relationship between the SVZ and GBM tumors. We particularly focused on cell population and proliferation changes in the SVZ as a consequence of GBM proximity to the LV.

MATERIALS AND METHODS

Experimental Animals

All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee of Mayo Clinic. Mice were housed in a fully AALAC-accredited facility in accordance with all federal and local regulations. Male athymic immunosuppressed J:NU mice (The Jackson Laboratory, strain 007850) were maintained at Mayo Clinic Jacksonville with a 12-hour light-dark cycle and *ad libitum* feeding. Animals were utilized for experiments at an age between 6-8 weeks.

Primary GBM Cell Xenograft and Euthanasia

We utilized a primary cell line of human GBM cells (GBM1A, also known as line 020913) (27). GBM1A cells were transduced with a GFP-luciferase lentivirus (RediFect™ Red-FLuc-GFP, Perkin Elmer CLS960003) and sorted for GFP positivity. Following cell transduction, intracranial implantation of tumors was performed as previously described (28–31). Briefly, mice were anesthetized and placed in a stereotactic frame. 5.0 x10^5 GBM1A-GFP luciferase+ cells were injected in 2 µL of DMEM/F12 into the right brain hemisphere. 3 injection sites were established in the following coordinates (in mm relative to bregma); LV-proximal: AP: 1.0, L: 1.2, D: 2.3, n = 17; LV-intermediate: AP: 1.5, L: 1.3, D: 3, n = 7; and LV-distal: AP: 1.0, L: 2.1, D: 2.3, n = 17. Tumor growth was monitored weekly by bioluminescence following luciferin injection. For survival experiments, mice were maintained until reaching humane endpoint criteria following GBM xenograft. For histology analysis, mice were maintained for 4 weeks after tumor implantation (n=7). Mice were then anesthetized and perfused with 4% paraformaldehyde. Brains were extracted and cryoprotected in 30% sucrose. Brains were sectioned using an HM 430 Freezing Microtome at 30 µm thickness. Sections were stored in 30% ethylene glycol, 20% glycerol, 0.05M PBS, pH 7.4 at -20°C until immunohistochemical processing.

Immunohistochemistry

Sections were permeabilized with 0.1% Triton in PBS (PBST) and blocked with 1% BSA and 10% normal horse serum. In the case of caspase-3 and Ki67 staining, antigen retrieval was performed using sodium citrate buffer (10 mM + 0.05% Tween) at 90°C for 25 minutes, followed by cooling in the sodium citrate buffer for 30 minutes before washing and blocking. Sections were then incubated overnight at 4°C in primary antibody at various concentrations (**Table 1**) diluted in 0.2% normal horse serum in PBST. Sections were washed and incubated in the dark for 1 hour at room temperature with secondary antibodies (**Table 2**) at a concentration of 1:500 in 2% normal horse serum in PBST. Sections were washed and counterstained with DAPI as a nuclear dye. At least three sections per animal were used per staining condition.

Imaging

Immunohistochemical preparations were visualized using a confocal microscope (Zeiss LSM800). Tumors were visualized by GFP+ cells and imaged with 10X, 25X, 40X or 63X objectives. ZEN® Blue Edition software (Zeiss) was then used to process the image. All sections for the same antibody combinations were imaged in the same way using the same exposure levels.

Volumetric Analysis

Tumor area data was obtained using ZEN® Blue Edition software. GFP+ tumors were traced using the "Draw Spline Contour" tool in ZEN software to obtain the area of each tumor section. Morphometric volume was then calculated

TABLE 1 | Primary antibodies used.

Antibody	Species	Detection	Dilution Factor	Catalog
GFP	Mouse	GFP+ GBM cells	1:500	Abcam (ab1218)
Human Nuclei (HuNu)	Mouse	Human GBM cells	1:200	Millipore (MAB1281)
Ki67	Rabbit	Proliferating cells	1:200	Thermo (RM-9106-S0)
	Mouse			Novocastra (NCL-L-Ki67-MM1)
phosphohistone H3 (pH3)	Rabbit	Proliferating cells	1:200	Cell Signaling (9701S)
GFAP	Rabbit	Astrocytic cells	1:200	Dako (Z0334)
SOX2	Rat	Undifferentiated cells	1:500	Thermo (14-9811-82)
OLIG2	Rabbit	Oligodendrocyte precursors	1:500	Millipore (AB9610)
Cleaved caspase-3 (Asp175)	Rabbit	Apoptotic cells	1:200	Cell Signaling (9661)
Doublecortin (DCX)	Goat	Neuroblasts	1:200	Santa Cruz (SC-8066)

TABLE 2 | Secondary antibodies used.

Secondary Antibody Wavelength	Species and Reactivity	Dilution Factor	Catalog
Alexa Fluor 568	Donkey anti-rabbit	1:500	Invitrogen (A10042)
Alexa Fluor 647	Chicken anti-rabbit	1:500	Invitrogen (A21443)
Alexa Fluor 488	Donkey anti-goat	1:500	Invitrogen (10246392)
Alexa Fluor 555	Donkey anti-mouse	1:500	Invitrogen (A31570)
Alexa Fluor 594	Donkey anti-rat	1:500	Invitrogen (A21209)

using the Cavalieri principle, which allows an accurate estimation of the volume (V) of a structure independently of its shape and size (32). This is calculated by finding surface area (A) of a number (n) of parallel sections spaced at a constant distance (t) and inserting into the following equation: $V = t * (A_1 + A_2 + A_3 \ldots + A_n)$.

Cell Quantification

ZEN® Blue software was used to estimate the number of cells expressing the human nucleus marker, HuNu, and the proliferation marker, Ki67 in the different groups, both in the tumor as in SVZ. In addition, cells positive for cleaved caspase-3, phosphohistone H3, SOX2, SOX2/GFAP, DCX and OLIG2 present in SVZ were quantified. For cell quantification of the SVZ, the ipsilateral and contralateral SVZ were imaged in 20X tiles using a confocal microscope. Using the ZEN® Blue software, the SVZ region was manually traced using the "Draw Spline Contour" tool to specifically isolate the cells of the SVZ for the subsequent analysis. Signal background was removed by adjusting the channel histogram to the peak of the curve and remaining cells were considered positive and counted. Cell quantification was performed from planes with no tumor cell presence in order to avoid changes in cell proportion, The cell density (number of cells per square millimeter) was calculated for each image.

Statistical Analysis

All data is represented as the mean ± the standard error (SEM) unless otherwise indicated. Statistical analysis and graphical representation were performed using GraphPad Prism® 6 software. Normal distribution of data was assessed using the Shapiro-Wilk normality test. To compare among multiple groups, analysis of variance (ANOVA) with Tukey's post-hoc correction was performed. For independent comparisons between two groups the student's t-test was performed. The level of significance was determined as $p < 0.05$.

RESULTS

GBM Proximity to the LV Contributes to Tumor Growth and Survival Outcome

We first evaluated the effect of the LV proximity on tumor growth in our animal model. Patient-derived GBM cells transduced to express GFP and luciferase were implanted at locations proximal, intermediate, and distal to the LV (**Figure 1A** and **Supplementary Figure 1**). Following a 4-week period, tumors were evaluated for volume, cellular density, proliferation, and apoptosis. When tumors were injected into locations proximal and intermediate to the LV, we observed a trend towards increased tumor volume compared to LV-distal tumors (LV-proximal: 2.55 mm^3; LV-intermediate: 3.74 mm^3; LV-Distal: 1.19 mm^3; **Figure 1B**) with no difference in tumor cell density (**Supplemental Figure 2**). In order to determine whether LV proximity induced differences in proliferation or apoptosis, we performed immunofluorescence staining for Ki67, cleaved caspase-3, and human nuclei (HuNu)+ GBM cells. In tumors injected in LV-proximal and LV-intermediate locations we observed a significantly higher percentage of Ki67+/HuNu+ GBM cells than in LV-distal tumors (LV-proximal: 23.36%, LV-Intermediate: 23.48%, LV-Distal: 11.41%; **Figures 1C–F**), indicating an increase in GBM proliferative index dependent on proximity to the LV. Additionally, the percentage of cleaved caspase-3+/HuNu+ cells was significantly decreased in LV-intermediate tumors compared to LV-proximal tumors (LV-Proximal: 0.014%, LV-Intermediate: 0.0037%, LV-Distal: 0.015%; **Figures 1G–J**).

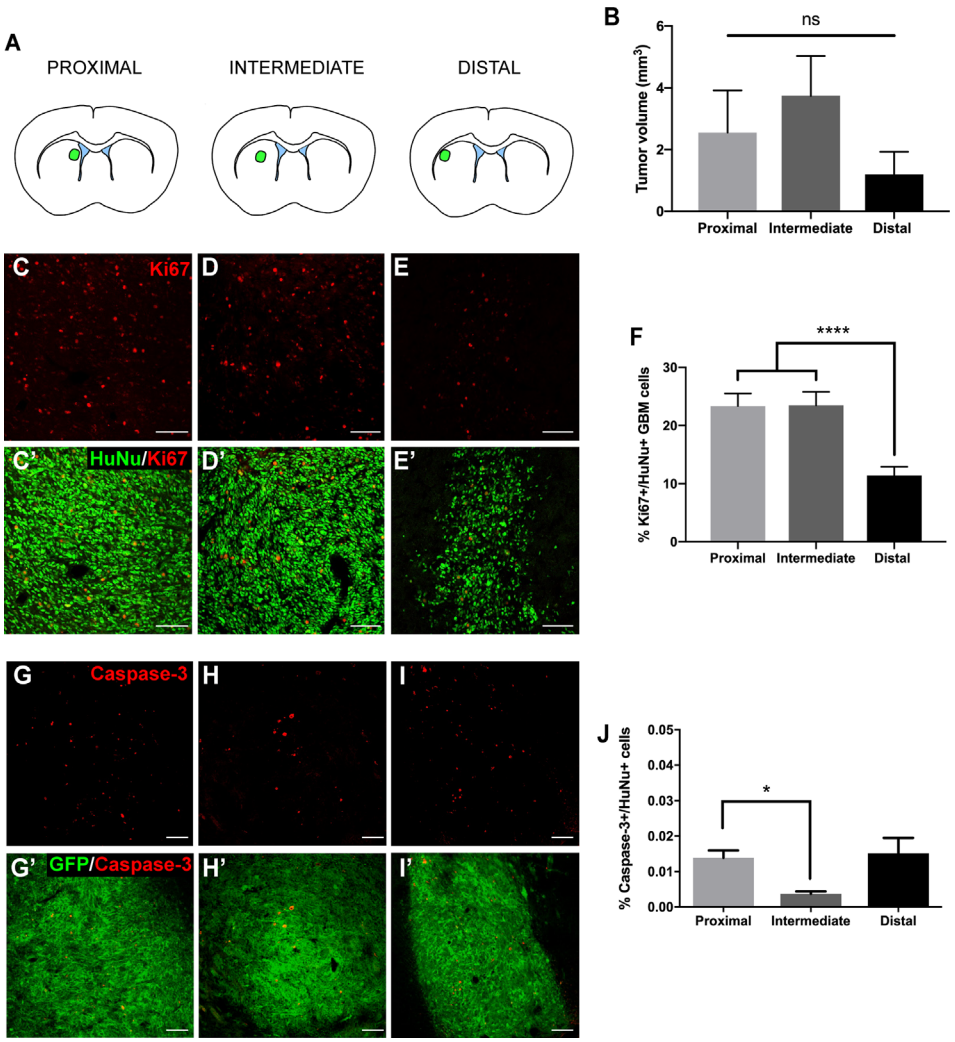

FIGURE 1 | GBM proximity to the lateral ventricle induces increased tumor growth. **(A)** Schematic illustration of the LV-proximal, LV-intermediate, and LV-distal injection sites. **(B)** Quantification of GFP+ tumor volume in LV-proximal (n = 6), LV-intermediate (n = 5), and LV-distal (n = 5) groups. **(C–E)** Representative Ki67 immunohistochemical staining in **(C)** LV-proximal, **(D)** LV-intermediate, and **(E)** LV-distal GBM. Merged with HuNu staining (green, C'-E'). Scale bar = 100 µm. **(F)** Quantification of the percentage of Ki67+/HuNu+ cells within the GBM tumor between LV-proximal (n = 6), LV-intermediate (n = 5), and LV-distal (n = 5) groups. **(G–I)** Representative cleaved caspase-3 (cleaved C3) immunohistochemical staining in **(G)** LV-proximal, **(H)** LV-intermediate, and **(I)** LV-distal GBM. Merged with GFP staining (green, G'-I'). Scale bar = 100 µm. **(J)** Quantification of the percentage of cleaved caspase-3+/HuNu+ cells in the GBM tumor between LV-proximal (n = 7), LV-intermediate (n = 3), and LV-distal (n = 5) groups. The data are presented as mean ± SEM. $^{*}p < 0.05$, $^{****}p < 0.0001$. NS, not significant.

We then evaluated the differences in tumor growth *via* bioluminescence imaging (BLI) and long-term survival outcome. Due to similar Ki67+ staining in LV-proximal and LV-intermediate tumor locations, we only used the LV-proximal tumor site for survival analysis. Tumor growth measured by increase in total flux (photons per second) was significantly higher in LV-proximal tumors than LV-distal tumors at 5 weeks post-xenograft (**Figures 2A, B**). Additionally, mice with LV-proximal tumors exhibited decreased median survival compared to their LV-distal tumor-bearing counterparts (LV-Proximal: 36 days, LV-Distal 52 days; **Figure 2C**). These findings show that we are able to effectively model several of the clinical differences of LV-proximal GBM compared to LV-distal GBM, such as increased tumor burden and decreased survival, in an immunocompromised rodent model.

Proliferation Levels in GFP-/HuNu- Cells Within the SVZ Are Decreased as a Result of GBM Tumor Proximity

The LV contains the SVZ, the largest neurogenic niche in mammals (11–14). Previous studies have indicated that the cellular populations of the SVZ are altered due to the presence of GBM (26), but do not explore the effect of tumor proximity on different neurogenic cell populations. We observed that SVZ size was not altered by the presence of tumors when compared between groups and between sides ipsilateral and contralateral

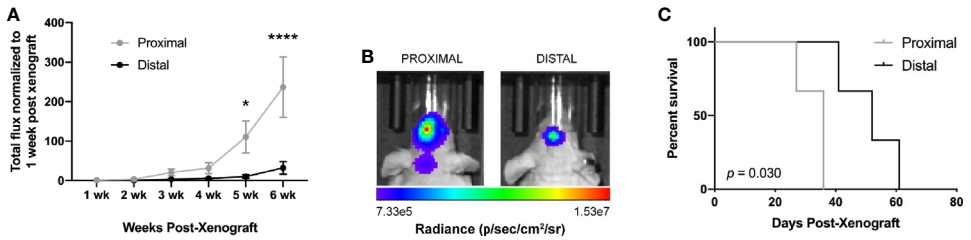

FIGURE 2 | GBM proximity to the lateral ventricles impacts long-term outcome in rodents. **(A)** Quantification of BLI total flux fold change over time in the LV-proximal and LV-distal GBM tumor conditions (n = 7). **(B)** Representative BLI images in radiance (photons/second/centimeter/steradian) of immunosuppressed athymic nude mice bearing orthotopic patient-derived GBM at LV-proximal (left) and LV-distal (right) locations at five weeks post injection. **(C)** Kaplan-Meier survival curve of mice bearing tumors in LV-proximal and LV-distal sites (n = 3). The median survival for LV-proximal or LV-distal tumor bearing mice were 36 and 52 days, respectively. The data are presented as mean ± SEM. $^{*}p < 0.05$, $^{****}p < 0.0001$.

to the tumor (data not shown). To explore the effect of GBM proximity on mouse SVZ cell proliferation, we performed immunostaining for Ki67 and evaluated Ki67+/GFP-/HuNu- cells in the regions of the SVZ where the tumor growth was also present, in both ipsilateral and contralateral hemispheres. We determined that the proliferation rate of innate cells in the SVZ ipsilateral to the tumor site is significantly decreased compared to the contralateral SVZ in the presence of LV-proximal and LV-intermediate GBM, but not in LV-distal GBM (LV-proximal: 14.01 cells/mm^2 ipsilateral *vs.* 40.97 cells/mm^2 contralateral; LV-intermediate: 15.85 cells/mm^2 ipsilateral *vs.* 40.41 cells/mm^2 contralateral; LV-distal: 24.18 cells/mm^2 ipsilateral *vs.* 50.60 cells/mm^2 contralateral; **Figures 3A–G**), indicating that tumor proximity decreases SVZ cell proliferation.

To verify that SVZ cells have decreased levels of mitosis with increased tumor proximity, we also performed IHC for phosphohistone H3 (pH3), a marker of chromatin condensation with higher specificity for mitosis than Ki67. Again, we determined that the proliferation rate of HuNu- cells in the SVZ ipsilateral to the LV-proximal tumor is significantly decreased compared to the contralateral hemisphere (LV-proximal: 92.51 cells/mm^2 ipsilateral *vs.* 182.93 cells/mm^2 contralateral; **Supplemental Figures 3A–E**). In contrast, there was no decrease in the proliferation of the ipsilateral SVZ cells in LV-distal GBM when compared to the contralateral SVZ (LV-distal: 233.62 cells/mm^2 ipsilateral *vs.* 214.67 cells/mm^2 contralateral; **Supplemental Figures 3A–E**). Despite changes in proliferation, almost no cleaved caspase-3 labeling was seen in GFP- cells of the SVZ (data not shown). These findings further support a shift in the proportion of SVZ cell proliferation in response to tumor proximity.

SOX2+/GFAP+/HuNu- Cells Within the SVZ Are Decreased as a Result of GBM Tumor Proximity

The SVZ contains NSCs that differentiate into progenitor cells, ultimately leading to the production of new neurons and glia throughout life (15–18). To examine how tumor proximity affects these populations of cells, we performed immunohistochemical staining for a variety of markers of different neurogenic cell types. We evaluated the staining for SOX2, a marker of NSCs and progenitors (33), in response to tumor proximity. SOX2+/HuNu- cell density is significantly decreased in the ipsilateral SVZ of LV-proximal tumors compared to LV-intermediate and LV-distal tumors (LV-proximal: 1273.83 cells/mm^2; LV-intermediate: 2706.12 cells/mm^2; LV-distal: 2853.37 cells/mm^2; **Figures 4A–D**). Cells that are positive for both GFAP and SOX2 and negative for HuNu, that represent astrocytic NSCs of the SVZ (34), were also decreased in response to LV-proximal tumors compared to LV-intermediate and LV-distal tumors (LV-proximal: 393.20 cells/mm^2; LV-intermediate: 604.48 cells/mm^2; LV-distal: 673.43 cells/mm^2; **Figures 4E–H**), showing that there is a decrease in NSCs and progenitors in the SVZ in response to LV-proximal tumors.

GBM Proximity to the Lateral Ventricle Decreases Oligodendrocyte Precursor and Neuroblast Density in the SVZ

To examine changes in neurogenic progeny in the SVZ, we also analyzed the number of GFP-/oligodendrocyte precursor cells (OPCs) and neuroblasts in relation to GBM tumor proximity. The differentiation of NSCs to OPCs is accompanied by the expression of the transcription factor OLIG2. We found that the presence of GBM significantly decreases the number of GFP-/OLIG2+ cells in the ipsilateral SVZ compared to the contralateral SVZ among all groups (ipsilateral 261.95 cells/mm^2 *vs.* contralateral 353.96 cells/mm^2; **Figure 5A**). Additionally, there are significantly fewer GFP-/OLIG2+ cells in the ipsilateral SVZ of LV-proximal group than in the LV-intermediate or LV-distal groups (LV-proximal: 159.88 cells/mm^2; LV-intermediate: 339.27 cells/mm^2; LV-distal: 386.19 cells/mm^2; **Figures 5B–E**), indicating that GBM proximity to the SVZ significantly decreases OPC generation in the SVZ.

The differentiation of NSCs into neuroblasts, as well as their migration through the brain and incorporation into the olfactory bulb, is well-studied in rodents (16, 35). Previous studies have shown an increase in the levels of SVZ neuroblasts in the presence of GBM, as well as neuroblast migration to the tumor site (26). In order to examine how the neuroblast population was changed in response to tumor proximity to the SVZ, we performed immunohistochemical staining for doublecortin (DCX+), a widely used marker for migratory neuroblasts.

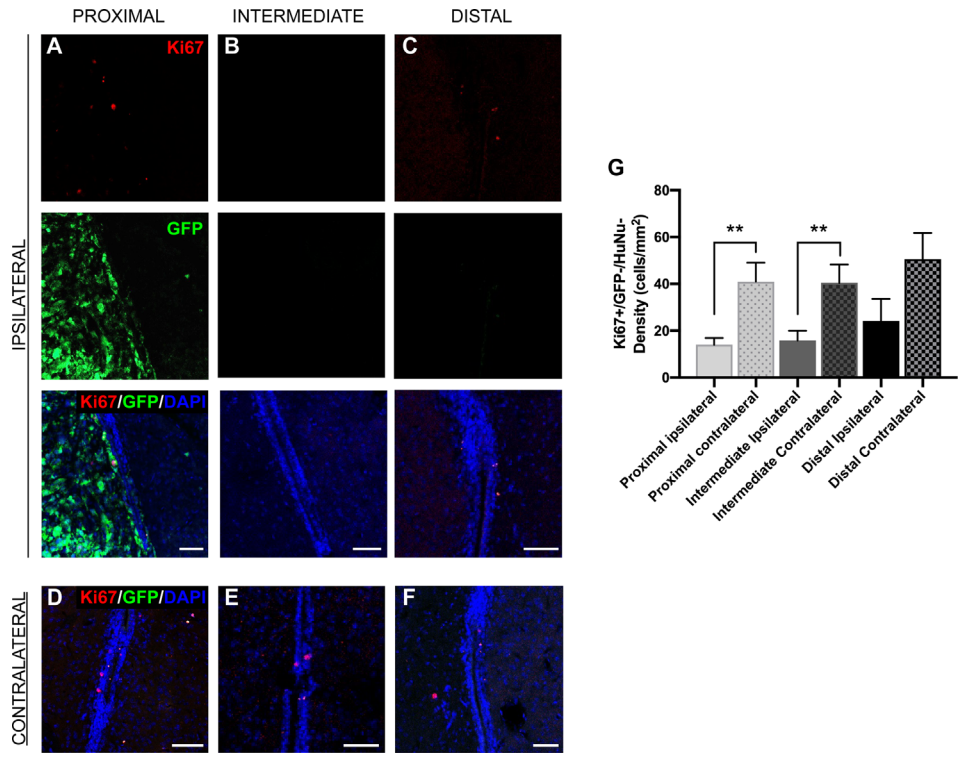

FIGURE 3 | GBM proximity to the LV negatively influences Ki67 expression in the SVZ. **(A–F)** Representative images of immunohistochemical staining for Ki67 in GFP- cells of the SVZ **(A–C)** ipsilateral and **(D–F)** contralateral to the tumor. Red = Ki67, green = GFP, Blue = DAPI. This was compared between **(A, D)** LV-proximal, **(B, E)** LV-intermediate, and **(C, F)** LV-distal GBM groups. Scale bar = 50 μm. **(G)** Quantification of Ki67+ cell density in the SVZ comparing between the SVZ ipsilateral and contralateral to the GBM in LV-proximal (n = 6), LV-intermediate (n = 5), and LV-distal (n = 5) mice. Data are presented as mean ± SEM. $^{**}p < 0.01$,

Interestingly, there was no significant change in GFP-/DCX+ cells among groups when measuring in the SVZ ipsilateral to the injection site (LV-proximal: 1608.86 cells/mm^2; LV-intermediate: 1936.69 cells/mm^2; LV-distal: 2484.84 cells/mm^2; **Supplemental Figure 4**), although there was a trend towards decreased GFP-/DCX+ cells with increased GBM proximity to the LV. These findings suggest that the proximity of GBM to the LV does not affect NSC differentiation down the neuroblast lineage, despite changes in the number and proliferation of NSCs. We did not observe any GFP-/DCX+ cells migrating to the tumor site (data not shown). While there were no significant changes in the ipsilateral hemisphere to the tumor, mice with LV-proximal tumors had significantly decreased DCX+ cells in the contralateral hemisphere than LV-intermediate conditions (LV-proximal: 1295.65 cells/mm^2; LV-intermediate: 2427.31 cells/mm^2; LV-distal: 1988.12 cells/mm^2, **Figures 6A–D**).

DISCUSSION

In this study, we highlight a two-way relationship between GBM tumors and SVZ biology dependent on tumor proximity to the LV in rodents. Our results indicate that human GBM cells respond to the LVs in a proximity-dependent manner by increasing their proliferation, ultimately resulting in decreased survival. Furthermore, we observed that tumor proximity to the LV decreases some aspects of neurogenesis in the SVZ, including proliferation as well as the density of NSCs and progenitors.

GBM tumors are more malignant in patients when located proximal to the LV than in LV-distal counterparts. The increased malignancy is evidenced by increased tumor size, increased distal recurrence, and decreased survival independent of extent of resection (5, 6, 8, 9, 36). Our work is the first to study the proximity-dependent interaction between GBM and the SVZ in a rodent model. This model recapitulates several of the features of LV-proximal GBM in patients, including increased tumor growth, increased proliferation, and decreased survival. It remains unclear the reason for increased malignancy in these tumors. Despite previous studies describing worse prognosis in patients with GBM close to the LVs, there is no substantial evidence tying these clinical findings to a molecular signature of GBM. Although some studies have linked LV-proximal GBM to characteristics such as molecular subtype and the expression of stem cell markers, others have found no association of LV-proximal GBM with a molecular signature (37–39). This may indicate that the increased malignancy of LV-proximal GBM may not be a cell-intrinsic factor, but a product of the SVZ microenvironment. This is supported by our previous studies as well as this work, where tumors derived from the same cell line become more malignant in response to the LV microenvironment.

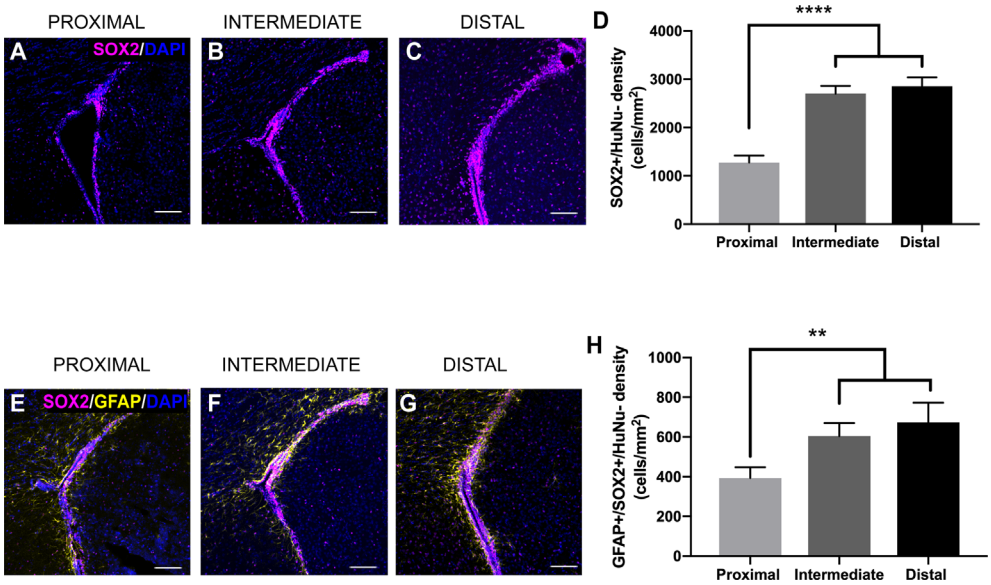

FIGURE 4 | SVZ SOX2+ progenitor number is altered by LV-proximal GBM. **(A–C)** Representative images of SOX2+ cells in the GFP-/HuNu- cells of the SVZ in **(A)** LV-proximal, **(B)** LV-intermediate, and **(C)** LV-distal groups. Scale bar = 100 μm. **(D)** Quantification of SOX2+ cell density in the SVZ of LV-proximal, LV-intermediate, and LV-distal GBM mice. **(E–G)** Representative images of GFAP+/SOX2+ cells in the SVZ of **(E)** LV-proximal, **(F)** LV-intermediate, and **(G)** LV-distal groups. Scale bar = 100 μm. **(H)** Quantification of GFAP+/SOX2+ cell density in the SVZ of LV-proximal (n = 7), LV-intermediate (n = 5), and LV-distal (n = 5) GBM mice. Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$.

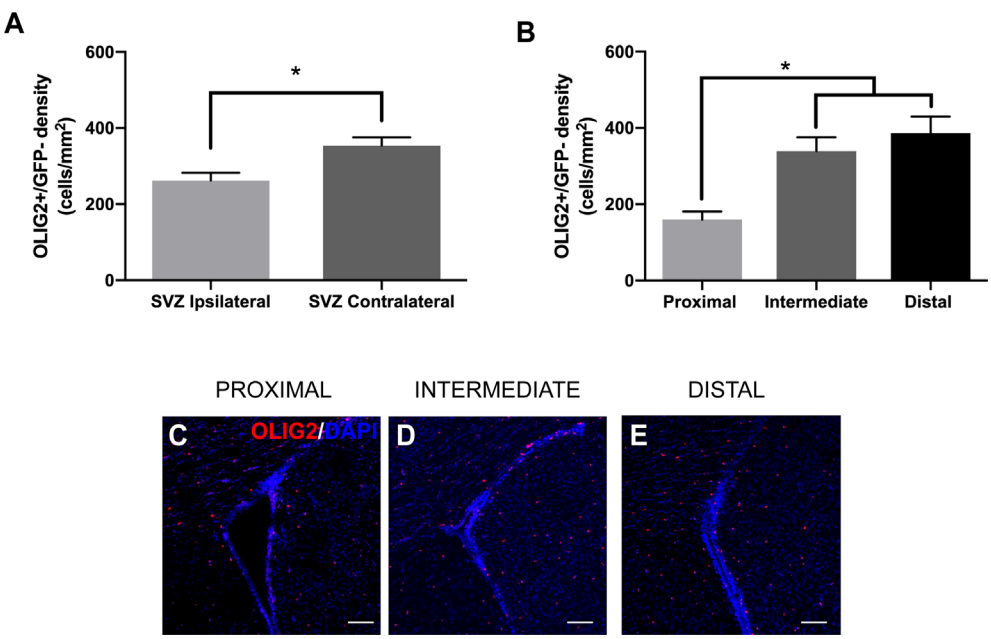

FIGURE 5 | LV-proximal GBM reduces the number of OLIG2+ progeny in the ipsilateral SVZ. **(A)** Quantification of OLIG2+ cell density in the ipsilateral and contralateral SVZ of all groups. **(B)** Quantification of OLIG2+ cell density in the SVZ ipsilateral to the tumor in LV-proximal, LV-intermediate, and LV-distal GBM mice. **(C-E)** Representative images of the ipsilateral SVZ in **(C)** LV-proximal (n = 7), **(D)** LV-intermediate (n = 5), and **(E)** LV-distal (n = 5) groups. Scale bar = 100 μm. Data are represented as mean ± SEM. $^{*}p < 0.05$.

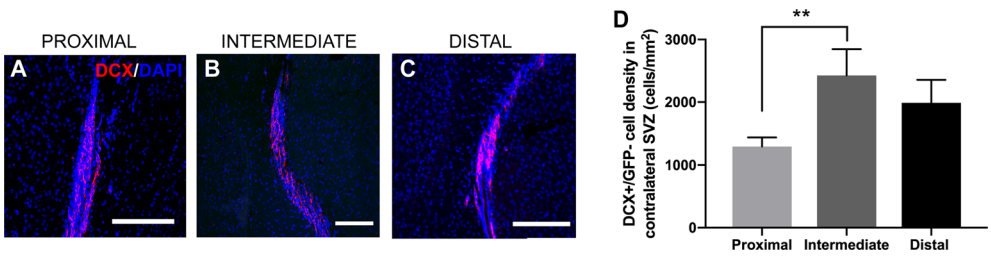

FIGURE 6 | LV-proximal GBM decreases the number of neuroblasts in the contralateral SVZ. **(A–C)** Representative images of DCX+ cells in the contralateral SVZ to the GBM in **(A)** LV-proximal (n = 7), **(B)** LV-intermediate (n = 3), and **(C)** LV-distal (n = 5) groups. Scale bar = 100 μm. **(D)** Quantification of DCX+ in the contralateral SVZ to the tumor between groups. Data is presented as mean ± SEM. $^{**}p < 0.01$.

Though this work does not identify the components responsible for increased malignancy in these tumors, there are several potential sources of neurogenesis-supporting factors that may contribute to GBM growth. The SVZ contains many NSC and progenitor cells that may interact directly with GBM cells, thereby increasing proliferation. Additionally, the SVZ contains many soluble factors which contribute to neurogenesis of SVZ NSCs that GBM cells may take advantage of. These factors may be released from the NSCs themselves or be contained within the nearby cerebrospinal fluid (CSF), ultimately contributing to GBM malignancy (40–43). Our previous work has revealed several CSF-induced transcriptomic changes in primary GBM cells, including upregulation of SERPINA3, MYC, and SPP1 (40, 41). Gene ontology analysis has indicated an upregulation in cell viability, movement, and migration pathways induced by CSF (40), all of which may contribute to the malignancy-promoting pathways in LV-proximal tumors. These *in vitro* findings warrant the study of transcriptomic changes in SVZ and GBM cells *in vivo* using a model similar to the one presented here. The elucidation of the bidirectional mechanisms supporting GBM tumor growth requires further unbiased transcriptomic studies in both animal models and navigation-guided tumor biopsy samples.

We observed decreased proliferation of SVZ cells in the presence of LV-proximal and LV-intermediate GBM tumors compared to LV-distal tumors. These findings agree with previous work, which found decreased proliferation of SVZ cells in the presence of GBM in an syngeneic intracranial C6 rat glioma model (26). However, other studies have found that signaling from the tumor increases SVZ proliferation, resulting in hypertrophic, hypercellular areas and increased levels of stem cell markers such as Nestin (44). One potential reason for these contradictive findings is a different mechanism of interaction by GBM cells and resident SVZ cells dependent on tumor proximity to the LV. Soluble factors that are expressed by GBM, such as PDGF-A, have the ability to increase the levels of proliferation in NSCs, resulting in hypertrophic areas of the SVZ that share some features of gliomas (45, 46). However, a different effect is seen when wild-type NSCs are directly placed in co-culture with *Ink4a/Arf-/-*, EGFRvIII mutated NSCs that generate tumors *in vivo* which recapitulate many features of human GBM (47, 48). Here, direct contact with glioma-like cells results in decreased levels of proliferation and increased levels of quiescence in NSCs primarily mediated by increased Notch signaling activation (48). This suggests that GBM proximity to the LV may differentially affect resident NSCs, where LV-proximal tumors induce decreased proliferation and increased quiescence through cell-cell contact *via* Notch signaling, while LV-distal tumors may signal to NSCs primarily through secreted components.

The SVZ of mice with LV-proximal tumors have decreased neurogenic capability compared to those bearing LV-intermediate or LV-distal tumors, measured through decreased SOX2+ stem cells and progenitors, decreased SOX2+/GFAP+ NSCs, and decreased numbers of OLIG2+ cells in the ipsilateral SVZ. Though there is no significant difference in the numbers of DCX+ neuroblasts in the ipsilateral SVZ among groups, there is also a decrease in DCX+ neuroblasts in the contralateral SVZ of LV-proximal tumor mice compared to LV-intermediate tumor mice, suggesting decreased neurogenesis in the contralateral hemisphere of LV-proximal mice. Interestingly, previous data shows that GBM tumors increase neuroblast density in the SVZ (26), which differs from our present findings. SOX2+ NSCs are able to give rise to new neural cells through their multipotent potential (49). Therefore, by decreasing the number of stem cells or the rate and number of cell divisions, it is expected that we will find a lower rate of neuronal renewal in SVZ, which is a direct alteration in neurogenesis (50). Decreased neurogenesis in the ipsilateral SVZ may be due to the previously mentioned increase in NSC quiescence *via* Notch signaling through cell-cell contact. Increased quiescence of NSCs results in both decreased proliferation and decreased differentiation into progenitors (51). The decrease of neuroblasts in the contralateral hemisphere, however, may suggest the secretion of a circulating factor that is able to affect SVZ neurogenesis in the hemisphere contralateral to the tumor. The identification of factors that decrease SVZ neurogenesis secreted by GBM cells or other cells in response to the presence of GBM, such as ependymal cells or cells of the choroid plexus, need to be further explored.

Alternatively, the decrease of neuroblasts in the contralateral hemisphere may be related to decreased CSF volume or flow throughout the ventricular system without directly altering secreted factors. Neurogenesis is regulated in part by both the flow and the contained chemokines within the CSF. Both the proliferation of NSCs and the migration of newly differentiated neuroblasts down the rostral migratory stream are regulated in a flow-dependent manner (43, 52). The decrease of CSF flow and the

loss of chemokine gradient may affect neurogenesis, particularly stem cell proliferation and neuroblast migration. In support of this scenario, glioma-bearing mice have reduced CSF circulation and output compared to non-tumor controls (53), which could implicate a loss of flow-dependent regulation in GBM. The contribution of CSF flow to neurogenesis and GBM malignancy in this animal model require further studies to fully understand.

Interestingly, there are significant decreases in the level of cleaved caspase-3 labeling in the tumors LV-intermediate tumor group. GBM tumors have quite low levels of caspase-3 labeling in humans (54), so further decrease in apoptosis of the tumor may be related to increased growth. This, accompanied by increased Ki67+ GBM cells, may indicate that LV-intermediate tumors were located in a "sweet spot" where the tumors are able to take advantage of neurogenic factors contained within the SVZ niche without leading to significant neurogenic disruption. The signaling pathways between the SVZ and GBM that regulate cell proliferation and apoptosis need to be further studied in order to determine the molecular contributors to this phenomenon.

In summary, this study provides the development of a novel rodent model of LV-proximal GBM. Due to the limitations of using human cells in an immunocompromised rodent model, it will be necessary to further evaluate and validate these observations in immunocompetent murine models. The proximity of the tumor to the LV results in increased tumor proliferation, increased tumor growth, and decreased survival. Additionally, we have determined that GBM proximity to the LV also negatively impacts the number of NSCs and downstream progenitors in the SVZ. This model will be invaluable for future studies to describe the interactions between the SVZ and GBM tumors, as well as for the investigation of novel therapeutics to target signaling between these two sites. Ultimately, these findings encourage future studies to elucidate the bidirectional molecular signaling between GBM and the SVZ, particularly the identification of pathways contributing to tumor progression in LV-proximal GBM patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Mayo Clinic.

AUTHOR CONTRIBUTIONS

EN, CR-P, and HG-C, conceptualized, lead the project, and analyzed the data. LR, EN, RB-V, SJ, ML-V, AC, NZ, CV-R, CR-P, and HG-C performed the experiments and analyzed the data. AQ-H performed tissue collection. HG-C provided funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.650316/full#supplementary-material

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