Working Towards Identifying the Cell of Origin of IDH wild-type Glioma

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Abstract

Glioma stem cells (GSCs) are thought to be capable of initiating and maintaining isocitrate dehydrogenase-wildtype glioblastomas. GSCs share properties with neural stem cells (NSCs) present in the subventricular zone (SVZ) of the human brain—properties such as the capacity for self-renewal, differentiation, and migration. Therefore, NSCs are candidates for the origin of GSCs. We hypothesize that cells derived from the SVZ of a glioma patient will express markers of GSCs and NSCs; furthermore, these cells will align closer to the NSCs' genotype and bear less mutational load when compared to GSCs derived from the tumor. To test this hypothesis, we study stem cells derived from the SVZ and the tumor of the same patient by molecularly and functionally characterizing each subset and performing whole genome sequencing to verify their mutational load. Our findings demonstrate that SVZ and tumor-derived cells have the genetic expression and functional characteristics of GSCs, as well as the genetic expression of NSCs. While SVZ-derived cells express more NSC markers and show more resistance to chemotherapy when compared to tumor-derived cells, the mutational load in both cell populations is similar. Our findings suggest that both cell lines are GSCs that arise from a common source but represent different clones. Whether or not GSCs derived from the SVZ align more proximally within the NSC lineage—when compared to GSCs derived from the tumor—was not conclusively demonstrated in our study.

Resumé

De nombreuses études s'accordent sur le fait que les cellules souches de glioblastome (CSG) sont en mesures d'initier et de maintenir l'isocitrate déshydrogénase (IDH) de type sauvage dans les glioblastomes. Les CSG partagent les mêmes propriétés que les cellules souches neurales (CSNs) présentes dans la zone sous-ventriculaire (SVZ) du cerveau humain, tels que la capacité de se renouveler et de se différencier, ainsi que le pouvoir de migration cellulaire. Pour toutes ces raisons, les CSNs sont les candidats parfaits pour être l'origine des CSG. Nous avons pour hypothèse que les cellules dérivées du SVZ des patients atteints de glioblastomes vont exprimer autant les biomarqueurs des CSG que des CSNs. De plus, ces cellules seront génétiquement plus proches des CSNs et auront moins de mutation en comparaison avec les CSG dérivées des tumeurs. Pour vérifier cette hypothèse, nous allons analyser l'aspect moléculaire et fonctionnel ainsi que la signature des CSG dérivées du SVZ et les comparer avec les CSG dérivées des tumeurs d'un même patient en utilisant le séquençage du génome en entier. Nos études démontrent que les cellules dérivées des SVZ et des tumeurs ont tous deux les mêmes capacités fonctionnelles et expressions génétiques que celles des CSG. Mais aussi la même expression génétique des CSNs. Les cellules dérivées du SVZ expriment plus de biomarqueurs similaires aux CSNs et sont plus résistantes à la chimiothérapie en comparaison avec les cellules dérivées des tumeurs, quoique la quantité de mutation est la même dans les deux populations. Nos recherches suggèrent que toutes les CSG proviennent de la même source, mais représentent différents clones. Toutefois, nous ne pouvons pas encore conclure si les CSG dérivées du SVZ sont plus semblables aux lignées des CSNs lorsqu'elles sont comparées aux CSG provenant des tumeurs.

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Contribution of Author

The work presented in this thesis has been prepared and written by Mariam Alrashid, with the exception of the following experiments described in results: FACS in Figure 1 is prepared by Shamini Ayyadhury, TMZ assay in Figure 5 prepared by Dr. Phuong Le, Salma Baig, and Redouane Allach and Circos plot in Figure 6 prepared by Dr. Mathieu Bourgey.

Rationale of Study & Hypothesis

IDH-wildtype glioblastoma is the most common and the most lethal malignant brain tumor in adults. So far, the tumor has no effective treatment, and the life expectancy of affected patients is 12.4 months. Over the last decade, comprehensive genomic analysis has allowed us to better understand the pathophysiology of this disease; however, the origin of glioblastoma remains elusive. Identifying the cell of origin of IDH-wildtype glioblastoma would not only further increase our understanding of gliomagenesis, but it would also enable the development of advantageous, targeted therapy in the future. A rare cell population referred to as 'glioma stem cells' is thought to be the source of glioblastomas and the cause for treatment failure. Ongoing evidence is showing similarities between the biological properties of glioma stem cells and those of neural stem cells (NSCs) residing in the subventricular zone (SVZ) of the human brain. This has led to the proposition that glioma stem cells arise from a malignant transformation of NSCs. In this manner glioma stem cells maintain the stemness and migratory characteristics of NSCs while acquiring additional mutations that enable them to evade the tight control of the SVZ niche and that lead to uncontrolled proliferation and tumorigenesis. Nevertheless, there is not enough direct evidence supporting this hypothesis. Therefore, we hypothesize that cells derived from a seemingly normal SVZ of a glioma patient will express markers of GSCs and NSCs; furthermore, these cells will align closer to the NSCs' genotype and bear less mutational load when compared to glioma stem cells derived from the tumor. This would further support the proposition that NSCs are the cell of origin of IDH-wildtype glioblastomas.

List of Figures and Tables

List of Abbreviations

SOX2: SRY-Box 2

OLIG2: Oligodendrocyte Transcription Factor 2

CD133: PROM1: Prominin-1

CD15: SSEA-1: Stage-Specific Embryonic Antigen 1

CD44: Cell Surface Glycoprotein CD44

O4: Oligodendrocyte Marker O4

NG2: Neuron Glial Antigen 2

SALL2: Spalt Like Transcription Factor 2

GLAST: Glutamate/Aspartate Transporter 1

POU3F2: POU Class 3 Homeobox 2

PAX6: Paired Box 6

NR2E: Nuclear Receptor Subfamily 2 Group E Member 1

DCX: Doublecortin

DRR: Down-Regulated in Renal Cell Carcinoma 1

BLBP: Brain Lipid Binding Protein 7

EphA2: Ephrin Type-A Receptor 2

Literature Review:

Overview of Glioblastomas:

Gliomas are the most common brain cancer in adults. The World Health Organization (WHO) previously classified gliomas into four grades — two low-grade gliomas (LLGs) and two high-grade gliomas (HGGs) — based on histopathological features supplemented with few genetic parameters; in this classification, the higher the grade of the tumor, the more aggressive its behavior and the poorer the prognosis [1]. Irregular hyperchromatic nuclei and rare mitotic events are histological features of LGGs; while hyper-cellularity, prominent mitosis, vascular endothelial proliferation, and necrosis are pathognomonic of HGGs. LGGs constitute grade 1 and 2; HGGs constitute grade 3 and 4 gliomas. Grade 1 gliomas are generally well-circumscribed lesions with low proliferative capacity and the possibility of a cure after complete surgical resection. Grade 2 are diffuse lesions that tend to recur and progress to higher grades of malignancy. Grade 3 is applied to lesions with clear histological evidence of malignancy. Grade 4 gliomas are infiltrative malignant gliomas with rapid evolution and a fatal outcome; these gliomas are known as glioblastomas (GBs). GBs arise either *de novo* or from lower-grade precursors. More than 90% of GBs arise *de novo*, these are termed “primary GBs” and typically affect the elderly; conversely, GBs arising from lower-grade gliomas are termed “secondary GBs” and affect a younger population [1].

In 2016, this histopathological classification has been revised and replaced by a new classification that considers, in addition to the above histological features, well-

established genetic mutations found in gliomas. Gliomas are now categorized into two distinct groups, based on the presence or absence of a mutation in an enzyme known as isocitrate dehydrogenase (IDH): IDH-mutant and IDH-wildtype gliomas [2] (Figure 1). IDH mutations occur in the genes that encode the enzyme isocitrate dehydrogenase 1 or 2. Wildtype IDH converts isocitrate to α-ketoglutarate. An IDH mutation alters the function of the enzyme and produces a neo-enzyme that converts α-ketoglutarate to R-2 hydroxyglutarate (2-HG). 2-HG is a metabolite that remodels the methylation landscape of the genome of gliomas resulting in a distinct phenotype known as the CpG hyper-methylator phenotype (G-CIMP) [3]. IDH1 mutations occur exclusively in codon 132, and IDH2 mutations occur in codon 172. IDH mutation characterizes a distinct entity of gliomas that appear as grade 2 gliomas and then progress with time to a higher-grade, giving rise to secondary GBs — as opposed to IDH-wildtype gliomas, which are essentially primary GBs. Therefore, IDH-mutant gliomas offer a better prognosis; overall patient survival is in the range of 5-25 years, while IDH-wildtype gliomas correlate with an overall patient survival of less than 2 years [4, 5]. Other genetic alterations found in secondary GBs include: tumor protein 53 (TP53) mutations (81%), alpha thalassemia/mental retardation syndrome X-Linked (ATRX) mutations (71%), and Loss of chromosome arm 10q [2]. As for primary GBs, we find the following the genetic alterations: telomerase reverse transcriptase (TERT) promoter mutations (80%), homozygous deletion of Cyclin Dependent Kinase Inhibitor 2A/Cyclin Dependent Kinase Inhibitor 2B (CDKN2A/CDKN2B) (60%), loss of chromosomes 10p (50%) and 10q (70%), epidermal growth factor receptor (EGFR) alterations (55%), and phosphatase and tensin

homolog (PTEN) mutations/deletion (40%) [2]. Recently, in 2021, this classification underwent further modification that considered the prognostic implications of different gliomas (Figure 2). While diffuse gliomas are still categorized into two distinct groups, based on the presence or absence of IDH mutation, the term “glioblastoma” has been reserved exclusively to IDH-wildtype gliomas and therefore all IDH-wildtype glioma are considered WHO grade 4 tumors [6]. The diagnosis of GB can now be established by the presence of one of the following genetic alterations, even if the histopathological criteria of GB isn’t met: CDKN2A/2B deletion, EGFR amplification, or TERT promoter mutation [6]. IDH-mutant astrocytomas are grouped in a separate category and will be graded as 2, 3 or 4 based on histopathological features or genetic alteration [6].

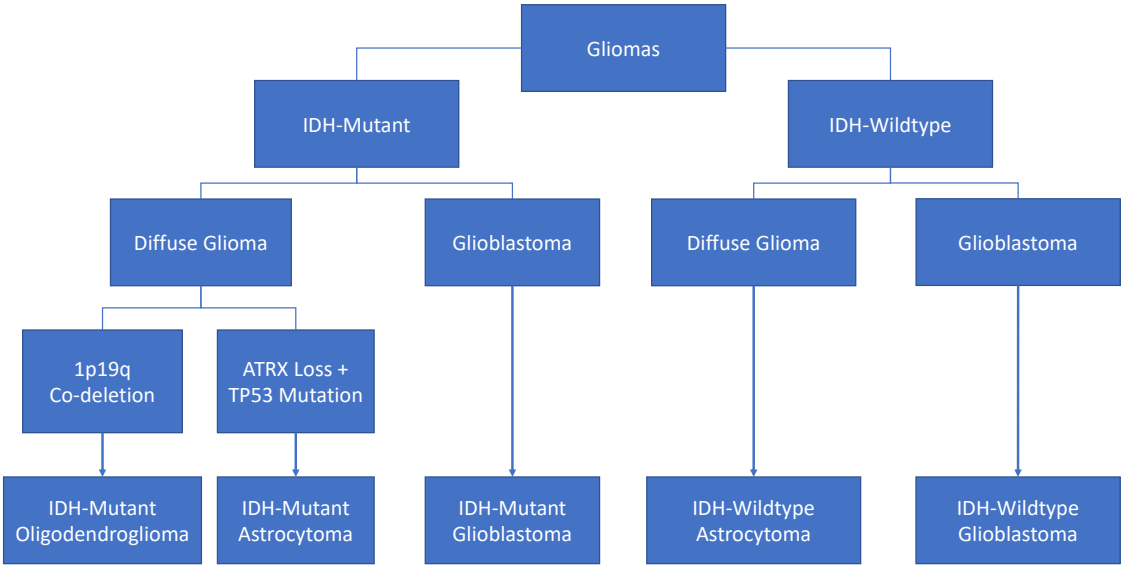
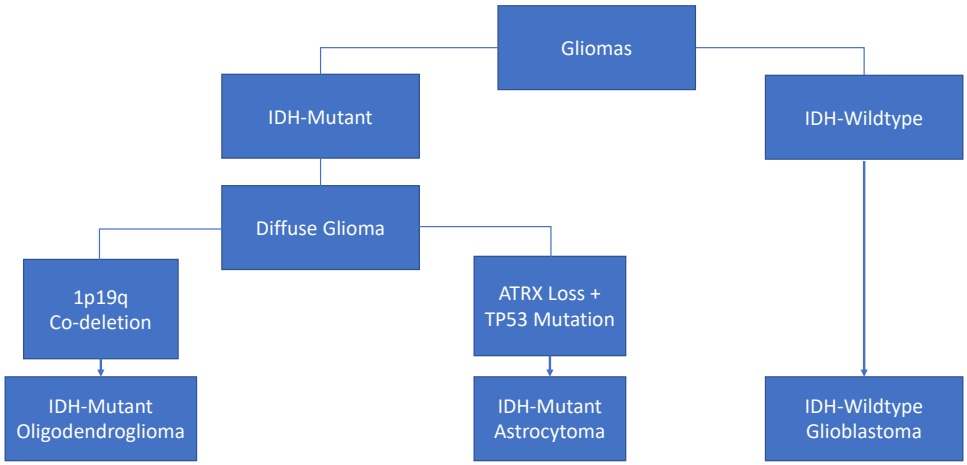


Figure 1: The 2016 WHO glioma classification. Source: Data adapted from David N. Louis, Hiroko Ohgaki, Otmar D. Wiestler, Webster K. Cavenee (Eds): WHO Classification of Tumors of the Central Nervous System (Revised 4th edition). IARC: Lyon 2016



*The diagnosis of Glioblastoma is established histologically or, if the histological criteria not met, when one of the following genetic criteria is fulfilled: CDKN2A/2B deletion, EGFR amplification, or TERT promoter mutation
**If genetic testing is inconclusive or not done then, the tumor is diagnosed based solely on the histopathological features and labelled as NOS (e.g. Astrocytoma NOS)

Figure 2: The 2021 WHO glioma classification. Source: Data adapted from Rushing, E.J.: WHO classification of tumors of the nervous system: preview of the upcoming 5th edition. Magazine of European Medical Oncology, 2021.

Furthermore, The Cancer Genome Atlas Consortium (TCGA) performed high dimensional profiling and genomic classification of nearly 600 GBs. This comprehensive genomic characterization revealed four distinct groups of GBs based on transcriptional profiles; these are referred to as proneural, neural, classical, and mesenchymal [6]. However, these GBs subtypes have been validated and recategorized into three groups instead: proneural, classical, and mesenchymal [7, 8]. The proneural subtype is distinctively characterized by the presence of IDH mutation, TP53 mutation, and platelet-derived growth factor receptor A (PDGFRA) mutations or amplifications [5, 7, 9]. Moreover, DNA methylation studies showed that the proneural subtype was enriched with the G-CIMP methylation profile, a CpG island hyper-methylation phenotype that has been associated with a better survival outcome [5, 9, 10]. Clinically, this subtype typically

affects younger patients, and demonstrates a less aggressive behavior compared to the classical and the mesenchymal GB subtypes [5, 9]. Interestingly, the proneural subtype exhibits a normal expression of EGFR, an intact PTEN and NOTCH activation; genes that are characteristically mutated in other GB subtypes [5, 9]. The classical subtype is characterized by EGFR amplification, lack of TP53 mutations and homozygous deletion of CDKN2A [5, 7, 9]. Moreover, the most common genetic abnormalities found in GBs; chromosome 7 amplification and chromosome 10 deletion are found highest in the classical subtype [5]. Similar to the proneural subtype, the classical subtype lacks the genetic abnormities that characterize other GB subtypes such as IDH, TP53, PDGFRA and NF1 mutations [5]. The predictive value of the DNA methylation of O-6-methylguanine-DNA methyltransferase (MGMT) gene promoter to treatment response has been shown to hold true only for patients who harbored the classical subtype of GBs [10]. The mesenchymal subtype is characterized by Neurofibromin 1 (NF1) and PTEN mutations [5, 7, 9]. It particularly shows overexpression of angiogenesis genes such as CD31, vascular endothelial growth factor (VEGF), and vascular endothelial growth factor receptor 2 (VEGFR2) and inflammatory markers such as fibronectin and COX2 [5, 11]. It also uniquely expresses microglia markers CD68, PTPRC, and TNF [7]. These genetic features are highlighted by the presence of extensive necrosis and inflammation along with a predominant presence of immune cells in these mesenchymal GBs. Therefore, some have argued that mesenchymal subtypes may have favorable responses to immunotherapy — as opposed

to traditional chemotherapy and radiotherapy, given their strong associations with the activation and recruitment of immune cells [11].

Yet, the prognostic value of these molecular subtypes remains arguable despite initial studies linking mesenchymal GBs with poorer prognosis, especially in the context of tumor recurrence [9, 12, 13]. Of more significance was the identification of all these molecular subtypes with variable degrees in individual tumors, accounting for what is known as inter-tumor heterogeneity [14-17]. This finding might explain why different cells in an individual tumor have different properties and possess different levels of treatment resistance that yield variable treatment responses. Moreover, the proneural signature is found to be enriched with genes associated with an oligodendrocytic signature; while the classical signature is enriched with genes associated with an astrocytic signature [5, 10]. This confirms that all gliomas express markers of distinct neural lineages, suggesting that the cell of origin is somewhere along the neural stem cell (NSC) hierarchy. Interestingly, recent studies have shown that while IDH-mutant gliomas express two distinct neural lineage markers — astrocytes and oligodendrocytes — IDH-wildtype gliomas express three distinct neural lineage markers — astrocytes, oligodendrocytes, and neurons [18, 19]. These findings can further support the hypothesis that while the cell of origin in gliomas lie along the NSC hierarchy, different neural/glial precursor cells give rise to either IDH-mutant or IDH-wildtype gliomas.

The current standard treatment for all GBs includes maximum safe surgical resection, concurrent radiation and chemotherapy in the form of temozolomide (TMZ), followed by adjuvant TMZ for 6-12 months [20]. Despite this multimodality approach,

about 70% of GB patients will experience disease progression within one year of diagnosis, with less than 5% of patients surviving five years after diagnosis [21, 22]. The poor response to treatment is related to the nature of the disease and the prescribed treatment. GBs commonly involve eloquent areas of the brain posing a challenge to an extensive surgical resection and, when a radical resection is feasible, infiltrating tumor cells invariably remain within the surrounding brain, making the procedure not curative. Radiation is very effective, but a dose escalation beyond 60 Gy often results in an increased toxicity without additional survival benefits [23]. The chemotherapeutic drug, TMZ, is an oral alkylating agent that delivers a methyl group to purine bases of DNA, commonly at the O6-guanine & N7-guanine positions. This methylation consequently damages the DNA and triggers apoptosis in tumor cells. However, some tumor cells are able to repair this type of DNA damage, by expressing a protein O^6-alkylguanine DNA alkyltransferase encoded in humans MGMT gene; thus diminishing the therapeutic efficacy of TMZ [24]. But, we observe a clear advantage in patients who receive radiation plus TMZ; those have a median survival of 14.6 months, compared to 12.1 months in patients who received radiation alone [22]. Also, a higher proportion of long-term survivors in the radiation/TMZ group was noted when these were compared to a group that only received radiation; with survival rates at 27% versus 11% at two years, and 10% versus 2% at five years, respectively [20]. In the past two decades many new therapeutic agents and modalities have been explored as an adjunct treatment to the above described GBs' standard-of-care therapy. Unfortunately, only a handful of these have shown to have a positive impact on the progression-free survival (PFS) or the overall survival (OS) of GB

patients. Lomustine, a chemotherapeutic alkylating agent, was found to significantly improve the OS of newly diagnosed GB patients who harboured the MGMT promoter methylation, when combined as adjunct treatment to the standard-of-care therapy of GB [25]. Yet, because of several weaknesses in this small, sampled randomized controlled trial, further validation with larger samples is required in future studies. Another innovative modality that is explored as an adjunct treatment of newly diagnosed GBs is tumor-treating fields (TTFeilds) [26]. TTFields produce low-intensity, alternating electric fields that are delivered by transducer arrays applied to shaved scalp. The rationale behind TTFields is that it selectively affects rapidly dividing GB cells and induce mitotic arrest and apoptosis. In a randomized controlled trial, the addition of TTFields to maintenance temozolomide chemotherapy showed improvement in OS of newly diagnosed GB patients [26]. The median OS was 20 months in the TTFields group compared to 16 months in the stand-of-care therapy group [26]. Moreover, with the current understanding of the crucial role of the immune system in GB's pathogenesis, immune checkpoint inhibitors have been explored as adjunct treatments. Nivolumab is a monoclonal antibody that blocks the human programmed death receptor-1 (PD-L1) from binding to PD-1, allowing for T cell-mediated tumor elimination. Despite initial phase I and II clinical trials showing Nivolumab as a promising and durable treatment option for GB, two randomized controlled trials, one exclusively with patients with MGMT promoter methylation and the other exclusively in patients with non-methylated MGMT promoter, showed there was no significant effect in the PFS or the OS of patients who received Nivolumab as adjuvant therapy [27, 28].

Surgical resection and additional radiation may be options for some patients upon recurrence of GB, but both options are limited because of the risk imposed by each treatment. Chemotherapy and corticosteroids may be used to palliate symptoms and improve quality of life; however, objective response rates are dismal, and progression-free survival for standard cytotoxic agents is only 3 to 6 months [29]. The following options may be pursued, as single agents or in regimens: TMZ, carboplatin, etoposide, irinotecan, and nitrosourea-based chemotherapy. Bevacizumab, a humanized monoclonal antibody that targets vascular endothelial growth factor, a protein necessary for angiogenesis has demonstrated superior progression-free survival, though no meaningful improvements have been noted with respect to overall survival [29, 30]. Molecular-targeted therapy such as tyrosine kinase and signal transduction inhibitors have been investigated, but so far failed to demonstrate any clinical benefit [31-34]. Immunotherapy in the form of a vaccine targeting EGFR variant III has also failed to confer any survival benefit [35]. Of late, the monoclonal antibody, pembrolizumab, has shown in a small trial to significantly improve OS when administered and continued as *neoadjuvant* therapy following surgery, as opposed to when it is administered as *adjuvant* therapy only after surgery [36]. The median OS in the neoadjuvant therapy patients was almost 14 months compared to 7.5 months in the adjuvant therapy patients [36].

Glioma Stem Cells:

A small group of cancer cells called glioma stem cells (GSCs) are thought to be responsible for tumor recurrence and progression [37-41]. These cells can be generally defined as glioma cells that share properties similar to normal stem cells—properties that

include self-renewal capacity, proliferation, multi-lineage potency, and migration capacity [42-44]. It is noteworthy that the nomenclature of these cells remains debatable; and, other than being referred to as GSCs, they are at times called “brain tumor-initiating cells” or “brain tumor-propagating cells” [45]. This nomenclature discrepancy reflects the lack of consensus when defining these cells: while the term “stem cell” is used, this does not necessarily entail that the cell of origin is a transformed stem cell [43, 45]. Nevertheless, it is accepted that, regardless of the term used, these cells should at least exhibit key functional properties that differentiate them from other tumor cells; this includes the capacity to self-renew, differentiate into heterogeneous types of tumor cells, and sustain a tumor growth *in vivo* [43, 45, 46].

There are numerous markers that have been shown to allow for the identification and isolation of GSCs. Of these markers are transcription factors, structural proteins and cell surface proteins that also mark normal stem cells. Examples of these commonly used markers are: SOX2, OLIG2, Nestin, CD133, CD15, and CD44 [38, 42, 47-50]. Unfortunately, these markers are neither completely sensitive nor specific in detecting the GSCs’ population and, to date, there is no single molecular marker available to accurately identify these cells [47, 51-53]. Therefore, functional validation remains essential when enriching for GSCs. These validation experiments include *in vitro* neuro-sphere formation assays, limiting dilution assays and *in vivo* tumor formation [54-56]. While the *in vitro* assays validate the capacity of self-renewal, proliferation and, stem cell frequency, the *in vivo* validate the most important functional criteria of GSCs: the capacity to initiate tumor [43, 45, 46]. This tumor-initiating capacity qualifies as the ability of a small number of cells

to initiate a tumor that recapitulates the cellular heterogeneity present in the parental tumor upon xeno-transplant [42, 44]. Recently, there have been technological advances, such as single-cell RNA sequencing (scRNA-seq), that allow for a less biased identification of GSCs in their native environments without relying on *in vitro* culture—this culture has been shown to alter the genotype of these cells [57]. In any case, these technologies are limited by reliance on cellular markers deeming the *in vivo* functional validation necessary [14, 18].

Similar to normal stem cells, the survival of GSCs relies not solely on their intrinsic metabolic/signaling pathways, but also on their interactions with their microenvironment [58-60]. As a result, GSCs relate closely to a perivascular niche that includes vasculature, neuronal cells, stromal cells, and immunological components—such as macrophages and T-cells that support their stemness [58, 60]. Moreover, glioma cells are thought to be structured in a hierarchical manner similar to normal tissues, ranging from the most primitive tumor-initiating cells that reside at the apex of the hierarchy, to the most differentiated non-tumor-propagating cells that reside at the bottom [14, 18, 40, 61]. GSCs are thought to possess unlimited capacity for self-renewal while dividing to generate a more proliferative progenitor population that is self-sustaining but has limited self-renewal capacity. Moving down the hierarchy, these progenitor cells generate a non-proliferative population that possess no, or low, tumorigenic potential eventually forming the main bulk of the tumor [37, 40, 61] (Figure 3). While the hierarchal model was initially popular, an alternative model, known as the stochastic (clonal) model has emerged. In the stochastic model, there is no sequential hierarchy of distinct sub-clones. Instead, it proposes that

normal cells over time acquire genetic mutations that transform them into potential tumorigenic cells; but, of these “potential tumorigenic cells”, additional genetic mutations are acquired, eventually conferring a selective advantage to a particular cell clone—allowing it to out compete other potential tumorigenic forming clones [37, 40]. This tumor cell expands clonally to form identical copies with identical tumorigenic potential. The implications of this model is that most glioma cells are capable of initiating and maintaining tumor growth and that the cell heterogeneity observed within the glioma reflect sub-clones at different stages of neoplastic transformation; therefore, an effective treatment requires the elimination of all clonal cells which constitute most of the tumor [40, 62, 63]. In contrast, the hierarchal model suggests that a rare cell population (GSCs) capable of initiating and maintaining tumor growth and that the cell heterogeneity observed within the tumor reflects the aberrant differentiation of the GSC. Thus an effective treatment requires the elimination of a rare set of cells, the GSCs [62]. These two models are not necessarily mutually exclusive and—although the hierarchal model emphasizes the importance of the stem cells in driving the tumor and the stochastic model emphasizes the importance of the microenvironmental influences—the combination of both models might represent how glioma cells come to exist, survive, progress, and evade treatment [40, 62, 64]. In other words, the concept of cellular plasticity—defined as the cell capacity for interconversion between differentiated and stem-like states—may explain how stochastic events are able to generate novel, hierarchically organized cell populations and how a hierarchically organized cell population reveals a wide spectrum of transitory differentiation states [64, 65].

The origin of GSCs is not yet established, but, just as there are two prevailing models for tumor propagation and heterogeneity, there are two prevailing theories on the cellular origin of GBs: the first hypothesizes that they arise from neural stem cells (NSCs) residing in the sub-ventricular zone (SVZ); the second, from de-differentiated glial cells normal or cancer [66-73].

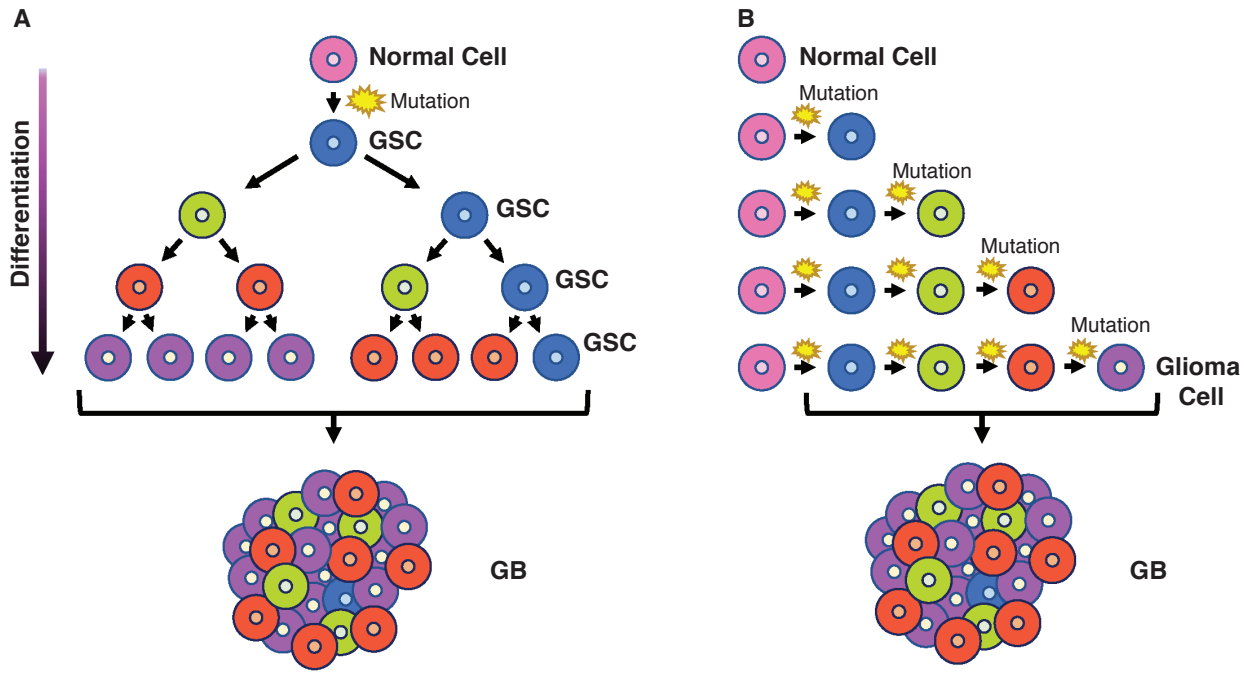


Figure 3: **Models of gliomagenesis**. (A) The hierarchical model proposes that a small cell population, known as GSCs, is responsible for originating and maintaining GBs. These GSCs are pluripotent cells that divide asymmetrically to maintain themselves while also giving rise to progenitor cells. The latter give rise to more differentiated cells. The bulk of the tumor is constituted mainly of differentiated cells that have low tumorigenic potential and a small percentage of GSCs. (B) The clonal model proposes that a normal cell undergoes a series of mutations that eventually transform it into a glioma cell. This glioma cell then multiplies exponentially (i.e. expands clonally), giving rise to the bulk of the tumor. Adapted from Bradshaw et al. [37].

NSCs as the Cell of Origin:

In recent years, numerous studies have shown the similar physiological processes that underlie both neurogenesis and gliomagenesis [16, 48, 60, 67, 74-77]. Some of these findings will be discussed below, but it is important to recognize that most of these findings and studies have been observed and conducted in the rodent brain; more specifically, in the SVZ of the rodent brain. Though the SVZ in the human and rodent species share similar characteristics, their cytoarchitecture and function are organized differently [69, 78-80]. Nevertheless, the rodent brain has proven to be "a good surrogate to the human brain", and several basic mechanisms of neuronal cellular activities have been preserved across the two species [80].

NSCs in the adult mammalian brain can be found in the following locations: the SVZ, the dentate gyrus of the hippocampus, and the subcortical white matter. The SVZ, the microscopic area lateral to the lateral ventricles, is thought to host the majority of NSCs [81-84] (Figure 4). The adult rodent SVZ consists of four cell types: ependymal cells (type E cells), astrocytic cells (type B cells), transient amplifying progenitors (type C cells), and neuroblasts (type A cells) [85]. Type B cells are divided into two subtypes, B1 and B2 cells. B1 cells are the true NSCs in the region, and type C cells are progenitor cells [86, 87]. Type A cells are highly migratory and organized into chains that leave the SVZ through cellular tunnels, formed by astrocytic cells in the rostral migratory stream, to reach the olfactory bulb. These cells are arranged in complex pinwheel structures and vary in density and organization throughout the ventricular walls [87].

The human SVZ differs slightly from its murine counterpart. The adult human SVZ consists of three anatomically distinct layers: the ependymal layer, the hypo-cellular gap, and the astrocytic ribbon [78, 88] (Figure 4). The hypocellular gap contains the processes of the underlying glial fibrillary acidic protein (GFAP)-positive astrocytes and few cell bodies. The astrocytic ribbon contains GFAP-positive astrocyte-like stem cells, also known as NSCs—similar to type B1 cells in mice [69, 78, 88]. Along with the presence of the hypocellular gap in the adult human SVZ, the absence of the neuroblast chains constitute the main differences between the murine and human SVZs [78, 88]. NSCs that are present in the SVZ of both species maintain contact with the surface of the lateral ventricle, as well as blood vessels [88].

A critical component of the SVZ is the “neurogenic niche”. This refers to the complex microenvironment that consists of ependymal cells, astrocytes, pericytes, microglia, macrophages, and neurons along with blood vasculature and a specific ECM [58]. The niche—through cell–cell interactions, the secretion of soluble factors (such as nitrite oxide, growth factors or neurotransmitters) and extracellular matrix—provides a special environment that sustains the self-renewal capacity and proliferation (or quiescence) of NSCs—coincidentally, two hallmark properties of GSCs [60].

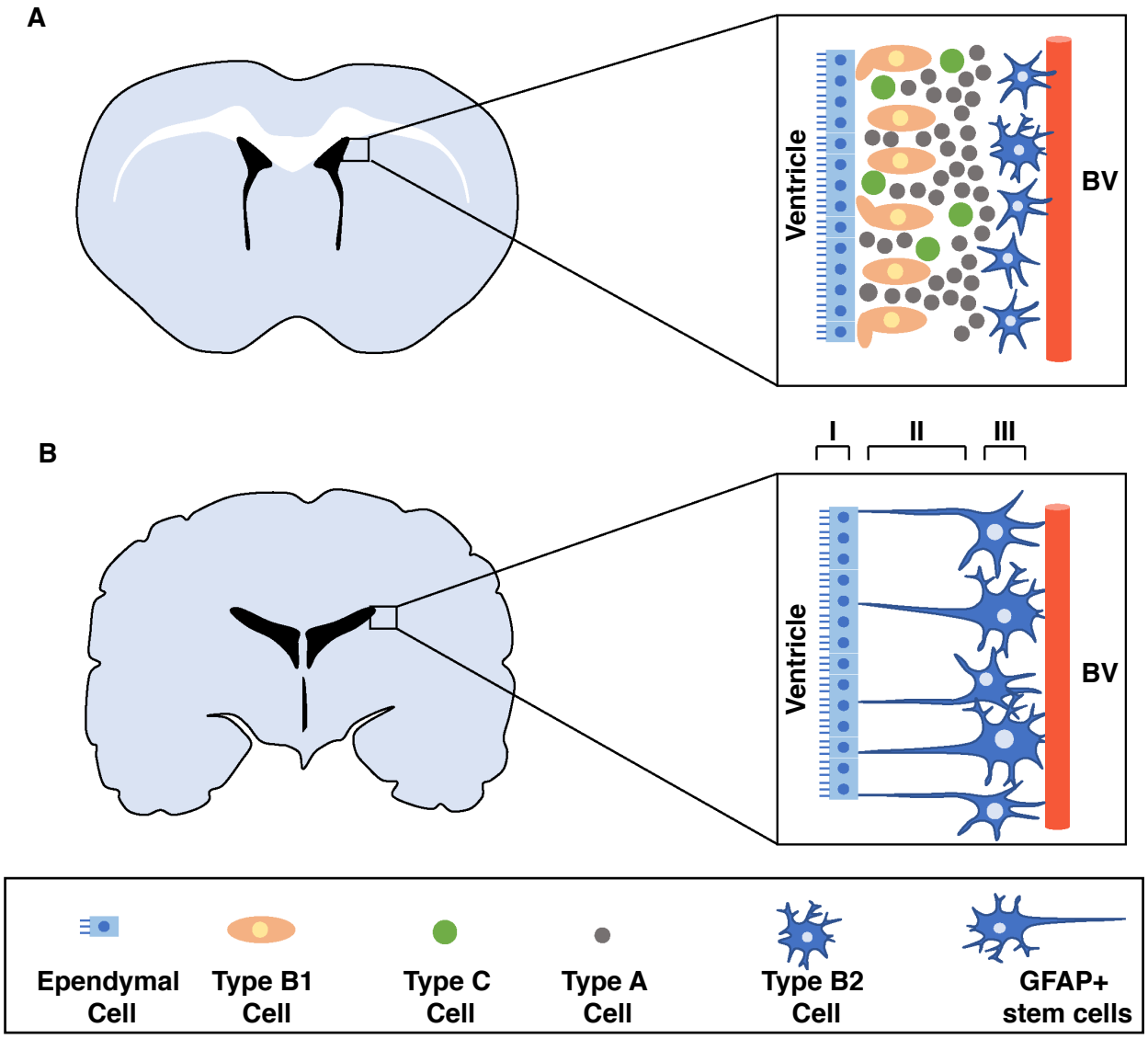


Figure 4: **The cytoarchitecture of the SVZ in the mouse and human brain.** (A) Diagram depicting a coronal view of the mouse brain. The enlarged area illustrates the cytoarchitecture of the SVZ in the mouse brain and cell types found there. (B) Diagram depicting a coronal view of the human brain. The enlarged area illustrates the cytoarchitecture of the SVZ in the human brain organized in 3 layers: the ependymal, thy hypocellular gap, and the astrocytic ribbon. In the diagrams, 'BV' refers to 'blood vessel'.

The proposition that GSCs are malignant versions of NSCs stems from three fundamental biological similarities between these cells: their genetic expression profiles, the signaling pathways that maintain their stemness and give rise to differentiated progeny, and their propensity to migrate long-distance into different regions of the brain [43, 60, 89]. Despite the general similarities that are observed between GSCs and NSCs, there are distinct genetic and epigenetic alterations in GSCs that underpin their malignant growth. For instance, while NSCs and GSCs express similar stem cell markers such as Nestin, SOX2, CD15, and CD133, the extent of expression of some of these markers is significantly different [42, 47, 74]. One such marker is SOX2; it is significantly overexpressed in GSCs when compared to NSCs, amplifying the GSCs' capacity for migration, as well as proliferation and self-renewal [10, 90, 91]. Interestingly, SOX2 together with POU3F2, OLIG2, and SALL2 are transcription factors that have been shown to be sufficient in transforming differentiated glioma cells into GSCs [49]. These differentially-expressed markers usually link to specific signaling pathways that play a key role in maintaining stem-cell properties while regulating proliferation and migration. Of these signaling pathways that are essential in neurogenesis—and yet commonly dysregulated in gliomas—are Notch, Wnt, Sonic hedgehog (Shh), Transforming Growth Factor–b (TGF-b), NF-κ B, and Epidermal Growth Factor (EGF) [43, 92-94]. For example, Notch signaling has been shown to be highly active in GSCs, consequently contributing to undifferentiated aggressive glioma phenotype by suppressing cell differentiation and sustaining the stem cell properties of these cell [94]. In addition, the activation of Notch in addition to Kras signaling was sufficient to generate tumors in the SVZ [95]. Another

commonly dysregulated signaling pathway in GSCs is Wnt, which has the effect of increasing the proliferation of these cells [93]. Inhibiting the Wnt signaling pathway has shown to decrease the proliferation of these cells [93]. In the neurogenic niche, the dysregulation of several neurotransmitter signaling pathways has been also implicated in gliomagenesis. For instance, the GABA signaling, through the activation of $GABA_A$ receptors, inhibits the proliferation of NSCs; but in GB these $GABA_A$ receptors are downregulated, and this reduces the growth-inhibitory effects of GABA signaling on GSCs [60]. Moreover, the diazepam-binding inhibitor (DBI)—overexpressed in GB—modulates the actions of GABA by directly inhibiting $GABA_A$ receptor signaling [60]. One study shows that functional $GABA_A$ receptors are almost absent in GBs, and that the expression of $GABA_A$ receptors correlates negatively with tumor grade [96]. To add, gliomas alter the neurotransmitter glutamate signaling in a manner that facilitates their proliferation [60].

Migration is a fundamental feature of NSCs and, unlike their differentiated counterparts, these cells are capable of leaving their niche and migrating to their final destinations, where they integrate with other neural cells [60]. In a similar fashion, invasion is a key feature of GBs and is the main reason surgical resection is not curative and why local chemoradiation is not always effective. Moreover, though GSCs are hypothesized to originate from the NSCs that are present in the SVZ, only 40-54% of GBs are found in the periventricular region upon diagnosis [97, 98]. This suggests that GSCs possess migration capabilities early on, allowing them to invade the brain and give rise to GBs in different regions of the brain. Studies have shown that GSCs employ many of the migratory mechanisms utilized by NSCs: dynamic flexibility in cell volume and

morphology through similar cytoskeletal protein and ion channels' modification; utilization of myelin tracts and blood vessels as scaffolds; and the facilitation of cell migration by similar chemo-attractive and repulsive cues in one's niche [60, 99, 100]. For example, both GSCs and neural progenitor cells (NPCs) express Na^+-K^+-Cl^- co-transporter 1, a fundamental co-transporter that plays a principal role in the accumulation of intracellular chloride and, subsequently, the regulation of cell volume [101]. The synchronous opening of the chloride channels and aquaporin 4 lead to ion and water efflux followed by cell volume shrinkage, enabling cell mobilization through the dense extracellular space [101-103]. Another example is the role of Rho signaling in the regulation of migration in NSCs and glioma cells [100]. Low levels of RhoA has been shown to be essential for migration in neurogenesis, in the same manner that low levels of myosin II—a contractile protein that is activated downstream of the Rho-ROCK pathway—is essential for migration in gliomagenesis [100, 104]. What is more, mysoin II has been shown to be essential for soma translocation in NPCs during neurodevelopment [105]. Also, though pleiotrophin—a growth factor and a cytokine functioning as a chemical cue in the niche—guides neuroblast migration during neurodevelopment, it nevertheless attracts glioma cells to the SVZ leading to its invasion [60, 106-108]. Interestingly, pleiotrophin accomplishes this through the activation of Rho/ROCK signaling pathway [107]. And given that glioma cells generally overexpress chemokine receptors and growth factor receptors (when compared to normal brain cells), they are more susceptible to chemotactic cues found in the niche [109].

Mature Astrocytes as the Cell of Origin:

The other proposed theory in regard to the origin of gliomas is thought to be the result of an alteration (mutation) in the astrocyte de-differentiation process. Normally, after brain injury in adults, mature astrocytes (and various types of neural and non-neural cells) acquire the ability to proliferate, resulting in what is known as "reactive gliosis" [110]. Few studies have demonstrated that a subset of these cycling-astrocytes de-differentiate into a status similar to NSCs, acquiring the ability to produce neurons and astrocytes [111-116]. It has been shown that the reversion of astrocytes to a neural stemness state makes them vulnerable to cancerous transformation when compared to mature astrocytes [71, 73]. In one study the transforming growth factor-alpha (TGF)-α, a protein commonly seen in gliomas, induced progressive astrocyte de-differentiation [111]. These de-differentiated astrocytes when exposed to an environmental stress, ionized radiation, were capable of producing gliomas in mice [73]. Similarly, platelet-derived growth factor receptor (PDGF-R), a protein commonly expressed in glioma cells, induced de-differentiation of astrocytes and neurons, making them prone to oncogenic transformation, too [117]. Another study has shown that mature astrocytes and mature neurons can be transformed by the loss of NF1 and TP53 genes and produce malignant gliomas in mice [71].

In addition, a malfunctioning de-differentiation process has been suggested as a major contributing factor for treatment resistance—a hallmark of GBs. Environmental stimuli such as hypoxia and acidic stress, and therapeutic agents such as TMZ, have been demonstrated to predispose the interconversion of non-GSCs into GSCs, thus contributing to treatment resistance [66, 118, 119]. Interestingly, these environmental

exposures not only elicit changes in the proportion of GSCs and non-GSCs, but they also elicit changes in the properties of these cells (e.g., proliferation capacity and quintessence) [62, 66]. In contrast to the above observations, a recent study showed that the deletion of the most commonly mutated tumor suppressor genes (NF1, TP53 and PTEN) in the more differentiated neural cells is not sufficient to induce malignant transformation in these cells, despite the phenotypical changes it triggers [120]. This study concluded that the more the cell is restricted to lineage along the glial differentiation axis, the less susceptible it is to undergo malignant transformation [120]. Additionally, the historical argument that mature astrocytes are likely the cell of origin of GBs—due to their histological resemblance to astrocytes and their expression of astrocytic markers such as GFAP and GLAST—has long been refuted by the well-established expression of these same markers in NSCs and NPCs [83]. Astrocytes were also proposed as the cell of origin because of their proliferation capacity throughout adulthood and their abundance in the brain; and this is reflected by the fact that GBs (grade IV astrocytomas) are the most common malignant tumors in adults. But this opinion, too, has been challenged by the discovery of neurogenesis in the adult brain and the competing hypothesis that committed glial precursor cells, such as oligodendrocyte precursor cells (OPCs), are the cell of origin of glial tumors—given their abundancy in the adult human brain and their capacity for proliferation and migration [16, 54, 121, 122].

OPCs as the Cell of Origin of IDH-mutant Gliomas:

It is noteworthy that, for IDH-mutant gliomas, the hypothesis that OPCs are the cell of origin has been gaining momentum [19, 122-126]. Of relevance, there has been recent evidence that these cells might also be the origins in IDH-wildtype GBs, too [16]. OPCs are committed glial precursor cells that can further differentiate into mature oligodendrocytes; but it is worth noting that, despite their name, OPCs can be diverted from an oligodendrocyte fate by astrocytic signals [113, 122]. They are abundant and preserve their capacity for self-renewal throughout adulthood, and this makes them susceptible to oncogenic mutations and, therefore, malignant transformation [122]. To add, they typically express the following markers: NG2, OLIG2, PDGFRα and O4—all of which are commonly expressed in almost all gliomas [127]. One study has shown that the direct introduction of TP53 and NF1 mutations into NSCs leads to glioma formation in mice only upon differentiation into OPCs [124]. Another study showed that the deletion of NF1, TP53 and PTEN in the OPCs in adult mice give rise to proneural GBs [128].

Whether different cells of origin are susceptible to certain mutations—and therefore lead to specific glioma subtypes—is not yet established. But so far, and as already mentioned, most findings suggest that NSCs are strong candidates for the cell of origin of IDH-wildtype GBs. For one thing, they are widely susceptible to a variety of mutations; and, more importantly, the striking biological similarities between the neurodevelopmental and the GBs physiological and molecular processes explains why GBs are capable of surviving and evading current treatment despite the seemingly dysregulated growth and the chaotic mutations observed in GBs. GBs appear to adapt a

highly complex and precise developmental system that promotes relentless proliferation and cell invasion, while also acquiring repairing mechanisms similar to the neuro-developmental system. In short, these tumors seem to emerge by hijacking a normal system and then tweaking it in a manner that ensures their own survival.

Hypothesis:

If the NSC is the cell of origin of IDH-wildtype glioblastoma, then cells derived from a seemingly normal SVZ of a glioma patient will express markers of GSCs and NSCs; furthermore, these cells will align closer to the NSCs' genotype and bear less mutational load, as determined on the basis of DNA copy number variability and single nucleotide variations, when compared to GSCs derived from the tumor.

Results:

SVZ and tumor-derived cells exhibit classical GSCs' properties

To determine whether the cells derived from the SVZ and the tumor have a distinctive proliferative and sphere-formation capacity, we established cell cultures from dissociated single cells of brain tissue samples harvested from the same patient. Cells labelled as SVZ-derived cells were isolated from a radiologically and intra-operatively normal-looking SVZ, and cells labelled as tumor-derived cells were isolated from a histopathologically-confirmed IDH-wildtype GB (Figure 4). Both cell lines were grown in serum-free media containing EGF and FGF2 in two different culture conditions: adherent and suspension (Figure 4). Serum-free media are thought to better promote stem cell proliferation and maintain the gene-expression of the original sample compared to serum media [43, 129]. SVZ and tumor-derived cells were capable of forming free-floating neuro-spheres and maintained this capacity constantly with higher passages. Also, cells that were initially grown in adherent cultures maintained the capacity to generate spheres when they were later expanded in suspension culture. To assess the cells' unlimited proliferation capacity, steadily expanding cell lines were established from SVZ and tumor-derived cells, and those were expanded for more than 25 passages. Based on growth properties, we have observed that SVZ-derived cells initially produced slower-expanding lines compared to tumor-derived cells which in turn produced faster-growing lines. Both cell populations were cultured in identical cell-seeding densities, but during the first two months SVZ-derived cells would only reach a confluence of 25% as opposed to tumor-derived cells where they would reach consistently a confluence of more than 75%. After

passage 5, the growth rate of the SVZ-derived cells abruptly increased and became similar to the tumor-derived cells' growth rate. This observed in-vitro behavioral change that SVZ-derived cells manifest with higher passages will be a recurring theme as will be demonstrated below.

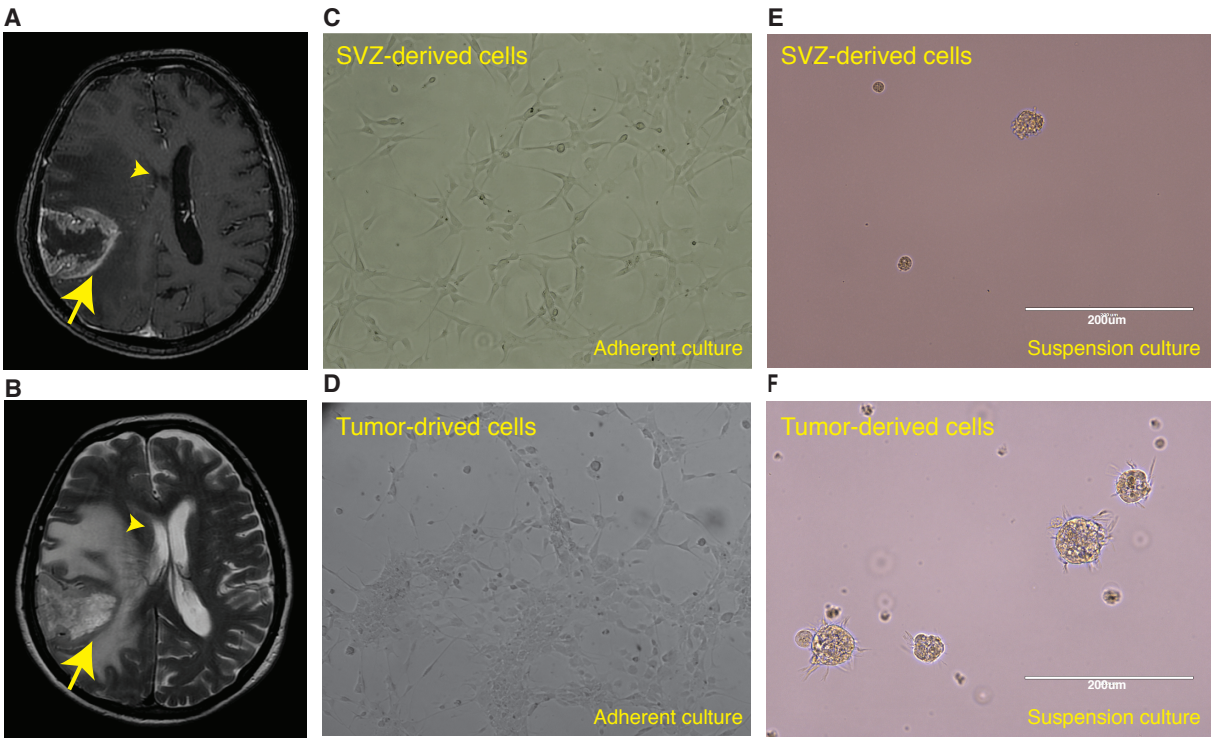


Figure 5: SVZ & tumor-derived cells exhibit self-renewal and sphere-forming capacity. (A) MRI of the brain showing the location of the samples obtained of the tumor (arrow) & the SVZ (arrow head). One cell line was derived from each sample. (B) Cells grown on laminin in serum-free media exhibited self-renewal capacity. (C) Cells grown in suspension in serum-free media exhibited capacity for neuro-sphere formation. Self-renewal & sphere-forming are characteristic qualities of NSCs & GSCs.

SVZ & tumor-derived cells express classical markers of NSCs & GSCs

Since both SVZ and tumor-derived cell lines exhibited characteristic in-vitro features of GSCs, we went on to further ascertain their GSC identity and the possibility of these two different clones sharing a common ancestry, the NSC. We did that by studying whether or not these cells expressed classical markers of GSCs and NSCs.

At first, we performed a literature review and compiled a list of all the markers that have been shown to be expressed in either a GSC or an NSC. Out of this list of markers we only chose the molecular markers that have been shown to be essential in either the pathogenesis or maintenance of GSCs and NSCs. As a result, a marker panel constituting of 16 molecular markers was compiled, the details of which are shown in (Table 1). Then, via western blot, immunofluorescence, and fluorescence-activated cell sorting (FACS), we studied both cell lines for the expression of these markers. We found that both, SVZ and tumor-derived cells consistently expressed all 16 molecular markers regardless of the number of cells' passage they were at when they were analyzed (Figure 5). Out of these 16 markers, 9 markers displayed a statistically significant differential expression between the two cell populations. Western blot immunoreactivity's quantification of POU3F2, GFAP, Nestin, Cytosol DRR, and EphA2 showed a higher level of expression in the SVZ-derived cells when compared to tumor-derived cells (Figure 5). Flow cytometric quantification of CD15 expression in SVZ-derived cells was 50%, as opposed to 3% in tumor-derived cells (Figure 5). Western blot immunoreactivity's quantification of OLIG2 and Nuclear DRR showed a higher level of expression in tumor-derived cells when compared to SVZ-derived cells (Figure 5). Flow cytometric quantification of CD133

expression in tumor-derived cells was 39% as opposed to 1% in SVZ-derived cells (Figure 5).

CD133 is thought to be one of the important markers in identifying GSCs [42, 43, 45]. Detection of this marker can be challenging given the nature of this protein with its numerous variants and changing glycosylation status [130]. Since flowcytometric quantification showed a significantly high fraction of CD 133 expression in tumor-derived cells, as opposed to its almost non-existent expression in SVZ-derived cells, we further investigated the expression of this marker through immunofluorescence.

By utilizing a different anti-CD133 antibody from the one used in FACS, we probed for the presence of CD133 expression in tumor and SVZ-derived cells. Again, tumor-derived cells were immunoreactive to the different anti-CD133 antibody while SVZ-derived cells failed to show any immunoreactivity to that same anti-body (Figure 5). Interestingly, the CD133 mRNA expression in tumor-derived cells was not in concordance with its protein expression. QRT-PCR showed that CD133 mRNA expression was modestly higher in SVZ-derived cells when compared to tumor-derived cells (Figure 6).

We next investigated the mRNA expression of all markers in both cell populations, in order to analyze which differentially-expressed markers showed consistency between their mRNA and protein expressions. QRT-PCR showed that the mRNA expression of the following markers: POU3F2, GFAP, EphA2 and CD15, was similar to their protein expression, in that they were significantly higher in SVZ-derived cells. GFAP and CD15 mRNA expression was increased more than two-fold and POU3F2 and EphA2 mRNA expression was increased less than two-fold. As for the proteins Nestin and DRR, while

these showed higher level of expression in SVZ-derived cells, their mRNA expressions through QRT-PCR were not significantly different in SVZ and tumor-derived cells.

In SVZ-derived cells there was also significant two-fold increase in the mRNA expression of classical NSC markers: BLBP, PAX6 and TLX (Figure 6). The increase in expression of these markers was not observed at the protein level.

However, the most striking differential expression between these cell lines was the expression of OLIG2, where it was expressed seven-fold more in the tumor-derived cells when compared to SVZ-derived cells. This significant increase in the expression of OLIG2 in tumor-derived cells was not only shown at the protein level (through western blot and immunofluorescence) but also at the mRNA level (Figure 6). QRT-PCR showed that the level of OLIG2 mRNA expression in tumor-derived cells was higher than it was in SVZ-derived cells, but this humble increase in expression was less than two-folds (Figure 6). Another interesting observation can be made: similar to the change observed in the growth rate of SVZ-derived cells, the level of expression of OLIG2 changed with higher passages and was upregulated until it became noticeably similar to its level of expression in tumor-derived cells.

Gene	Description	Significance	GSC	NSC
Nestin	Type VI intermediate filament protein	Expressed in activated NSCs that give rise to the neural progenitor cells. Required for the survival & renewal of NPCs. Upon terminal neural differentiation, nestin expression is down-regulated and ultimately lost [86, 131] [132]. Not expressed in quiescent NSCs. Consistently expressed in GSCs.	[133, 134]	[86, 131, 135-137]
SALL2	Transcription Factor	Interact with key transcription factors (e.g. SOX2) for pluripotency maintenance in GSCs & NSCs. Has been associated with neurogenesis and with neuronal cell differentiation [138]. It is one of the four core transcription factors together with SOX2, POU3F2 and OLIG2 that can reprogram differentiated GBM cells into GSCs [49].	[49]	[137, 139]
CD133	Trans-membrane glycoprotein	Often expressed in NSCs, where it is thought to function in maintaining stem cell properties by suppressing differentiation (gene cards). Despite being a GSC marker, it's not consistently expressed in GSCs [47].	[42, 133]	[137, 139]
CD44	Cell-surface glycoprotein	Expressed in NSCs & GSCs. Functions by mediating cell-cell and cell-matrix interactions. Plays an important role in cell migration, tumor growth and progression.	[133]	[135]
GLAST	Glutamate/ aspartate transporter	Expressed in RGCs that give rise to NSCs. In adult NSCs it is co-expressed with nestin, sox2 & GFAP [132]. Preventing glutamate mediated excitotoxicity in the CNS by terminating the postsynaptic action of glutamate. Anti-GLAST immunotherapy promotes anti-tumor cytotoxicity[140].	[141]	[86, 135, 136, 142, 143]

Gene	Description	Significance	GSC	NSC
CD15	Catalyzer	Expressed in embryonic stem cells & adult SVZ. Their expression correlates well with the prevalence of NSCs. Expressed in GSCs, too [47].	[47]	[137, 139]
GFAP	Class-III intermediate filament	Marker of RGCs that give rise to NSCs. GFAP progenitor cells show the capacity to generate new neurons during adult neurogenesis [132].		[136], [86, 137],[131, 139]
POU3F2	Transcription Factor	Involved in neural differentiation & play role in regulation of neocortical layers development[132]. It is one of the four core transcription factors together with SOX2, OLIG2 and SALL2 that can reprogram differentiated GBM cells into GSCs [49].	[49]	
PAX6	Transcription Factor	Regulate NSCs proliferation & differentiation [132].		[135, 136, 143]
TLX	Orphan nuclear receptor	Present in Quiescent & active NSCs. Important in regulating NSC self-renewal & proliferation [132].	[144]	[86]
DCX	Microtubule-associated protein	Expressed in migrating neuroblasts & immature neurons. DCX is a label for post mitotic neuronal progenitor cells & small percentage are expressed in Nestin positive cells [132].		
SOX2	Transcription Factor	Frequency marker for NSCs and thought to be critical for NSCs proliferation & differentiation. [132] It is one of the four core transcription factors together with OLIG2, POU3F2 and SALL2 that can reprogram differentiated GBM cells into GSCs[49].	[49, 133]	[86, 131, 135, 136, 143]

Gene	Description	Significance	GSC	NSC
OLIG2	Transcription Factor	Plays a critical role in glial progenitor proliferation & oligodendrocyte development. It has an important role in gliomagenesis and tumor phenotype plasticity[145]. It is one of the four core transcription factors together with SOX2, POU3F2 and SALL2 that can reprogram differentiated GBM cells into GSCs [49].	[49]	[136]
DRR	Stress-inducible actin-binding protein	Expressed in GSCs and is an active driver of GBM invasion and proliferation [146].	[146]	
BLBP	Cytoplasmic protein	Expressed in NSCs, RGCs & in the astrocyte lineage [132]. Involved in the maintenance & self-renewal of NSCs & proliferation of astrocytes [82]. Involved in the proliferation & invasion of GBMs [147]. The combination of nestin & BLBP is thought to be a unique GSC marker by enriching for calcium signaling genes that make them highly sensitive to calcium channel blockers[134].	[133, 147]	[86, 131, 135, 136, 139, 142, 143]
EphA2	Receptor tyrosine kinase	Expressed in GSCs and has effects on proliferation, invasion, and neovascularization[148].	[148]	

Table 1: Marker panel of common NSCs & GSCs molecular markers.

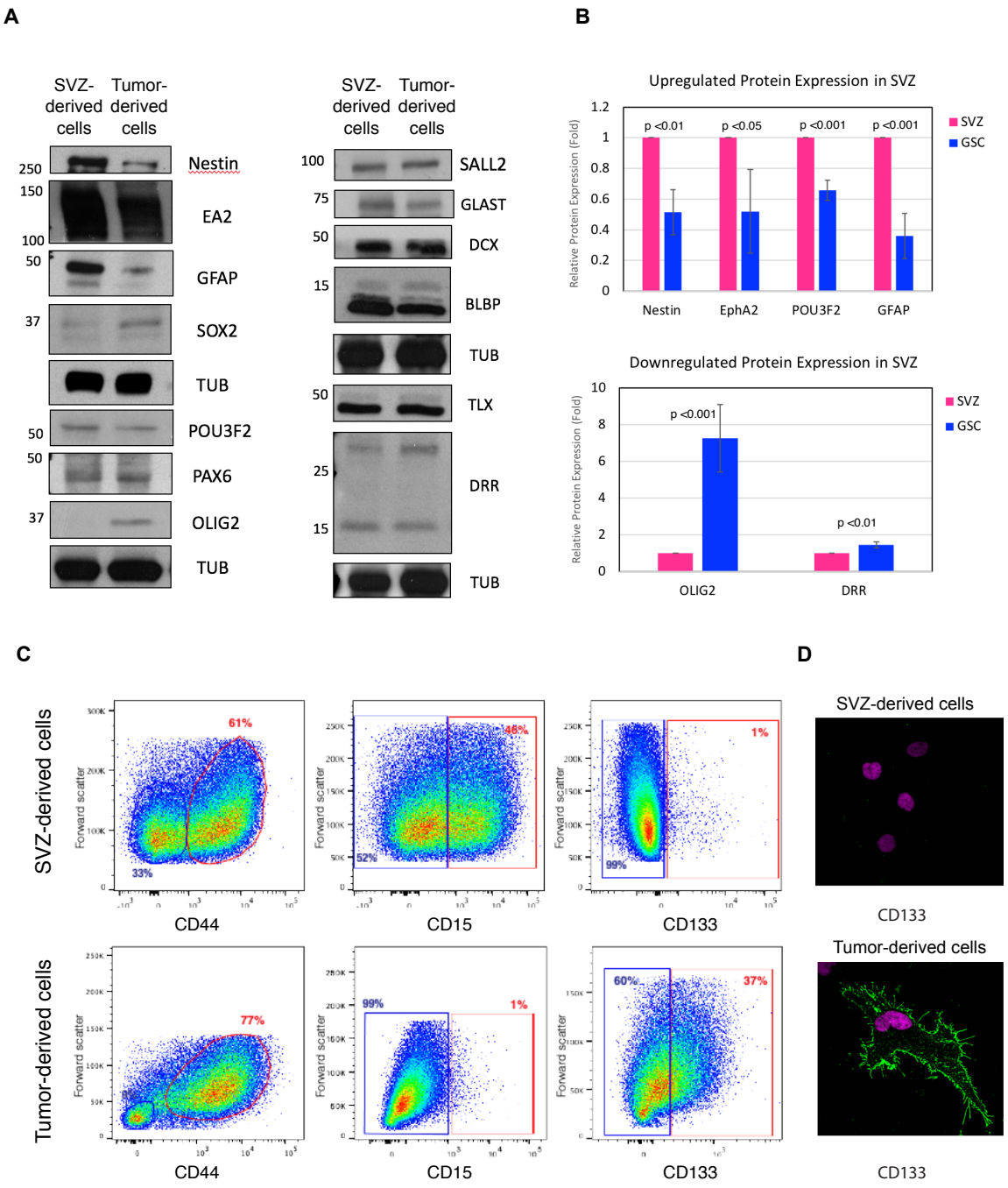


Figure 6: Cells derived from the SVZ & the tumor express classical markers of NSCs & GSCs. (A) Western blot analysis of cells derived from the SVZ & the tumor show similar expression of classical NSCs & GSCs markers. Western blot quantification of the differentially expressed markers is shown in (D). Only the statistically significant differential expression is shown with their respective P-values. (B) Immunofluorescence & (C) FACS showing the expression of CD133, CD44, & CD15 in both cell lines. CD 133 is expressed more prominently in cells derived from tumor in contrast CD15 is expressed prominently in cells derived from the SVZ.

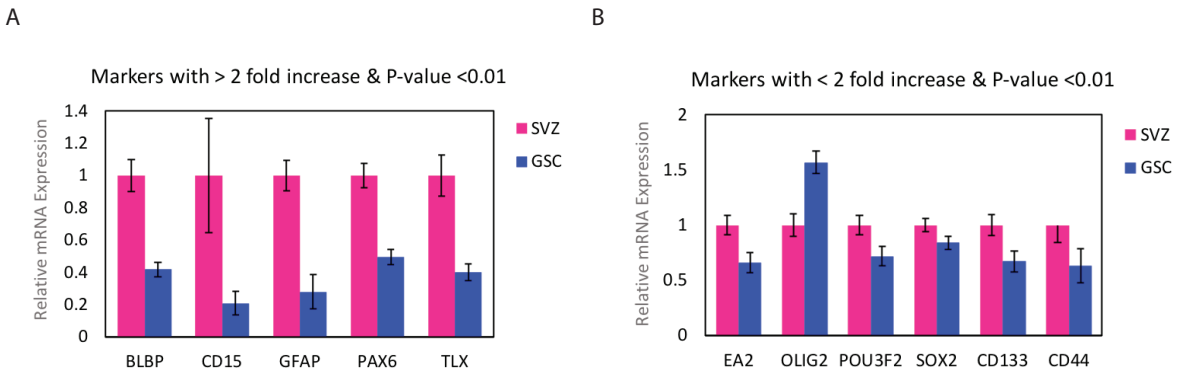


Figure 7: mRNA expression of the classical NSCs markers is upregulated in SVZ-derived cells when compared to tumor-derived cells. (A) Diagram showing the increased mRNA expression of the following markers: BLBP, PAX6, CD15, TLX and GFAP in SVZ-derived cells, all in which are considered more of classical NSCs markers. (B) Diagram showing the increased mRNA expression of Olig2 in tumor-derived cells and the slightly decreased mRNA expression of CD133.

SVZ & tumor-derived cells carry unique genetic aberrations that exist in GB and yet each represent a different clone

SVZ and tumor-derived cells expressed classical GSCs' markers but we wanted to further confirm that both cell populations were indeed GB cells; since SVZ-derived cells were harvested from a normal looking SVZ and expressed significantly more markers of NSCs than tumor-derived cells. Through deep whole genome sequencing (WGS) the copy number variability (CNV) and single nucleotide variation (SNV) were examined in both samples. The WGS was performed in a triple-matched sample that included the SVZ-derived cells, tumor-derived cells and normal tissue (blood). Analysis of the copy number variability (CNV) in both samples revealed that they were genetically aberrant cells, and few of these aberrations were characteristic of GBs such as chromosome 7 amplification, chromosome 10 deletion and focal deletion of 9p21 [149, 150] (Figure 7).

This further confirmed that cells derived and grown from SVZ & tumor not only express GSCs markers, but they also have the classical genomic aberrations of GB cancer cells and are therefore truly GB cancer cells. The number and pattern of CNVs in both samples were almost identical with minor differences (Figure 7). Both shared 11 CNVs, of which chromosome 7 amplification and focal deletion of 9p21 were present in both cell lines, and chromosome 10 amplification was present only in the SVZ-derived cells. The differences between these cell lines were more prominent when the single nucleotide variations (SNVs) were analyzed, as will be demonstrated later.

In order to test our hypothesis—that SVZ-derived cells will bear less mutations than its counterpart, the tumor-derived cells—we calculated the mutational load of each sample and compared them to each other. When compared to the blood results, we identified 10,218 single-nucleotide variations (SNVs) and 10,089 SNVs in SVZ-derived cells and tumor-derived cells, respectively. Of those, only the non-synonymous SNVs were analyzed and used to calculate the mutational load (Figure 7). Although, SVZ-derived cells had 75 somatic mutations compared to 80 somatic mutations in the tumor-derived cells, this difference was not statistically significant to conclude that SVZ-derived cells have less mutational load than tumor-derived cells.

Since both cell lines seemed to share similar number of SNVs, the nature of the somatic mutations was analyzed in order to determine if they represented the same clone. The matched pair shared almost 80% of the SNVs and showed divergence in 20% of the SNVs suggesting, along with the subtle difference in CNVs, that both arise from a common source and yet constitute different clones.

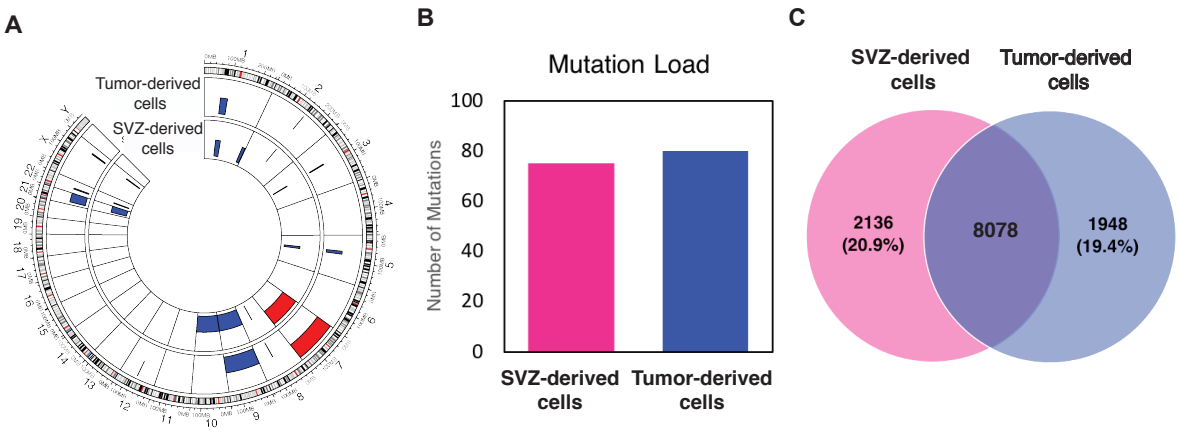


Figure 8: SVZ & tumor-derived cells carry similar CNVs but have a 20% divergence of SNVs. (A) Circos plot visualizing the CNVs. The changes for each sample are shown with the outer track representing tumor-derived GSCs and the inner track SVZ-derived cells. Regions marked in blue identify deletion event while regions marked in red identify a duplication event. Both cell lines have similar CNVs with minor differences. (B) Histogram showing the mutational load in each cell line (p-value 0.1768). (C) Venn diagram showing the number of shared somatic mutations between the two-cell line with 20% divergence rate.

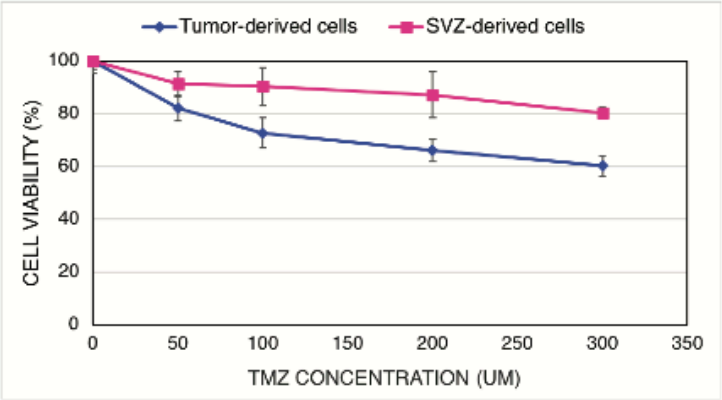
SVZ-derived cells are more resistant to TMZ when compared to tumor-derived cells

So far, there were striking similarities between the phenotype and genotype of SVZ and tumor-derived cells, so we proceeded to investigate the functional properties of these cell lines in order to explore the potential presence of a functional hierarchy among these cells.

Glioma recurrence has been repeatedly attributed to GSCs' capacity for resisting treatment and possibly treatment-driven mutation that yield a more aggressive tumor on recurrence [39]. Therefore, the effect of TMZ chemotherapy on both cell lines was studied in vitro (Figure 8). SVZ and tumor-derived cells were treated with TMZ using a dose-escalation strategy ranging from 50 to 300μM/L. For control a 300μM/L of DSMO was used. A significant difference in response to TMZ treatment was observed between SVZ

and tumor-derived cells (Figure8). While the difference in cell response to TMZ was modestly different at 50μM/L, it became more pronounced with higher dosages. At a concentration of 300μM the percentage of cell viability of SVZ-derived cells was 80% compared to 60% in tumor-derived cells.

Figure 9: Differential response of SVZ and tumor-derived cells to TMZ. Diagram showing that SVZ-derived cells are more resistant to TMZ treatment than tumor-derived cells (P-value 0.0004).



Discussion:

The concept that NSCs are the cell of origin of GBs arose from the striking similarities between the biological properties of GSCs and NSCs. To further provide evidence to this concept we hypothesized that cells derived from a seemingly normal SVZ of a glioma patient will not only express markers of GSCs and NSCs but will also align closer to the NSCs' genotype and bear less mutational load when compared to GSCs derived from the tumor.

We started by verifying that the cells we isolated from the SVZ and the tumor exhibited indeed stem cells properties in-vitro. We have shown that SVZ and tumor-derived cells exhibited self-renewal and sphere-forming capacity, two hallmark properties of stem cells [54, 55]. Our choice to study the self-renewal capacity of these cells in different culture conditions stems from the understanding that each has its own limitations. Suspension culture has long been the standard culture condition to enrich for NSCs and to assess for stem cell properties, but it has been repeatedly shown that the spheres grown in this condition may contain progenitor and more differentiated cells that are capable of producing spheres and undergoing a limited number of passages in this system [44, 54, 55, 151]. On the other hand, adherent culture provides a uniform access to growth factors which in turn suppresses differentiation, making it superior in terms of promoting a more homogenous purified clone of stem cells [136, 143, 152]. Furthermore, GSCs do not exist in isolation in-vivo but, rather, are informed by the tumor microenvironment, making the adherent culture system more comparable to the brain environment than the sphere culture [152, 153]–more so when studying cells isolated

from the SVZ, since it has been shown that NSCs residing in the SVZ are affected by their microenvironment [60, 87, 154]. Therefore, after confirming the self-renewal and sphere-forming capacity of the SVZ and tumor-derived cells in sphere culture condition, we chose to proceed to analyze the cells expanded in the adherent culture.

We then went on to characterize the SVZ and tumor-derived cells by studying their expression of GSCs and NSCs' molecular markers. We developed a marker panel that allowed us to further verify the stemness identity of the cells we isolated, and to demonstrate a biological relation between the genotype of the cells that we have isolated and the genotype of NSCs normally residing in the SVZ. Each marker was specifically selected based on previously published evidence that its expression was essential in maintaining the proliferative and migration properties of NSCs and by extension GSCs. The significance of each marker has been summarized in table 1 but the specific rationale for choosing each one of them is explained below. The cell surface proteins CD133, CD44, and CD15 were chosen because the combination of the three is acknowledged by most to be the "classical markers of GSCs" [37, 42, 43, 45, 47, 50, 55, 155-157]. SALL2, POU3F2, OLIG2, and SOX2, as mentioned earlier, are neurodevelopmental transcription factors that have been shown in combination to be sufficient enough to reprogram a differentiated glioma cell into GSCs [49]. In addition, SOX2 has a role in maintaining the undifferentiated state of stem cells (GSCs and NSCs included) and silencing it in GSCs has been proposed as a novel therapeutic approach [91, 138]. EphA2 have been shown to be co-expressed with CD133 in GSCs and was found to be play an active role in maintaining the GB cells in a stem-like state by negatively regulating the MAPK pathway

[148, 158, 159]. DRR, downregulated in renal cell carcinoma, is a structural protein that has been found to be highly expressed in the human NSCs and recently has been shown to play an important role in the invasion and regulation of the stem cell properties of GSCs [146, 160, 161]. Additionally, DRR has been demonstrated to regulate the expression of the four core transcription factors (SALL2, POU3F2, OLIG2 and SOX2) that were implicated in re-programing differentiated GB cells into GSCs [160]. GFAP, GLAST, PAX6, and BLBP were included in the marker panel because they are the hallmarks of type B1 cells, the cells that are considered NSCs in the adult brain [84, 86, 136, 139, 143, 162, 163]. TLX a marker that is also uniquely expressed in type B1 cells, has been shown to be crucial in neurogenesis [164]. Nestin is frequently acknowledged as a marker of NSCs both in embryo and in adult brain, and its expression fades away once cells enter the path of differentiation [86, 134, 162, 165]. DCX, a protein that facilitates microtubule polymerization, is considered a marker of neuroblasts (premature neurons) rather than NSCs but have been shown to be critical in the movement of these cells in the adult brain and since glioma cells are known for their invasive properties, we thought it would be a significant marker [166, 167]. Besides, it has been shown that there might be a small window were DCX-positive and Nestin-positive cells overlap [168].

Based on the expression of the cell surface markers, CD133, CD44, and CD15, and the intracellular stem cell markers Nestin and SOX2, we have shown that the SVZ and tumor-derived cells express markers that enrich for GSCs. We also have shown that both cell populations express not only GSCs' markers, but also NSCs' markers. Moreover, the differential expression of these markers suggests that the SVZ-derived

cells align closer to the NSCs' genotype while the tumor-derived cells align closer to what is known as the "classical" genotype of GSCs. The expressions of Nestin and GFAP, both markers of NSCs, were significantly higher in the SVZ-derived cells. CD15, a protein that is considered one of the markers of GSCs, is also known to be expressed in NSCs that are present in the SVZ of the adult human brain and its expression correlates with the prevalence of NSCs [169]. Our data show that CD15 was expressed in 50% of the SVZ-derived cells and in just 3% of tumor-derived cells. In addition, SVZ-derived cells showed double the expression of TLX, PAX6, and BLBP transcripts when compared to tumor-derived cells. TLX is a transcription factor that is expressed in adult NSCs and controls the expression of a broad network of genes that maintain the NSCs in an undifferentiated, proliferative state [170-172]. It also plays a role in governing the localization of NSCs to the neurogenic niche [173]. PAX6, another transcription factor that is expressed in adult NSCs, has been shown to play a critical role in the self-renewal and neurogenesis of NSCs through Wnt/ß-catenin and ß-catenin/Pax6 signaling pathways [174, 175]. It is also thought to regulate migration of newborn neurons [176]. BLBP, a cytoplasmic protein that is responsible for the fatty acid intake and transportation, is a marker of the astrocytic-ribbon niche that contains the NSCs in the postnatal human brain [136, 143, 162, 177]. The expression of BLBP in the NSCs has been shown to correlate with its mitotic activity and neurogenesis [177]. The differential expression observed in TLX, PAX6, and BLBP transcripts in SVZ-derived cells did not fully correlate with their protein expressions. Despite an adequate protein expression in SVZ-derived cells, these markers failed to show a statistically significant differential expression when compared to tumor-derived

cells. This modest correlation between the mRNA and protein expression has been frequently documented and could be related to the inherent difference of the techniques utilized to detect each, as well as the variations in sample preparations that each technique requires [178-180]. Also, the choice of primers when it comes to QRT-PCR might dictate the splice variant of the mRNA detected and whether or not this variant translates into a protein. Similarly, the choice of antibodies when it comes to western blot determines its ability to detect a protein in different configurations [181]. Moreover, the variations in cell-to-cell states (dividing cell versus a steady-state cell) and the post-transcriptional modifications an mRNA undergoes before translating into a protein are other layers that might contribute to an imprecise correlation between the ratio of mRNA and proteins expression [182]. Nevertheless, SVZ-derived cells showed differential expressions of NSCs markers such as Nestin, GFAP, and CD15 proteins on top of the differential expressions of TLX, PAX6, and BLBP transcripts when compared to tumor-derived cells, suggesting that the SVZ-derived kind aligned closer to the NSC's genotype.

In contrast, tumor-derived cells had an increased expression of CD133 and OLIG2 when compared to SVZ-derived cells. CD133, a cell membrane glycoprotein, has been famously known as the first marker that allowed the identification of GSCs and to date is still considered by most researchers a reliable marker for the enrichment of GSCs [42, 55, 155-157, 183]. The function of CD133 protein in stem cells is not very clear but it has a role in regulating the proliferation and colony forming capacity of these cells [15, 16]. Our flow cytometry data demonstrated that CD133 was expressed in 39% of tumor-derived cells as opposed to 1% in SVZ-derived cells. This differential expression of

CD133 was reproduced by immunofluorescence, too, where tumor-derived cells displayed CD133 expression in the cell membrane while it was absent in the SVZ-derived cells. This increased protein expression of CD133 in tumor-derived cells was not in concordance with its mRNA expression in these same cell populations. CD133 transcript was expressed more in the SVZ-derived cells than in the tumor-derived cells. One explanation for this observation might be that the mRNA levels detected in the SVZ-derived cells did not reflect the CD133 expression levels localized in the plasma-membrane of these cells. This discrepancy between CD133 protein and mRNA expression has been observed previously in glioma cells and was explained by the variance of CD133 localization [155, 184]. The glioma cells that lacked the plasma membrane expression of CD133 showed exclusive cytoplasmic expression of CD133 [155, 185]. In other words, the increased expression of CD133 transcript in SVZ-derived cells might reflect the “cytoplasmic” CD133 rather than the “plasma-membrane” CD133. The cytoplasmic CD133 will not be detected by flow cytometry without the permeabilization of these cells, which might explain the almost lack of expression of CD133 in SVZ-derived cells when assessed by flow cytometry and immunofluorescence, despite its high mRNA expression. Interestingly, the localization of the CD133 in glioma cells affect its capacity to form tumors adequately. Glioma cells that expressed cytoplasmic CD133 have shown to be less proliferative and form tumors with a longer latency when compared to glioma cells expressing membranous CD133 [155]. However, once these cytoplasmic-CD133 glioma cells re-expressed the CD133 in the plasma membrane, they recovered the impairment in their self-renewal capacity and tumorigenic

potential [155]. OLIG2, a marker that we have shown to be expressed seven-fold more in tumor-derived cells, is a transcription factor that play a critical role in supporting the proliferation and the self-renewal capacity of GSCs [48, 145, 186-188]. It has been shown to be co-expressed with CD133 and is considered one of the most specific markers of GSCs [48, 188-190]. It also has been identified as one of the core transcription factors that can reprogram differentiated glioma cells into the GSCs, re-emphasizing the importance of OLIG2 in maintaining GSCs' stemness [49].

An additional indication that the cells we have isolated might have originated from NSCs is that the NSCs markers that were expressed more significantly in the SVZ-derived cells were retained in the tumor-derived cells. Not only that, but also these NSC markers that establish the identity of NSCs are some of the same proteins that GSCs rely on in maintaining their self-renewal and invasion capacity; these are the two hallmarks of GBs confirming what others have shown: that NSCs are strong contender as the cell of origin of GBs. For example, GSCs have been shown to express TLX and its upregulation has been shown to promote the formation of gliomas in the SVZ of mice [191-193]. Knocking down the expression of TLX has been also shown to inhibit GSCs tumorigenic potential in mice [144]. These findings indicate that TLX is as important a stem cell regulator in GSCs as it is in NSCs. BLBP, another NSC marker, has been found to be highly expressed in GSCs; the downregulation of its expression decreased the glioma cells tumorigenic potential and decreased the expression of DCX, a protein that is important in cell migration [147, 194]. PAX6, yet another NSC transcription factor that is persistently expressed in glioma cells, seems to play a different role in GBs acting as a tumor-

suppressor protein [195]. There is mounting evidence that PAX6 suppresses cell proliferation and invasion of GB cells as well as supressing angiogenesis of GBs [195-198]. Further supporting these findings, PAX6 has been found to be downregulated in GBs when compared to adjacent healthy tissue and, furthermore, when compared to lower grade gliomas [199]. In our study, tumor-derived cells (the cells that we have shown to align closer to the GSCs genotype) had a significantly lower expression of PAX6 transcripts compared to SVZ-derived cells based on the studies above that is essential for the proliferation of these cells, further supporting the proposition that SVZ-derived cells align closer to NSC genotype while tumor-derived cells align closer to GSCs genotype.

Nevertheless, SVZ-derived cells were harvested from a normal-looking SVZ and, as demonstrated above, many of the GSC and NSCs markers overlap; therefore, our WGS data was necessary, first, in proving that SVZ-derived cells are indeed GB cells and, second, in ensuring that the SVZ-derived and tumor-derived cells constitute two different clones. We have shown that both cell populations expressed defining genomic alterations of GBs, such as chromosome 7 amplification and focal deletion of 9p2, therefore confirming their GB identity [2, 149, 150, 200]. Chromosome 10 amplification, an additional pathognomonic aberration that is regarded as an early event of gliomagenesis, was only found in SVZ-derived cells [14, 201, 202]. We expected that, since SVZ-derived cells aligned closer to the NSCs genotype, that the latter would constitute the precursor cells from which the tumor-derived cells rose. Consequently, in theory, tumor-derived cells should at least harbour the major chromosomal aberrations that were present in the SVZ-derived cells especially in the light of an early genetic event

such as chromosome 10 amplification. In our study this was not the case; a possible scenario explaining this is the occurrence of an early separation of cell clones from a common tumor precursor cell (a transformed NSC) leading to parallel genetic evolution of these two cell populations. Indeed, despite the subtle variations noted in the CNVs between SVZ and tumor-derived cells, there is a sequence divergence among the SNV genes as high as 20%, further confirming that they constitute different clones of GB cells. Performing genomic sequencing of these cell populations allows us to compare their mutational load in order to investigate whether or not SVZ-derived cells, in addition to aligning closer to NSCs genotype, carried less mutational load, as we proposed in our hypothesis. Our analysis of the WGS data showed that tumor-derived cells had 129 extra SNVs when compared to SVZ-derived cells, suggesting that the mutational load is considerably higher in tumor-derived cells. However, for the purpose of our study, we chose to calculate the mutational load based only on the non-synonymous SNVs. Non-synonymous SNVs are nucleotide substitutions that alter the amino acid sequence and therefore its coded protein; whereas synonymous SNVs alter neither the amino acid sequence nor the protein and, as a result, sometimes yield what is thought of as silent mutations. When only the non-synonymous SNVs were calculated, the increase observed earlier in the number of somatic mutations in tumor-derived cells dropped to 5 instead of 129. This difference was not statistically significant, which does not allow us to conclude that SVZ-derived cells had a less mutational load compared to tumor-derived cells to support our hypothesis. Our choice to solely calculate the non-synonymous SNVs was based on the idea that, when it comes to cancer biology, only synonymous SNVs produce

silent non-consequential mutations; but recent studies have shown the significant impact of these synonymous SNVs on cancer splicing, RNA stability, and translation protein folding [203, 204]. In other words, synonymous SNVs in cancer (including GBs) can impact protein expression as well as mRNA secondary structure.

So far, we have shown that SVZ-derived cells exhibited similar properties and genetic expression to NSCs and, while it wasn't statistically significant, a lesser degree of mutational load when compared to tumor-derived cells. Yet, assessing the behaviour of these cells in-vitro and in-vivo constitutes the best validation to the stemness identity of these cells. In the same manner, it allows for assessing whether or not SVZ-derived cells align closer, *functionally*, to the cell of origin by comporting themselves similar to the cells lying on the top of the GSCs hierarchy. One such characteristic function that is attributed to GSCs is their inherent ability to resist treatment when compared to their counterparts the differentiated GB cells [38, 45, 205]. More specifically, GSCs resist the chemotherapeutic drug TMZ, which is a major component of the current standard treatment for GB patients [20, 22, 38, 45, 205]. Through the TMZ response assay, we were able to show that SVZ-derived cells were more resistant to TMZ treatment when compared to tumor-derived cells. When both cell population were treated with TMZ at concentration of 150μM, 90% of SVZ-derived cells were viable as opposed to 70% in tumor-derived cells, and this differential response to TMZ was maintained with higher concentrations. This finding further supported the possibility of SVZ-derived cells aligning closer to the cell of origin, the proposed NSC of the SVZ.

There have been a few limitations in our study that might have compromised our results and provided a less than ideal environment to confirm our hypothesis. By far, the most significant limitation of our study was that the cell lines we analyzed underwent serial passages in culture, which prompted a change in their genotype and behaviour over time. We tried to avoid this possible mishap by limiting our analysis to cells that underwent less than 16 passages in culture. Even then, we observed a significant alteration in the growth rate and genotype of the SVZ-derived cells. These alterations were observed in passages as early as passage 5. Our observation has highlighted what has been previously suggested: stem cells grown in in-vitro conditions will adapt to their environment over time [57, 206, 207]. Moreover, the critical transition that the cells undergo in their "new" artificial environment probably occurs around passage 3-5 [206, 207]. It is noteworthy that the cell adaptation to in-vitro conditions not only alter their genetic expression, but also, this in-vitro genomic cell evolution might change their response to functional assays such as tumour cell proliferation or drug sensitivity [57]. We have certainly observed in our study the in-vitro genomic evolution of SVZ-derived cells. We have noted how these cells, demonstrated a very slow proliferation rate; for months, they laid in culture forming only very few small spheres, but with later passages these cells started behaving like tumor-derived cells and picked up their proliferation rate. Similar to the altered proliferation rate of SVZ-derived cells with serial passages, the protein expression of the markers OLIG2 and SOX2 were altered after serial passages. During earlier passages, OLIG2 and SOX2 were barely expressed in SVZ-derived cells compared to tumor-derived cells, but, as we proceeded to recapitulate these findings with biological repeats of later passages, this

differential expression became less obvious and the levels of OLIG2 and SOX2 expressions in SVZ-derived cells started mimicking that of the tumor-derived cells. After passage 10 the expression of these markers was up-regulated in SVZ-derived cells. Interestingly, while SVZ-derived cell phenotype and genotype were changing with time in culture and mimicking tumor-derived cell genotype, tumor-derived cell genotype and phenotype remained stable. One might think that these changes are related to in-vitro culture cross-contamination, but this scenario is unlikely because the results of the WGS that were conducted in cells that underwent 14 passages detected, first, the subtle difference in CNV and, second, the 20% SNV divergence between SVZ and tumor-derived cells, rendering them different cell clones. An alternative scenario would be that initially the SVZ-derived cells were more stem-like in their functional capacity compared to tumor–derived cells but, with time, their properties shifted and became similar to the progenitor population of tumor-derived cells. In other words, the SVZ-derived cells, which we have shown to align closer to NSCs and to functionally behave like a cell at the top of the GSCs' hierarchy in terms of treatment-resistance, was more regulated as opposed to tumor-derived cells and, with time, SVZ-derived cells picked up more mutations that made them similar to the tumor-derived cells. This scenario would have been confirmed have the WGS data revealed a less mutational load in the SVZ-derived cells compared to tumor-derived cells, which in our study was not statistically significant. This bears the question: whether conducting the WGS in cells freshly isolated from the SVZ might have detected the presence of less mutations in SVZ-derived cells, similar to what Lee et al. have shown: that SVZ-tumor free tissue had significantly fewer mutations (down to 1%)

compared to the tumor; but once the SVZ had visible tumor, their somatic mutations were very similar to the tumor [67]. Nevertheless, our study reiterates the drawbacks of utilizing an in-vitro culture in assessing the genomic expression and behavior of GSCs and the potential instability of GB-derived cell lines. This might reflect the aberrant proliferative advantage these GB cells possess, their capacity for adaptation to a new environment, and the intrinsic genetic evolution these GB cells undergo. It is therefore important to realize the limitations of in-vitro culture and acquire a methodology that allows the analysis of these cells as soon as they are isolated and avoid expanding them in culture for more than 2 serial passages in order to preserve their original genomic identity and what function it entitles.

Another limitation to our study is that our comprehensive analysis covered only a matched sample from one patient. It is true the study was conducted as a proof of concept but, acknowledging the well-known intra- and inter- heterogeneity of IDH-wildtype GBs, matched pairs from multiple patients would have strengthened our data and allowed for better detection of statistically significant differences between the SVZ and tumor-derived cells and provided more evidence that the differences we observed were not cell-cycle dependent nor culture induced [5, 14, 15].

One more limitation to our study is that the SVZ and tumor-derived cells were not properly assessed for their tumor-initiation capacity upon xeno-transplant, a fundamental functionality of GSCs [42, 44-46]. An in-vivo assay would have allowed us to further confirm their stemness identity along with exploring their differential behavior in-vivo.

Despite the above limitations, our genomic and chemo-response assays data show that the normal-looking SVZ harbors malignant cells at the time of diagnosis. We have also shown that SVZ-derived cells shared a similar genetic expression and functional characteristics to the cells present in the tumor. Interestingly, in our case, when we translate these findings clinically we find that the patient who harbored this tumor had a brain MRI that showed no tumor grossly detectable in the SVZ at the time of diagnosis, nor after 3 years, despite the presence of these glioma cells. The patient had a progression-free survival (PFS) of 3 years, which is very rare for a patient diagnosed with IDH-wildtype GB; the average PFS being 6 months and the overall survival being 14.2 months with GTR and adjuvant chemo-radiotherapy [20, 22]. Moreover, when the patient developed a recurrence, it was not at the surgical site, where more than 65% of cases occur, but at the area where we derived the SVZ cells, which was at least 2 cm away from the tumor margin and did not exhibit any abnormality radiologically in the MRI or intra-operatively [208] (figure 7). Some might argue, that the reason for the delay in recurrence can be due to the adjuvant treatment given at the time of diagnosis, except that the SVZ was out of the radiation field. Then the question remains, why would GSC-like cells be present in the SVZ and yet only form a tumor years later? Are these cells genetically dormant and activated subsequently over time to produce tumors? Does the cell of origin of IDH-wildtype GB arise from the SVZ and migrate to other areas of the brain, or do these GSCs arise from a different area in the brain and then migrate to a potential niche (the SVZ) to survive? There is growing evidence to support the importance of the proliferative niche and the bidirectional relationship between GSCs and their niche. On

the one hand, perivascular niches enhance stem-like properties of GSCs and promote their invasion and resistance to therapy. On the other hand, GSCs induce the remodeling of perivascular niches, generating endothelial cells and pericytes and inducing angiogenesis/vasculogenesis to support their growth [209]. Moreover, few studies have shown that irradiating the SVZ prophylactically increases the PFS and OS in a subset of GB patients [210, 211]. Therefore, in light of our findings and previous studies, the SVZ could be a potential therapeutic target in a subset of, if not in all, GB patients. Yet further studies are necessary to validate and assess the value of the SVZ as a therapeutic target in GB patients.

CONCLUSION:

We hypothesized that cells derived from the SVZ of a glioma patient will align closer to the NSCs' genotype and bear less mutational load when compared to GSCs derived from the tumor, suggesting that NSCs are the cell of origin of IDH-wildtype GB. We demonstrated that SVZ and tumor-derived cells have the genetic expression and functional characteristics of GSCs, as well as the genetic expression of NSCs. We also showed that SVZ-derived cells express more NSC markers and therefore align closer to the NSC's genotype. They also show more resistance to chemotherapy when compared to tumor-derived cells, a functionality attributed to the GSCs. The mutational load in both cell populations was similar. Our findings suggest that both cell lines are GSCs that arise from a common source but represent different clones. Whether or not GSCs derived from the SVZ align more proximally within the NSC lineage—when compared to GSCs derived from the tumor—was not conclusively demonstrated in our study.

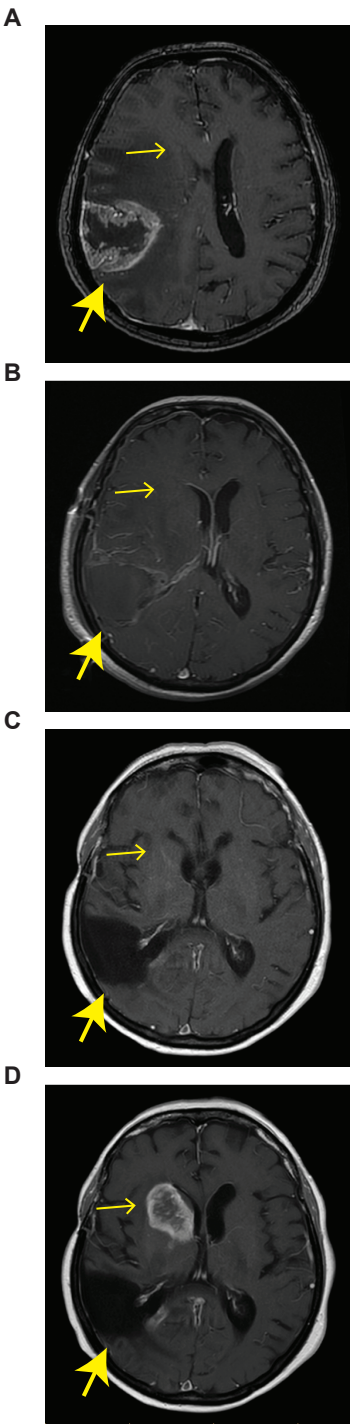


Figure 10: Tumor recurrence in the normal looking SVZ, 3 years from the time of diagnosis. Axial views of brain MRI with gadolinium showing the normal looking SVZ (yellow arrow) and the tumor (arrow head) at the time of diagnosis in (A) then post-surgical resection and adjuvant chemo-radiotherapy in (B) then 3 years from the time of diagnosis in (C) then 2 months from (C) in (D).

Methods & Materials:

Tissue Culture:

The tumor and SVZ samples were obtained, with the approval of the Montreal Neurological Hospital's research ethics board, from a consented patient. A neuropathologist confirmed the diagnosis of the tumor sample as grade 4 IDH-wildtype GBM. To isolate and enrich for GSCs, both tissues (tumor and SVZ) are washed three times in sterile Phosphate Buffered Saline (PBS) containing penicillin and streptomycin. Blood vessels are dissected out from the tissue and then it is mechanically dissociated using a pipette. Then they are digested in a collagenase solution containing DNAse and $MgCl_2$ for 1-2 hours at 37°C, with intermittent pipetting every 20 minutes. The digested specimens are washed again, three times with sterile PBS and the resulted single cells are counted. To expand SVZ and tumor-derived cells, both suspension and adherent culture were utilized. For suspension cultures, single cells were expanded as neuro-spheres in complete neurocult-proliferation media (Neurocult basal medium containing: Neurocult NS-A proliferation supplement at a concentration of 1/10 dilution, 20ng/ml recombinant EGF, 20ng/ml, recombinant bFGF, and 2µg/ml Heparin) from Stem Cells Technologies. When spheres appeared large enough for passaging (<300 µm in diameter), they were collected in a tube, and centrifuged at 1200r.p.m. for 3 minutes. To dissociate the spheres, 700microL of Accumax (Millipore) was added to the cell pellet and then incubated for 5 minutes at 37°C. Cells were then washed with PBS, centrifuged and re-suspended in complete Neurocult-NS-A proliferation medium and seeded at a concentration of 100, 000 cells/flask. All cells were maintained at 37°C in a 5% CO_2

humidified atmosphere. For adherent cultures, flasks are coated with poly-ornithine (Sigma) and laminin (Sigma) for 3 hours at 10ug/ml prior to use. Single cells are then plated in the coated flasks in an expansion media identical to the one described above. When cells appeared, confluent, Accutase (Sigma) was used to detach the cells and these cells were collected in a tube and centrifuged at 1200r.p.m. for 3 minutes. Further dissociation was carried out similar to neuro-spheres as described above Medium was replaced every 2-3 days in both culture conditions.

Immunoblotting

Preparing protein samples from cells

Once confluent, the desired cells were washed with PBS, pelleted, lysed with 0.1% NP-40 and sonicated on ice. BCA protein assay (ThermoScientific, 23225) solution was prepared by mixing BCA protein assay reagent A and B in a 50:1 ratio respectively. NP-40-BSA ladder (2mg/ml) ladder was prepared through a sequential dilution with a total volume of 10ul per well. Cell lysate samples were added in duplicates next to the ladder, 10ul per well. 200ul of BCA protein assay solution was then added to both the ladder and cell lysate wells and incubated at 37°C for 15 minutes. Protein concentrations were analyzed using Fisher Scientific's BioTek Epoch Microplate Spectrophotometer and Gen 5 1.11 Program. Results were plotted in an Excel spreadsheet and the loading volumes were determined based on calculated protein concentrations.

Western Blot

Two 10-well 12% SDS-PAGE gels were made using 30% Acrylamide, 4X Tris-SDS pH 8.8 and pH 6.8, 0.1% APS, water, and TEMED. The amount of protein loaded per well

was determined from the BCA Protein Assay. The gels were run in a 1X running buffer at 80V for 2.5 hours. The protein transfer was done using Bio-Rad nitrocellulose membranes, which was run in a 1X transfer buffer at 100V for 1.5 hours. After the transfer, the membranes were placed in ponceau to visualize the protein bands and then rinsed with water. The membranes were then blocked in 0.1% TBST-BSA for 30 minutes and the primary antibodies (Table 2) were added and incubated overnight at 4C. Membranes were washed once with distilled water and three times with 0.1%TBST, 15 minutes per wash. Secondary antibodies conjugated to HRP were then added and incubated at room temperature for 1 hour, shaking. Membranes were then washed once with distilled water, twice with 0.1% TBST, and three times with distilled water, 15 minutes per wash, changing the wash frequently. ECL western blotting substrate (Thermo Scientific, 32106) was used as per the manufacturer's instructions.

Immunofluorescence

Cells were grown on cover slips coated with laminin for 48 hours then they were fixed with 4% PFA for twenty minutes. The cells attached to the cover slips were then washed with PBS, blocked with 0.5% bovine serum albumin (BSA), and permeabilized with 0.5% Triton X-100 when detection of intracellular antigens is required. Then the cells were immunolabeled with the primary antibodies for CD133 (Miltenyi) and incubated overnight at 4C humid chamber. The following day the cells were washed with PBS and blocking buffer and incubated with secondary antibodies for 30 minutes at room temperature. Cover slips were mounted on glass slides using ProLong™ Diamond Antifade Mountant with DAPI (Molecular probes) to counterstain cell nuclei. Fluorescent images were

acquired using ZEISS LSM 700 laser scanning confocal microscope with a 20X or 63X objective.

Marker	Dilution Used	Company
Nestin	1:1000	Millipore, ABD69
SALL2	1:1000	Bethyl Laboratories, A303-208A
Ephrin A2	1:500	Santa Cruz, SC-924
GLAST	1:1000	Miltenyi Biotec, 130-095-822
GFAP	1:1000	Chemicon International
DCX	1:1000	Abcam, ab167400
Pax6	1:200	Santa Cruz, SC-32766
POU3F2	1:1000	Abcam, ab94977
TLX	1:1000	Persus Proteomics, PP-H6506-00
Sox2	1:1000	Millipore, AB5603
Olig2	1:1000	M Temecula California
DRR	1:250	Covance
BLBP	1:1000	antibodies-online.com, ABIN1574112

Table 2: Table showing the antibody dilution used and the source of each antibody used in the marker panel.

Fluorescent-activated cell sorting

Multi-parametric flow cytometry was carried out by labeling cells with CD15 (Santa Cruz), CD44-AF700 (BD), and CD133-PE or PE/Vio770 (eBioscience and Miltenyi). After leaving aside 1 x 10^5 cells as unstained control, cells were re-suspended in PBS at a concentration of 1 x 10^6/mL. Aqua live/dead dye (Molecular Probes) was added at 1:1000 and incubated for 25 minutes on ice, protected from light. Cells were washed, and 1 x 10^5 cells were kept aside for fluorescence minus-one (FMO) controls and 1e6 cells were used for complete staining with antibodies. FMO controls were prepared for all colors except aqua(live/dead). All cells were completely stained with antibodies at a final dilution of 1:50-1:20. FMO controls were used to identify for positive/negative staining.

Quantitative real-time PCR

The CFX ConnectTM Real-Time System (Bio-Rad) was used for all qPCRs. Cells were collected, washed twice with PBS, and pelleted prior to lysis. RNA was extracted using the Aurum total RNA mini kit (BioRad), according to manufacturer's protocol. RNA concentration was measured using Nanodrop, prior to cDNA synthesis with iScript Reverse Transcription Supermix (Bio-Rad). Synthesized cDNA was used for quantitative Real-Time PCR (RT- qPCR) using SsoFast EvaGreen Supermix (Bio-Rad). All reactions were run in triplicates. Reactions with no template and reactions with no Reverse Transcriptase were included in each experiment as controls. The following primers used for this study, including the sequences for control primers, are as indicated in Table 3.

GENE	FWD PRIMER	REV PRIMER
BLBP	CCA GCT GGG AGA AGA GTT TG	CTC ATA GTG GCG AAC AGC AA
CD15	TGC CAG CCA CCG AAT AAA	CAT GTG GAA TCC CGG TAA CA
PAX6	GGG CAA TCG GTG GTA GTA AA	CTA GCC AGG TTG CGA AGA AC
TLX	GCT AAC ACT CTA CTG GCT GTA TC	GAG CCA CCA CCT CTT GTA AA
OLIG2	GCT GCG TCT CAA GAT CAA CA	CAC CAG TCG CTT CAT CTC CT
POU3F2	ATG TGC AAG CTG AAG CCT TT	CTC ACC ACC TCC TTC TCC AG
SOX2	TTG CTG CCT CTT TAA GAC TAG GA	CTG GGG CTC AAA CTT CTC TC
CD44	CAT CTA CCC CAG CAA CCC TA	GGT TGT GTT TGC TCC ACC TT
CD133	TTG TGG CAA ATC ACC AGG T	TCA GAT CTG TGA ACG CCT TA
GFAP	GGC CCG CCA CTT GCA GGA GTC C	CTT CTG CTC GGG CCC CTC ATG A

Table 3: Table showing the primer sequence used in the QTR-PCR.

Deep WGS

Genomic DNA was extracted with either the QIAamp mini DNA kit (Qiagen) for freshly frozen brain tissues or the Wizard Genomic DNA Purification Kit (Promega) for blood following the manufacturers' instructions. Each sequenced sample was prepared according to Agilent library preparation protocols (Agilent Human All Exon 50 Mb kit). Libraries underwent paired-end sequencing on an Illumina HiSeq 2000 and 2500 instrument (average read depth of 391Å~) according to the manufacturer's protocol. The tumor-pair analysis procedure was done using the GenPipes analysis framework [212]. It entails trimming raw reads derived from whole-genome followed by alignment to a known reference, post-alignment refinements, and variant calling. Trimmed reads are aligned to a reference by the Burrows-Wheeler Aligner, bwa-mem [213]. Refinements of mismatches near insertions and deletions (in-dels) and base qualities are performed using GATK in-dels realignment and base recalibration to improve read quality after alignment [214]. Processed reads are marked as fragment duplicates using Picard Mark Duplicates and single-nucleotide polymorphisms and small in-dels are identified using

either GATK Mutect2 [214]. Copy Number Analysis have been performed using the combination of BVAtools (https://bitbucket.org/mugqic/bvatools/src/master) and ScoNEs (https://bitbucket.org/mugqic/scones/src/master) tools. Alignment refinement and variant identification utilize both tumor and normal samples together in order maximizing the information and improve the detection of somatic events.

TMZ

Cell Viability Assay

The following GSCs cell line: SVZ & OPK 126 were seeded in a 96-well plate at a density of 5000 cells (passage 8) per well, in triplicates per condition. TMZ was dissolved in DMSO and diluted in NCC. Cells were treated with increasing concentrations of TMZ (0, 50, 100, 200, 300uM) for 5 days. As a control for TMZ, cells were treated with DMSO in the 0µM TMZ condition. Cells were then incubated with XTT Cell Viability Assay for 4 hours. Cells were then subjected to absorption reading at 480nm for XTT.

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