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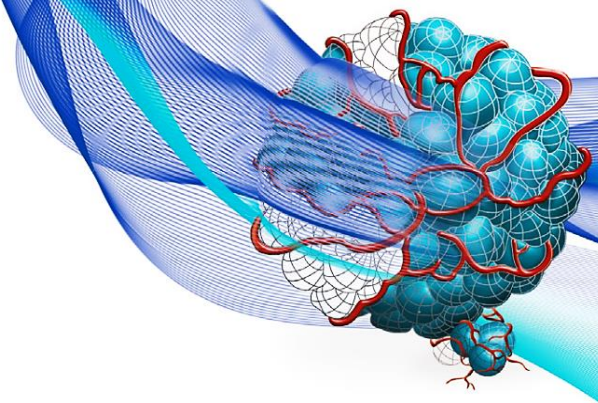

Role of Subventricular Zone-Released CXCL12 in Glioblastoma Invasion and Radioresistance.

Nicolas Goffart

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Thesis submitted to fulfill the requirements for the degree of

Philosophiae Doctor in Biomedical and Pharmaceutical Sciences

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Academic Year 2014-2015

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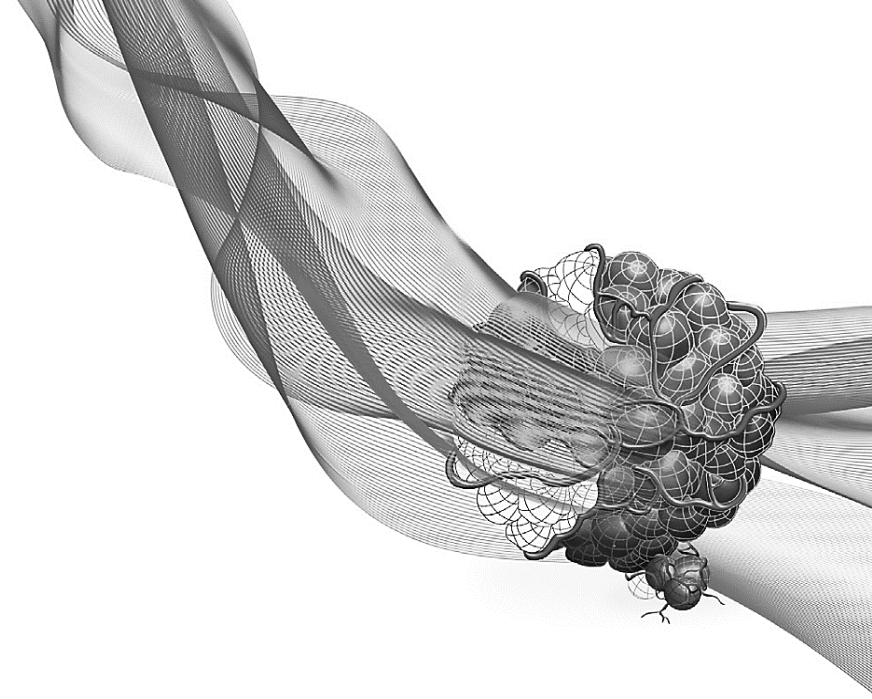

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« Bear in mind that the wonderful things you learn in schools are the work of many generations. All this is put in your hands as your inheritance in order that you may receive it, honor it, add to it, and one day faithfully hand it on to your children. »

- Albert Einstein

Acknowledgments

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Abbreviation List

- **BBB**: Blood Brain Barrier
- **BM**: Bone Marrow
- **CBTRUS**: Central Brain Tumor Registry of the United States
- **CC**: *Corpus Callosum*
- **CDK**: Cyclin-Dependent Kinase
- **CNS**: Central Nervous System
- **CRBL**: Cerebellum
- **CSC** : Cancer Stem Cell
- **DG**: Dentate Gyrus
- **EC**: Endothelial Cell
- **ECM**: Extra-Cellular Matrix
- **EGF**: Epidermal Growth Factor
- **EGFR**: Epidermal Growth Factor Receptor
- **EMT**: Epithelial-to-Mesenchymal Transition
- **FGF**: Fibroblast Growth Factor
- **GBM**: Glioblastoma Multiforme
- **GFAP**: Glial Fibrillary Acidic Protein
- **GFP**: Green Fluorescent Protein
- **GPCR**: G Protein-Coupled Receptor
- **GSC**: Glioblastoma Stem Cell
- **GTR**: Gross Total Resection
- **Gy**: Gray
- **HSC**: Hematopoietic Stem Cell
- **IDH**: Isocitrate Dehydrogenase
- **KPS**: Karnofsky Performance Score
- **LOH**: Loss of Heterozygosity
- **MAPK**: Mitogen-Activated Protein Kinase
- **MSC**: Mesenchymal Stem Cells

- **NPC**: Neural Progenitor Cells
- **NSC**: Neural Stem Cells
- **OB**: Olfactory Bulb
- **OS**: Overall Survival
- **OPC**: Oligodendrocyte Precursor Cell
- **PBS**: Phosphate Buffer Saline
- **PDGF**: Platelet-Derived Growth Factor
- **PFS**: Progression-Free Survival
- **PI3K**: Phosphoinositide 3-Kinase
- **PTEN**: Phosphatase and TENsin homolog
- **Rb**: Retinoblastoma protein
- **SGZ**: Subgranular Zone
- **SVZ**: Subventricular Zone
- **SVZ-CM**: Subventricular Zone-Conditioned Medium
- **TCGA**: The Cancer Genome Atlas
- **TM**: Tumor Mass
- **TMZ**: Temozolomide
- **VEGF**: Vascular Endothelial Growth Factor
- **WHO**: World Health Organization

Summary

Patients with glioblastoma multiforme (GBM) display an overall median survival of 15 months despite multimodal therapy. This catastrophic survival rate is the consequence of systematic relapses which may arise from remaining glioblastoma stem cells (GSC) left behind after surgery. We and others demonstrated that GBM cells enriched in tumor-initiating abilities (or GSC) escape the tumor mass and specifically colonize the adult subventricular zone (SVZ) after transplantation in mice brains. This specific location, away from the initial tumor site, may therefore represent a high-quality model of clinical obstacle to therapy and relapses since GSC specifically retain the ability to form secondary tumors. Relying on recent findings demonstrating the existence of GSC in the human SVZ, we first focused on the molecular mechanisms underlying the oriented migration of these GSC toward the SVZ stem cell niche. In this context, several *in vitro* experiments strongly suggested the importance of SVZ-released CXCL12 in this original model of brain cancer invasion. Furthermore, interfering with the CXCL12/CXCR4 signalling significantly hampered the *in vivo* invasion of the SVZ, suggesting this signalling system to tightly regulate GSC migration abilities as well as their particular tropism toward the SVZ region.

Then, relying on the implication of GSC in resistance to therapy, we wondered whether the SVZ environment could endorse the role of a GSC reservoir potentially involved in malignant brain tumor relapses. In this context, we demonstrated SVZ-nested GSC to be specifically resistant to radiation *in vivo*. Interestingly, these cells also displayed enhanced mesenchymal hallmarks compared to GBM cells from the tumor mass. Of note, the acquisition of mesenchymal properties usually correlates with sharper therapeutic resistance. These mesenchymal traits were further shown up-regulated upon CXCL12 stimulation *in vitro*. Interestingly enough, SVZ-released CXCL12 was finally demonstrated to mediate GBM resistance to radiation *in vitro.*

Taken together, these data highlighted the critical role undertaken by CXCL12 in mediating the invasion of the SVZ environment by GBM cells enriched in tumor-initiating abilities (GSC). These findings also underpinned the adult SVZ stem cell niche as a potential environment involved in GBM extrinsic resistance to radiotherapy and strongly suggest the SVZ to play a role in GBM relapses. Further research is therefore mandatory to better characterize the relationship between GSC and the SVZ. This could potentially lead to the identification of new therapeutic targets disrupting this union and impairing with GSC intrinsic properties.

Table of Content

Introduction

Chapter I

1. Brain tumors

Anarchical cell proliferation inescapably leads to benign or malignant tumor formation. This is true for every single organ including the central nervous system (CNS). Brain tumors encompass neoplasms that originate from the brain itself (primary brain tumors) or involve the brain as a metastatic site (secondary brain tumors). Primary brain tumors account for 2.4% of all cancer mortality whereas metastatic tumors are described as the most frequent type of adult brain tumors and account for 30% of all cancers. Most of the CNS metastatic neoplasms are highly infiltrating carcinomas which may, for instance, derive from lung, breast and kidney. On the other hand, gliomas account for the majority of primary tumors arising within the brain parenchyma. The term "glioma" refers to tumors which display histologic features similar to normal glial cells (astrocytes, oligodendrocytes and ependymal cells). However, the cells of origin for most malignant primary brain tumors remain enigmatic. While traditional sources favor an origin from normal glial cells, more recent data suggest such tumors to arise from neural stem cells (NSC) or from neural/glial progenitors [1].

Historical attempts to develop a classification system for brain tumors date back to the 1830s. The German pathologist Rudolf Virchow first introduced the term "glioma" in 1865. Virchow was also the first to correlate microscopic and macroscopic features of CNS tumors [2]. Ever since Virchow's era, different classifications have been handed down. Brain primary neoplasms are nowadays mainly classified according to the tissue they originate, including neuroepithelial, hematopoietic, lymphatic, meningeal and embryonic tissue. Gliomas are typically described as neuroepithelial tumors and are classified based on the predominant cell type, histologic features, mutations and outcomes. In this context, the World Health Organization (WHO) employs a classification based on histological features, dividing astrocytic gliomas into four distinct groups, identified as grade I to IV in accordance with variable therapeutic and clinical outcomes [3]. Grade I is benign, rare and most of the time associated with long survival. Grade II is characterized by the formation of an infiltrative mass and displays low mitotic activities. Grade III shows high mitotic figures, invades other regions of the brain and tends to recur after surgical resection. Grade IV is the most aggressive and most malignant type of astrocytic tumors. It grows quickly, invades the nearby healthy tissue, is

mitotically active and displays large areas of necrosis and endothelial hyperplasia [3]. Taken together, these data allow to draw a simplified decision tree for the diagnosis of the disease, notably taking into account the type of glioma encountered, its nature and its WHO grade (Figure 1).

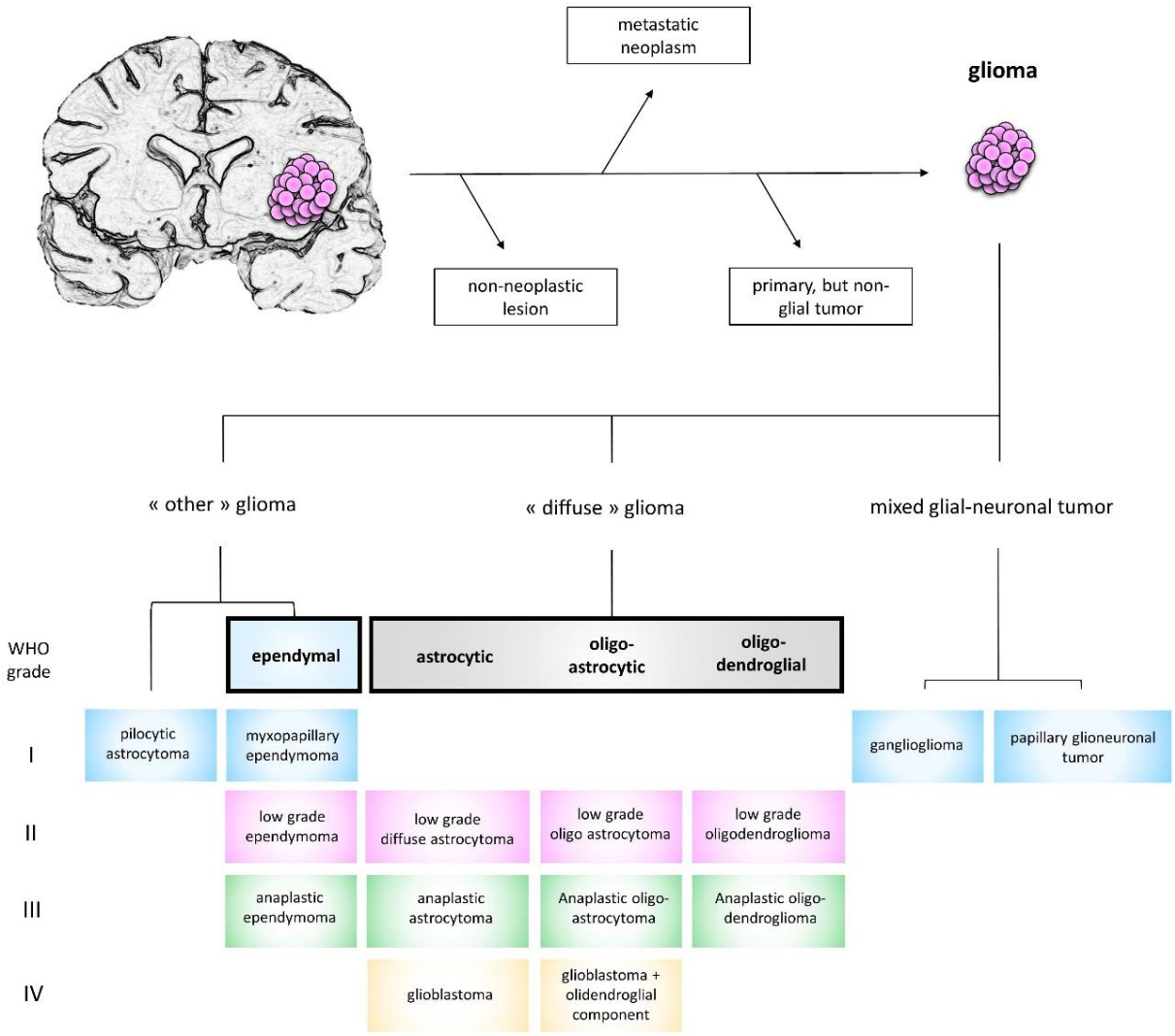


FIGURE 1. Simplified decision tree for histopathological diagnosis of gliomas. After excluding a number of differential diagnostic options, the neoplasm is further defined within some categories depending on its nature and graded according to specific histopathological features described in the manuscript. The term mixed glial-neuronal tumor underscores that gliomas are not just glial in nature. Of note, it is important to keep in mind that this scheme is not complete since the WHO classification recognizes multiple other entities in the group named "other glioma", including pilomyxoid astrocytoma (WHO grade II), angiocentric glioma (WHO grade I), chordoid glioma of the third ventricle (WHO grade II), desmoplastic infantile astrocytoma (WHO grade I) and pituicytoma (WHO grade I). The group named "mixed glial-neuronal tumor" also includes additional members such as desmoplastic infantile ganglioglioma (WHO grade I) and rosette-forming glioneuronal tumor of the fourth ventricle (WHO grade I). Discussion of these very infrequent glial tumors falls however outside the scope of the present work.

2. Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is the deadliest type of cancer and the most frequent primary malignancy of the CNS. Relying on the decision tree we just described, GBM are easily characterized as WHO grade IV diffuse gliomas exhibiting an astrocytic origin (Figure 1). This neoplasm carries an average survival rate of approximately 15 months from the time of diagnosis [4]. The standards of care for GBM patients include surgery, radiotherapy and chemotherapy with temozolomide (TMZ). Each treatment modality may be used alone or in combination with one another, most of the time depending on the tumor location, symptoms, the patient's general condition/age and the tumor's primacy or recurrence [5, 6]. The only purposes of these therapeutic options are to improve the patient's quality of life and survival but not to cure the patients yet. In the facts, GBM usually overcome classical treatments unfortunately leading to tumor recurrence. GBM are therefore considered as one of the nastiest scourge to fight in hospital settings, responsible for the early death of about 500 people each year in Belgium.

Grade IV brain tumors are characterized by extreme proliferation, invasion vascularization and therapeutic resistance [7, 8]. On the other hand, it is also a very complicated process to pinpoint one particular mechanism driving tumorigenesis since GBM are extremely heterogeneous in nature. These tumors are indeed made up of immature and progenitor cells but also express differentiation markers of all three major brain cell types including neurons, oligodendrocytes and astrocytes [9]. Because of their complex nature, GBM have recently been classified into distinct subtypes with regard to a wide panel of molecular signatures. Extensive genetic analyses indeed classified malignant brain tumors into neural, pro-neural, classical and mesenchymal subtypes based on data from The Cancer Genome Atlas (TCGA) [10]. A more detailed description of this GBM stratification is further discussed in the present manuscript (see Chapter I, section 5.5). Let's just keep in mind for the moment that this classification particularly helped to dive deeper in the tumor biology and could potentially be of clinical interest in predicting outcomes. As matter of fact, neural and pro-neural subgroups have been described as great responders to therapy whereas classical and mesenchymal tumors often display a poor prognosis [10, 11]. These recent transcriptional breakthroughs, highlighting the complex nature/heterogeneity of malignant brain tumors, also emphasize the fact that GBM can definitely not be treated with a one-size fits all approach. Innovative molecular targets specific

to each tumor subtype need to be identified and associated in multipronged therapeutic strategies in order to weaken the tumor and potentially overcome GBM deadly relapses.

3. Descriptive Epidemiology

Government cancer surveillance and strong health system records are mandatory in order to track the incidence of gliomas in a reliable manner. Gliomas indeed vary significantly according to the histologic type, age at diagnosis, gender, race and country [12]. Overall age-adjusted incidence rates for all gliomas range from 4.67 to 5.73 per 100 000, representing more than 80% of malignant brain tumors [13]. Glioblastoma is the most common glioma subtype and displays a 5-year relative survival of only 0.05-4.7% despite access to state-of-the-art therapeutic modalities [14]. The annual incidence of GBM ranges from 0.59 to 3.69 per 100 000 [13, 15] and increases with age, peaking in the 50-55 age group [16]. Examination of brain tumor incidence data from the Central Brain Tumor Registry of the United States (CBTRUS) reveals a slight but significant annual increase in incidence [17]. Moreover, a recent census of the annual age-adjusted incidence in Nordic countries between 1979 and 2008 found no clear trend in glioma incidence rates during the assessed period, though there was also a slight increase in brain tumor incidence rates overall [18].

Curiously, primary GBM tend to develop more frequently in males than in females (M:F ratio = 3:1) and are rarely seen in younger patients, constituting only 8.8% of all childhood CNS tumors [16, 19]. In the United States, gliomas are more common in non-Hispanic whites than in blacks, Asian/Pacific Islanders and American Indians/Alaska Natives [14, 20].

Malignant brain tumors could also be a component of several familial tumor syndromes associated with an increased incidence of GBM, the most important ones being the Li-Fraumeni syndrome (*TP53* germline mutations), neurofibromatosis 1 and 2 (mutations in the *NF1* or *NF2* genes respectively) and Turcot syndrome (APC and hMLH1/hPSM2 germline mutations) [7, 21]. The occurrence of brain tumors in individuals with hereditary syndromes supports the hypothesis of a genetic predisposition to brain tumors. However, syndromes resulting from mutations in high penetrance genes are rare [3].

Several occupations (physicians, firefighters and farmers), environmental carcinogens (vinyl chloride, several industrially used chemicals, polycyclic aromatic hydrocarbons) and diet

(N-nitroso compounds) have been associated with a plausible elevated risk of GBM development. Conversely, the only environmental factor unequivocally associated with an increased risk of brain tumor development is the exposition to therapeutic X-rays [22-25]. Children treated with X-rays for acute lymphoblastic leukemia have indeed been reported to present a significant higher risk of GBM and primitive neuroectodermal tumor (PNET) development up to 10 years after the initial therapy [26-28].

4. Histology/Pathology

GBM are preferentially located in the supratentorial and periventricular white matter [21, 29]. It is referred to as "multiforme" because the tumor exhibits heterogeneity in many aspects including clinical presentation, pathology, genetic characteristics and response to therapy [30]. GBM may develop spontaneously (*de novo* or primary) or by progression from pre-existing lower grade gliomas (secondary) [7, 31]. Primary GBM usually tend to occur in older patients and are characterized by the amplification of the epidermal growth factor receptor gene (*EGFR*), overexpression of MDM2 (an important negative regulator of the p53 tumor suppressor), mutations in the phosphatase and tensin homolog gene (*PTEN*) and/or loss of heterozygosity (LOH) of chromosome 10p [32]. On the other hand, secondary GBM tend to be less aggressive and usually occur in younger patients. Characteristically, and unlike primary GBM, secondary GBM display isocitrate dehydrogenase 1 (*IDH1*) and *p53* mutations, platelet-derived growth factor alpha (*PDGFα*) amplification, LOH of chromosomes 10q and 17p, loss of chromosome 19q and an increased telomerase activity and hTERT expression [32]. Among these genetic aberrations, *IDH1* mutations are the most appropriate to discriminate primary from secondary GBM and are found in over 80% of grade II and III astrocytomas [33].

Upon gross observation, GBM are not encapsulated and poorly delineated. Tumors often contain large areas of necrosis and discoloration from areas of hemorrhage [30, 34]. On a cellular level, GBM are characterized by abnormalities of both cellular and nuclear structures, diffuse borders, microvascular proliferation, high density of cells per unit area, "giant" cells with multiple nuclei and necrosis with regions of pseudopalisading cells (Figure 2) [7, 35].

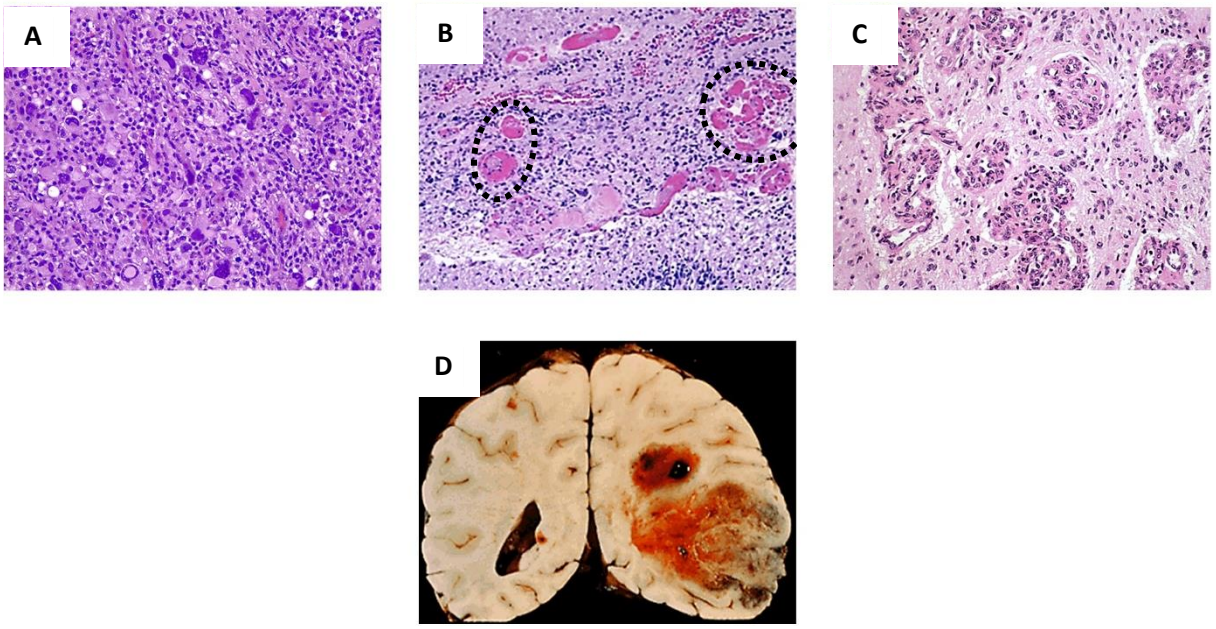


FIGURE 2. Common histological features observed in GBM. Microscopically, GBM show areas of high cellular density, large areas of necrosis and microvascular proliferation. Panel A shows cellular anaplasia in a large number of GBM cells. Panel B shows multiple areas of necrosis (as indicated by dotted circles). While areas of necrosis may be hypoxic, GBM are also highly vascularized. Tumor growth is indeed sustained with microvascular proliferation as shown in panel C. GBM are finally characterized by large areas of diffuse migration and angiogenesis. Panel D shows a coronal section of a brain containing a large and diffuse hemorrhagic GBM. Images from: *http://neuropathology-web.org/chapter7/chapter7bGliomas.html#gbm*

For pathologists the presence of microvascular proliferation and pseudopalisades surrounding an area of necrosis are usually the prerequisites for the classification as a grade IV astrocytoma. Both criteria are indeed regarded as reliable indicators of the tumor aggressiveness [34].

5. What is Wrong with my Genome?

Over the last 20 years, different studies have helped to identify the most common genetic alterations in GBM. The most significant breakthrough in the field was the elaboration of "*The Cancer Genome Atlas*" (TCGA). This gigantic library was originally meant to fully characterize different types of cancer both at the genetic and transcriptional level [36]. Malignant brain tumors were among the first type of neoplasms to be characterized, using over 200 pathologically diagnosed cases. One of the most compelling purpose of the TCGA was to

map core pathways with one another. This process notably allowed to uncover the most frequently observed genetic alterations in GBM and provided a network view of pathways altered in GBM development. This multidimensional analysis of genomic data outstandingly shed the light on a plethora of new unsuspected-before targets suitable for GBM treatment. This part of the manuscript is therefore dedicated to the description of the most common genetic alterations found in GBM and will then explain how the TCGA reformed the way we now look at grade IV malignant brain tumors.

5.1.Loss of Heterozygosity on Chromosome 10 and PTEN Mutations

LOH on chromosome 10 is the most common genetic abnormality found in GBM, occurring in 70% of the cases [19, 31]. As mentioned in the previous section, GBM can develop *de novo* (primary) or through the progression from a low-grade or anaplastic astrocytoma (secondary). Interestingly, primary GBM usually display an entire loss of chromosome 10 in contrast with secondary GBM which either show a partial or complete loss of chromosome 10q but no loss of 10p. This actually suggests that LOH on chromosome 10q is a major factor in the evolution of GBM as the common phenotypic end point of both genetic pathways, whereas LOH on chromosome 10p is largely restricted to primary GBM (Figure 3) [37]. Malignant Grade IV brain tumors with partial LOH on chromosome 10 display three major deletions (10p14-pter / 10q23-24 / 10q25-qter), suggesting the presence of multiple tumor suppressor genes [19]. Indeed, many genes known to be important in cancer are found on chromosome 10, including Mitogen Activated Protein Kinase 8 (*MAPK8*), Frequently Rearranged in T-cell lymphoma 1 (*FRAT1*), Murine leukemia viral oncogene homolog (*BMI1*) and Phosphatase and tensin homolog (*PTEN*) reported to be mutated in up to 40% of GBM [35, 38]. Loss of PTEN functions by mutations or LOH correlates with poor survival in anaplastic astrocytoma and GBM, suggesting PTEN to play a role in patient outcomes [39]. Interestingly, amplification of EGFR in the background of heterozygous *PTEN* knockout mice leads to the development of invasive GBM very similar to the human condition, demonstrating in this way the importance of PTEN in GBM progression [39]. In the same line, GBM cells mutated in both *p53* and *PTEN* genes (*p53*mut/*PTEN*mut) or *PTEN* alone (*p53*wt/*PTEN*mut) exhibit higher invasion rates and preferentially express F-actin in filopodia and lamellipodia compared to wild-type *p53* and wild-type *PTEN* or mutated *p53* and wild-type *PTEN* GBM cells [40, 41]. Apart from enhanced invasive abilities, inactivated PTEN has also been correlated with increased angiogenesis [42-

44], increased proliferation [45] and extensive centromere breakage/chromosomal translocations, suggesting a fundamental role of PTEN in the maintenance of chromosomal stability [46].

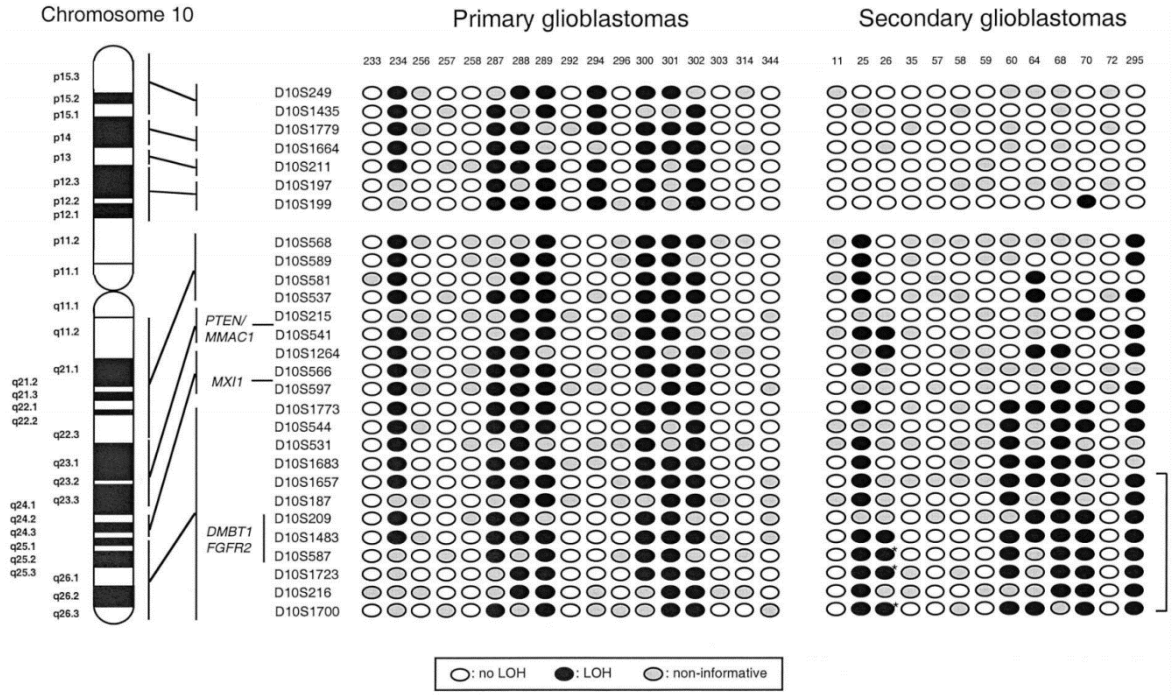


FIGURE 3. Allelic patterns of chromosome 10 in 17 primary and 13 secondary GBM. The overall frequency of loss of heterozygosity (LOH) on chromosome 10 is similar in both GBM subtypes but the extent of chromosomal loss differs. Primary GBM often show a complete loss of chromosome 10 (10p and 10q) while, in secondary GBM, LOH is typically restricted to the long arm (10q only) [37].

5.2.The Epidermal Growth Factor Receptor Overexpression

The second most common genetic aberration encountered in GBM is the overexpression of the epidermal growth factor receptor (EGFR, also referred to as ErbB1), occurring in 36% of primary GBM and in 8% of secondary GBM [7, 31]. EGFR is a cell surface receptor with a tyrosine kinase cytoplasmic domain [47] and belongs to the HER superfamily together with ErbB2, ErbB3 and ErbB4 [48]. Family members ErbB1 and ErbB3 are known to form heterodimers with ErbB2 whereas ErbB1 has the ability to form homodimers [49]. The most common ligands for HER receptors are the members of the EGF family of growth factors including transforming growth factor α (TGFα) and EGF among others [50].

Binding of the cognate ligand to HER receptors induces the receptors homo- or heterodimerization, resulting in a conformational change that will activate the intracellular tyrosine kinase domain of the receptor. This results in autophosphorylation of its cytoplasmic tail and activates downstream signaling pathways including the phosphatidylinositol 3-kinase (PI3K)/Akt and the ras-raf-mitogen-activated protein kinase (MAPK) pathways [48]. Several mutated variants of *EGFR* have been identified in GBM, with the most common being variant III (*EGFRvIII*). This mutation, within the ligand binding site of the receptor, leads to its constitutive activation and is found in about 50% of all GBM [7]. EGFRvIII overexpression has been shown to correlate with increased tumor initiation, invasion, proliferation and inhibition of apoptosis [51, 52].

The value of EGFR overexpression as a prognostic indicator is open to discussion. Different studies have indeed reported the *EGFR* amplification as an independent, significant, unfavorable predictor for overall survival (OS) in GBM patients [53-55]. On the other hand, other studies, conducted on larger cohorts, failed to show any correlation [38]. Relying on age stratifications among patients, Ohgaki and collaborators nevertheless defined the *EGFR* gene status as a strong predictive indicator in patients younger than 60 [38].

5.3.The Rb-CDK4/6-p16^{INK4A} Pathway

Disruption of the Retinoblastoma (Rb) pathway appears as a requested event in tumor formation, either achieved through inactivating alterations of Rb, amplification of the G1 cyclin-dependent kinases 4 or 6 (*CDK4/6*) or deletion/silencing of the *p16^{INK4A}* tumor suppressor [36, 56]. The Rb protein is indeed a negative regulator of the cell cycle. It controls the cells progression from G1 to S phase [47]. *Rb* alterations occur in all 4 GBM subtypes but loss of Rb expression is a more frequent event in the pro-neural group (see Chapter I, section 5.5) [57]. Loss of Rb expression in GBM correlates most of the time with methylation of its promoter region [58]. Methylation of the *Rb* promoter is not found in low-grade diffuse and anaplastic astrocytoma, suggesting the *Rb* promoter methylation as a late event during astrocytoma progression [58].

The cyclin-dependent kinase 4 (CDK4)/cyclin D1 complex is known to promote tumor growth by phosphorylating Rb. This consequently induces the release of E2F, a transcription factor that activates genes involved in the G1/S transition [19]. Pharmacological inhibition of

CDK4 (by the PD0332991 compound) was recently shown to inhibit tumor growth and prolong survival in both intracranial xenografts and GBM genetically engineered models [59, 60]. Clinical trial evaluation of PD0332991 in patients with recurrent Rb-positive GBM has just been completed but results haven't been released yet (NCT01227434 – *www.clinicaltrials.gov*). While waiting for the final conclusions of this phase II clinical investigation, Sarkaria and colleagues raised the fact that penetration across the blood brain barrier (BBB) may affect the efficacy of experimental therapies and PD0332991 is no exception to the rule. They indeed demonstrated that this compound was effective in GBM subcutaneous flank tumor models but ineffective in orthotopic models, suggesting limited penetration across the BBB [61].

Tumor suppressor $p16^{INK4a}$ binds to CDK4 and prevents the phosphorylation of Rb, therefore inhibiting the G1/S transition. Interestingly, approximately 31% of *de novo* tumors display $p16^{INK4a}$ deletions, allowing a stronger CDK4 activity and cell cycle progression [31]. Additionally, inactivating mutations in $p16^{INK4a}$ also lead to the expression of anti-apoptotic genes which, in turn, favor unrestrained cell proliferation [62]. Surprisingly, relying on many recent population based studies, using both univariate and multivariate analysis, no predictive value could be associated with $p16^{INK4a}$ deletions in GBM [63, 64].

5.4. p53-MDM2-p14^{ARF} Pathway

p53 is often described as the "guardian of the genome". The p53 pathway is indeed crucial in the proper maintenance of the cell cycle in response to cellular stress including radiation exposure, DNA strand breaks and toxins. p53 facilitates DNA repair by halting the cell cycle or, if damages are too important, induces cell death. Following DNA damages, p53 gets activated and triggers transcription of genes such as $p21^{Waf1/Cip1}$. This notably helps to block the cell cycle by inhibiting CDKs and allows DNA repair enzymes to do their job [65]. p53 is stabilized by $p14^{ARF}$ and degraded by MDM2 [66]. The genetic locus *INK4a/ARF* on chromosome 9p21 produces both $p14^{ARF}$ and $p16^{INK4a}$ by alternative splicing [65]. Since $p16^{INK4a}$ negatively regulates CDK4 (see Chapter I, section 5.3), $p14^{ARF}$ inhibits MDM2, consequently blocking the rapid ubiquitin-mediated degradation of p53 [19]. *p53* mutation are present in 28% of primary GBM and 65% of secondary GBM [31]. The type and distribution of *p53* mutations may differ between GBM subtypes. In secondary GBM, 57% of *p53* mutations are located in two hotspot codons (248 and 273) whereas mutations are more equally distributed

through all exons in primary GBM [38]. Amplification of *MDM2* occurs in less than 10% of GBM usually in tumors lacking *p53* mutations [19]. $p14^{ARF}$ expression is lost in 76% of GBM and has been correlated either with an homozygous deletion or methylation of the $p14^{ARF}$ gene promotor [19, 31]. No difference is found in the overall frequency of $p14^{ARF}$ alterations between primary and secondary GBM, but the $p14^{ARF}$ promoter methylation is actually more frequent in secondary GBM [67].

5.5. *Order brought to Chaos: Verhaak and The Cancer Genome Atlas*

Different studies have helped to expand our knowledge on GBM genetics but, until recently, very little was known on the molecular alterations which truly underlie tumor aggressiveness [68]. As previously mentioned, the TCGA study tremendously helped to identify genetic signatures associated with survival and GBM progression [36]. Indeed, this pilot project rapidly gave rise to a detailed catalogue of genomic abnormalities commonly found in GBM. In a study published in 2010, Verhaak and colleagues leveraged the full scope of TCGA data and painted a coherent portrait of GBM molecular subclasses. They provided a framework that actually unifies transcriptomic and genomic dimensions in order to better classify malignant brain tumors. They ended up with a four-point classification including classical, mesenchymal, pro-neural and neural subtypes (Figure 4).

- The classical subtype systematically correlates with an amplification of chromosome 7 and a loss of chromosome 10. As a result, *EGFR* gene amplification together with loss of $p16^{INK4A}$ and $p14^{ARF}$ genes are observed in 97% of classical tumors. Interestingly, the NSC marker Nestin as well as intermediates from the Notch (NOTCH3, JAG1) and Sonic hedgehog (SMO, GAS1, GLI2) signaling pathways are found highly expressed in this specific subtype.

- The mesenchymal subtype displays expression of mesenchymal markers such as CHI3L1 (also known as YKL40) and MET. Concomitant mutations in the *NF1* and *PTEN* genes are also characteristic of this subtype. Genes involved in tumor necrosis factor and NF-κB pathways are specifically found overexpressed in GBM mesenchymal tumors, potentially as a consequence of higher overall necrosis and associated inflammatory infiltrates in mesenchymal tumors.

- The pro-neural subtype is featured by two major alterations: the α-type platelet derived growth factor receptor (*PDGFRA*) amplification and isocitrate dehydrogenase (*IDH1*) point mutations. *p53* mutations and LOH are also frequent events in this subtype. Chromosome 7 amplification paired with loss of chromosome 10 were observed in 54% of the TCGA samples.

- The neural subtype is still nowadays poorly defined but can be typified by the expression of neuronal markers such as NEFL, GABRA1, SYT1 and SLC12A5.

From this study, Verhaak and colleagues concluded that aberrations of *EGFR*, *NF1* and *PDGFRA/IDH1* genes respectively defined the classical, mesenchymal and pro-neural subtypes and showed a trend toward increased survival rates in patients bearing pro-neural tumors [10].

Prior to Verhaak and the TCGA study, Phillips and colleagues already described three major groups to classify grade III and IV brain tumors based on gene expression [68]. Phillips' pro-neural and mesenchymal subtypes were similar to the ones described by Verhaak. Phillips also described the existence of a proliferative subtype which could partly be compared to Verhaak's classical subtype (Figure 4). In this study, mesenchymal and proliferative subtypes accounted for grade IV gliomas, while the pro-neural subtype covered both grade III and IV gliomas [68]. Since Verhaak and colleagues described frequent *p53* and *IDH1* mutations in pro-neural tumors and relying on the fact that these alterations are characteristic hallmarks of secondary GBM (see Chapter I, section 4) [69, 70], it is tempting to speculate that several tumors within this subtype could actually represent secondary GBM. Deeper investigations in the TCGA data indeed revealed that 75% of the tumors characterized as pro-neural by Verhaak were in fact secondary GBM [10]. Additionally, Phillips and colleagues elegantly correlated prognosis with subtypes and stated that tumors with a pro-neural signature are to predict a better prognosis compared to mesenchymal or proliferative tumors [68]. Apart from prognosis, GBM subtypes may also be correlated to patient outcomes and response to therapy [71-73]. Taken together, these findings specifically emphasize the importance of GBM molecular subtyping in the subsequent management of patients.

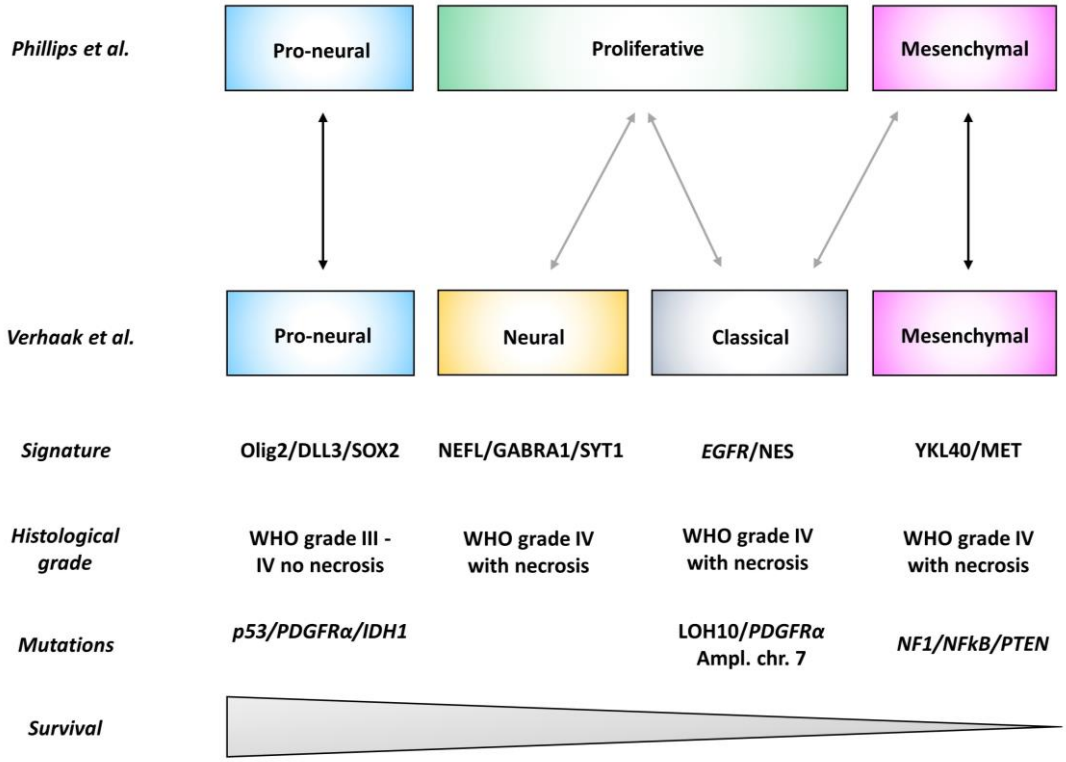


FIGURE 4. Molecular subtyping of GBM based on gene expression. The direct comparison between Phillips' and Verhaak's datasets shows a near complete agreement for pro-neural and mesenchymal GBM signatures (black arrows). There is however less concordance between proliferative and neural/classical GBM subtypes (grey arrows). Overall, Phillips and Verhaak both agree on the fact that survival decreases from the pro-neural toward the mesenchymal subtype. (Illustration modified from Woehrer et *al.*, 2010)

Evidence have demonstrated that GBM subtyping might be essential to tackle down the issue but one must always keep in mind that tumor samples used for scientific purposes only represent a small fraction of the whole tumor. Since GBM tumors are highly heterogeneous in nature, researchers have then reported different GBM subtypes to co-exist within the same tumor [74-76]. Moreover, transitions between one subtype to another upon recurrence has also been described and may complicate the choice for the most appropriate therapy [77-79]. However, as GBM subtypes correlate to some extent with prognosis and response to therapy, it might be worth to give this classification a try to the clinic in order to drive stratified patients toward the most optimal treatment.

Chapter II

1. Cancer Stem Cells: the Birth of a Myth

The cells responsible for the onset of malignant gliomas have been source of discord for many years and are still a matter of fierce discussion. Growing evidence support the idea that malignant tumors are initiated and maintained by a sub-population of tumor cells with similar biological properties as normal adult stem cells [80-82]. This concept was first described by Rudolf Virchow in 1863. Based on histological similarities between embryonic stem cells and cancer cells, Virchow proposed that tumors originally develop from "dormant" or quiescent cells located in the host tissue. From then on, the existence of such a fraction of cells has been demonstrated in many types of cancer [83, 84] including brain tumors [85-87]. Normal stem cells are defined by both their ability to make more stem cells, a property known as "self-renewal" and their ability to produce cells which differentiate, also known as progenitor cells. Stem cells are notably able to accomplish these two tasks by asymmetric cell divisions. Progenitor cells then give rise to several new identical progenitors through symmetric cell division before they become proliferative exhausted and begin to terminally differentiate [88, 89]. Relying on these features, the cancer stem cell theory postulates that only a specific minority of tumor cells display the ability to initiate tumors and that these cells may possibly arise from mutations in normal stem or progenitor cells [83, 90].

2. Glioblastoma Stem Cells: Who the Heck are You?

Brain tumor stem cells or glioblastoma stem cells (GSC) are thought to arise from oncogenic mutations in neural stem cells (NSC). This idea is fueled by the facts that GSC express putative markers of human NSC including Sox2, Nestin and prominin-1, also known as CD133 [91, 92]. CD133 has emerged over the years as a key marker for GSC identification, being essential for their maintenance and tumorigenic potential [93]. Silencing CD133 using shRNA knockdown strategies demonstrated that both GSC self-renewal and tumorigenic capacities were jeopardized [94]. In the same line, Singh and collaborators demonstrated that GBM cells expressing CD133 are able to recapitulate tumors in immunocompromised mice

while the CD133-negative fraction could not [95]. Ever since this demonstration, divergent perspectives have fed a matter of controversy that revolves around the fundamental nature of CD133 as a GSC marker. Several studies have indeed demonstrated that CD133-negative GBM cells also possess classical features of GSC including neurosphere formation, multipotency, self-renewal and recapitulation of the original tumor in xenotransplantation experiments [96, 97]. Care should thus be taken when using CD133 as a GSC marker. Unlike normal NSC, GSC cannot be pinpointed with a single surface marker. This sharply underlies the need to identify additional GSC markers in order to better fight malignant brain tumors [98]. Of note, integrin α6 has recently been found over-expressed in GSC, mainly localizing with CD133 in both GBM surgical biopsies and tumor-spheres [95]. Interestingly enough, targeting integrin α6 was shown to inhibit GSC self-renewal, proliferation and tumor formation abilities both *in vitro* and *in vivo* [99]. Consequently, using multiple markers to specifically target GSC in combination with radio- and chemotherapeutic paradigms may help building new significant therapeutic avenues.

Interestingly, several genetic reports, using murine glioma models and imaging analyses from clinical studies, have provided evidence that GBM may arise near the sub-ventricular zone (SVZ) stem cell niche [29, 82, 100, 101], suggesting NSC to be involved in the onset of the disease. The SVZ, located along the lateral ventricles, maintains the ability to produce neurons and glia throughout life [102-104]. This source of stem cells and progenitors in the adult brain will be further described in details (See Chapter III, section 1 and 2). Let's just keep in mind for the moment that NSC are hierarchically organized in the niche. Quiescent type B cells (which can be considered as "true" NSC) give rise to highly proliferative cells, also known as transit-amplifying progenitor cells (type C cells). These cells then differentiate into two lineage-restricted progenitor cells: neuroblasts (type A cells) and oligodendrocyte precursor cells (OPC) [105, 106]. Curiously, NSC residing in the neurogenic niche were reported to be more sensitive to malignant transformation compared to differentiated cells and hence more likely to form tumors upon mutagenic exposure or oncogene activation [107-110]. As an example, quiescent type B cells (notably characterized by the expression of GFAP) were shown to specifically pile up a large number of genetic mutations in a transgenic hGFAP-Cre/$p53^{flox/flox}$ mouse model [111]. Additionally, Wang and colleagues described in a classy manner that transit-amplifying type C cells are able to accumulate large strings of alterations leading to tumor initiation and that Olig2-positive type C cells were involved in gliomagenesis early stages [111]. In this fashion, another study demonstrated that intra-ventricular infusion of PDGF, one of the

numerous growth factors involved in cell division, actively induces PDGFRα-positive quiescent type B cells to proliferate, finally leading to large GBM-featured hyperplasia [112].

Together, these data strongly suggest GSC to potentially derive from NSC but, even though the cancer stem cell theory is increasingly being accepted, we still nowadays can't state loud and clear that this is the truth. It remains indeed an open question whether or not the cancer stem cell theory could explain the onset of malignant brain tumors on its own. It is still unclear whether GSC originate from normal undifferentiated cells such as NSC or eventually derive from de-differentiation of mature tumor cells and, as such, are the consequence of tumor progression instead of being the real initiators. Other theories co-exist aside from the cancer stem cell hypothesis (reviewed in Goffart *et al.* 2013 - see Appendix, Paper 3) [9] but the existence of GSC has tremendously helped to explain certain aspects of the tumor behavior and particularly reflects the cellular complexity of malignant brain tumors.

3. The Reign of Terror

Malignant gliomas represent one of the greatest challenge in the management of cancer patients worldwide. Although notable achievements have been made in the field, GBM patients nearly-systematically relapse within 15 months from the time of diagnosis, reflecting the failure of current therapeutic strategies [113, 114]. One potential explanation to this therapeutic fiasco is that current therapies fail to kill GSC. Therapies that kill non-tumorigenic cancer cells can definitely reduce tumors in size but will be ineffective to cure patients. Conversely, therapies which are designed to kill or to induce differentiation of cancer stem cells are thought to better improve outcomes [115, 116]. In the facts, the majority of cytotoxic therapies target fast dividing cells while GSC are preferentially spared as they are either quiescent or slowly cycling [117, 118]. Tumor dormancy is typically seen as a key limiting event in the treatment of malignant diseases but this state is not sufficient enough to fully explain GBM resistance to cytotoxic agents.

In this context, Chen and colleagues managed to corroborate the implication of GSC in tumor recurrence following chemotherapy. Using spontaneous murine glioma models, they showed that the first cell type to undergo proliferation when TMZ treatment was discontinued is the Nestin-positive population (GSC) [119]. Worryingly, GSC are usually found enriched in most recurrent gliomas suggesting GBM recurrence to be incriminated to GSC left behind after

traditional treatment [120]. In this line, an increased number of CD133 expressing cells was found specifically enriched in GBM cell cultures following radiation or primary chemotherapy [121]. Still not reassuring, GSC are also known to support therapeutic failure by expressing elevated levels of multi-drug resistance genes and DNA mismatch repair genes [122, 123]. In this way, GSC preferentially activate DNA damage checkpoints in response to radiation and display increased DNA repair capacities compared to non-GSC [124]. While GBM relapses contain an increased level of CD133 expressing cells [122], GSC isolated from recurrent tumors were demonstrated to give rise to more aggressive/invasive tumors compared to GSC isolated from primary tumors and of course deriving from the same patient [125]. These data indicate that GSC are highly dynamic in nature and could definitely contribute to tumor regrowth from minimal residual disease post-surgery and treatment.

However, in order to readdress the debate, we sought into the TCGA datasets and found absolutely no correlation between higher mRNA expression levels of potential GSC markers and resistance to radio/chemotherapy. Additionally, a very recent study assessed whether markers used to identify GSC (Nestin, CD133, SOX2, CD15, CD44) are of value to predict radio-sensitivity in primary glioma cultures derived from patient biopsies. Unexpectedly, these markers were generally not defining a more resistant, but rather a more sensitive group of GBM cells [126]. While profiling samples of different radio/chemotherapy-treated GBM patients for the expression of CD133, CD15 and Nestin, Kim and colleagues came to similar conclusions and stated that the expression of stem cell markers in GBM does not support any prognostic value [127]. In our opinion, these two very confusing studies deserve credit for calling into question the widely accepted theory that cancer stem cells are sources of therapeutic resistance. At least, the markers used to detect GSC should not be used without any skepticism.

4. Hijacking the Brain Blood Supply

Like normal cells in most human tissues, tumor cells are depending on blood vessels for oxygen and energy supply as well as removal of waste products. Solid tumors have a need to increase their vascular process in order to meet the metabolic demands of a growing population of tumor cells [128]. The outgrowth of new blood vessels from preexistent ones, also called angiogenesis, allows to achieve this goal. Angiogenesis refers to a complex process, including multiple coordinated steps, such as production and release of angiogenic factors, directional migration and proliferation of microvascular cells, proteolytic degradation of extracellular

matrix (ECM) barriers and the formation of new vessels [129]. The vasculature emerging from tumor angiogenesis is, most of the time, abnormal and leads to a vicious circle whereby the micro-environment remains poorly oxygenated, perpetuating in this way the consistent production of angiogenic and growth factors [130].

In this context, GSC are also regarded as key entities involved in the formation of new blood vessels within the tumor. This sub-population of cancer cells is indeed known to specifically produce elevated levels of angiogenic factors, including VEGF and CXCL12. These two factors are specifically involved in both angiogenic and migration processes of high grade gliomas [131, 132]. Hypoxia, a state in which oxygen supply is insufficient, stimulates the release of VEGF and CXCL12 through the activation of the PI3K/Akt signaling pathway in GSC [133-135]. Interestingly, inhibition of CXCR4, one of the two CXCL12 receptors, using siRNA or chemical inhibitors, decreases GSC tumorigenicity and angiogenesis [135]. More than ten year ago, Morrison and colleagues reported the first evidence that low O_2 tension supports NSC survival and proliferation by inhibiting their differentiation and maintaining stem cell characteristics [136]. Relying on similarities between NSC and GSC, Qiang and collaborators recently demonstrated that hypoxia also mediates the expansion and maintenance of GSC through the activation of Notch signaling [137]. Consistent with these facts, several studies suggested that hypoxia actively spearheads GSC tumorigenic capacities, self-renewal and survival through mechanisms that are shared between NSC and GSC [138, 139]. Hypoxia also plays a critical role in regulating tumor cell plasticity (Figure 5). As a matter of fact, it has recently been proposed that hypoxia specifically maintains non-GSC self-renewal abilities and promotes a stronger stem-like phenotype in this sub-population of cells via the up-regulation of critical stem cell factors including OCT4, Nanog and c-MYC [140]. This non-GSC surprising plasticity emphasizes the importance of developing therapeutic strategies specifically targeting the influence of the tumor micro-environment in addition to cancer stem cells targeted therapies (Figure 5).

In 2006, Bao and collaborators questioned the potential role of GSC in supporting tumor angiogenesis. To do so, they transplanted GSC into immunocompromised mice and identified widespread tumor angiogenesis and hemorrhage in GSC transplanted animals compared to the ones transplanted with non-GSC [131]. Using *in vitro* models of angiogenesis, Bao and colleagues then demonstrated GSC conditioned media to significantly increase endothelial cells (EC) migration and tube formation compared to non-GSC conditioned media. Interestingly, these pro-angiogenic effects (tube formation is regarded as the first step of vessels formation)

were markedly hampered by the use of VEGF neutralizing antibodies [131]. Reciprocally, EC secrete factors which maintain GSC self-renewal and survival through the activation of mTOR signaling [141]. It has also been reported that incubation of GSC with EC conditioned media rescues GSC from apoptosis and autophagy [142] and significantly upregulates the expression of CXCR1 and CXCR2, further propelling GSC invasion abilities [143].

As if it wasn't bad enough, *in vivo* experiments recently showed that GSC display the ability to transdifferentiate into pericytes and endothelial-like cells in order to participate to the formation of new tumor blood vessels [144-146]. As you have understood, a growing body of studies sustains the critical role of GSC in GBM neovascularization. The findings that GSC actively secrete angiogenic factors and transdifferentiate into pericytes and endothelial-like cells particularly shed the light on new original mechanisms involved in cancer progression. Targeting the win-win relationship between GSC and EC may therefore be essential for the development of therapies yet to come.

5. How to Fight the Monster?

If GSC are to be incriminated for tumor initiation, progression, angiogenesis, therapeutic resistance and tumor recurrence, these cancer cells therefore represent potential powerful targets for winning the upcoming battles in the war against GBM. Elimination of these GSC should ultimately lead to the tumor eradication. Nevertheless, next generation of therapeutic strategies should not forget about differentiated GBM cells. Indeed, as mentioned in the previous section, environmental conditions play a critical role in regulating tumor cell plasticity and have been reported to reprogram differentiated GBM cells toward a stem-like cell phenotype [140]. These findings potentially inform the development of anti-cancer stem cell therapies since gathering all the attention on GSC only may be insufficient to improve patient outcomes. Targeting both GSC and non-GSC should help to overcome the issue. This strategy could indeed prevent the generation of differentiated tumor cells on one hand and restraining the dedifferentiation risk of "mature" cells into new GSC on the other hand (Figure 5).

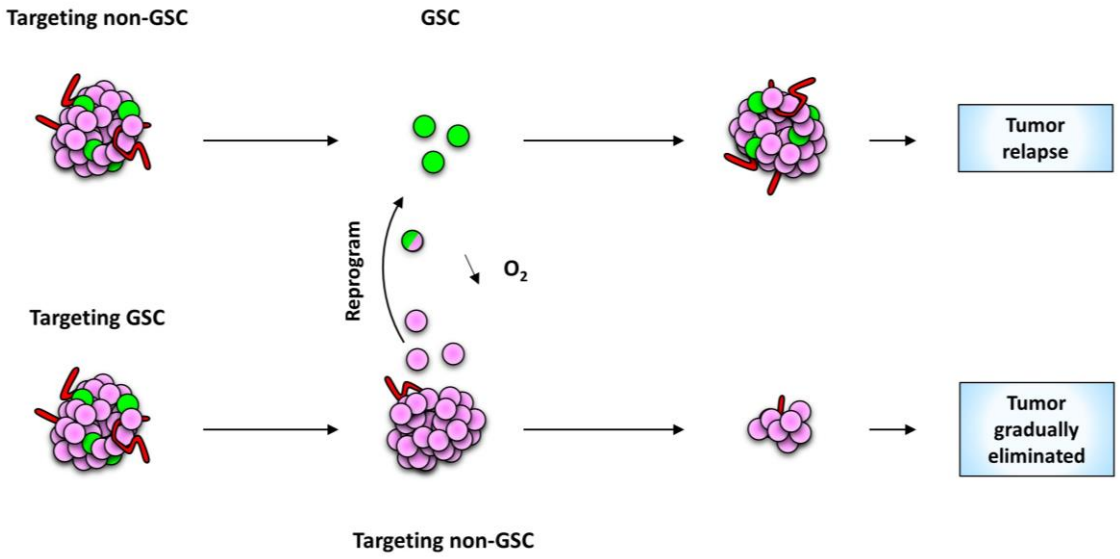


FIGURE 5. Therapeutic targeting of glioblastoma stem cells (GSC) and non-glioblastoma stem cells (Non-GSC). According to the cancer stem cell hypothesis, traditional therapy (top row) will only hit the tumor bulk leaving GSC the ability to trigger GBM relapses. Targeting GSC (bottom row) will lead to the gradual elimination of the tumor but will not avoid the risk of dedifferentiation of "mature" tumor cells into new GSC. Targeting both populations of GBM cancer cells (GSC and non-GSC) is therefore crucial to fully eliminate the tumor and prevent relapses. (Illustration adapted from Cheng et *al*., 2010)

There is an urgent need for the development of novel therapeutic strategies specifically fighting GSC without harming patients' healthy cells. Therapeutic effects might be achieved by forcing GSC differentiation and/or targeting specific surface markers or alterations in multiple signaling pathways. In this context, several groups have recently shed the light on new potential GSC therapeutic targets including transcription factors and stemness-related pathways such as Sonic Hedgehog, Wnt, TGF-β, BMP, EGFR and Notch signaling. A detailed overview of these new GSC-targeted approaches is provided in "Glioblastoma stem cells: new insights in therapeutic strategies" (Goffart N. *et al.*, Future Neurology, 2015 - see Appendix, Paper 4).

6. Glioblastoma Stem Cells as a Complex Adaptive System

Cancer is commonly accepted as a robust and complex adaptive system able to survive, adapt and proliferate despite perturbations resulting from anti-cancer drugs and the host immune system. [147-149]. It is essential that mechanisms underlying tumor robustness be formally studied, as without an in-depth understanding of these principles, strategies to overcome therapeutic resistance are unlikely to be developed [150]. In other words, more attention should be given to the tumor adaptive mechanisms in response to therapy, so as to nip them in the bud. It seems therefore mandatory to assess cancer as an adaptive system characterized by emergent and global properties that are produced by a requisite diversity of local interactions [151, 152]. These emergent properties confer the hallmarks of a complex adaptive system (organization, adaptability and survival) and can only be ascribed to the complex system itself, never to the properties of the individual components of the latter [147, 152]. Interestingly, GBM fit the elemental criteria of a complex adaptive system. These neoplasms are indeed heterogeneous, self-adaptive and self-organized (Figure 6). Moreover, evidence speak up for interactions between GSC and local environmental cues which could sustain GSC survival and proliferation [153]. As described in the previous section, diffusible factors secreted by the surrounding vasculature help to maintain GSC in a stem cell-like state and exert an influence on their proliferation and self-renewal as well (see Chapter II, section 4) [154]. On the other hand, GSC are able to secrete autocrine and paracrine factors that can potentially facilitate their invasion [155]. These secreted factors are conversely able to diffuse through the surrounding stroma and create a permissive micro-environment for malignant progression [155]. With the plethora of factors involved in GSC proliferation, invasion and survival, it is plausible that brain tumor stem cells may arise as complex adaptive systems which would interact through diffusible factors and adherence cues. As a matter of facts, it has recently been shown in a drosophila model that diverse, adjacent tumor cells tightly cooperate to produce emergent properties of tumorigenesis and invasion [156]. To what extent this occurs in human glioma has yet to be determined. With these considerations in mind, a paradigm shift in the way we intellectualize GSC must occur. We have to redefine the “old” stochastic and hierarchical models that have been used so far to understand GBM progression and heterogeneity [157]. The stochastic model states that every cell within the tumor is equally likely to be the cell of origin and facilitates tumor initiation and progression (Figure 6A). Conversely, the hierarchical

model relies on the paradigm that GSC represent a biologically distinct subset within the total cancer cell population (Figure 6B).

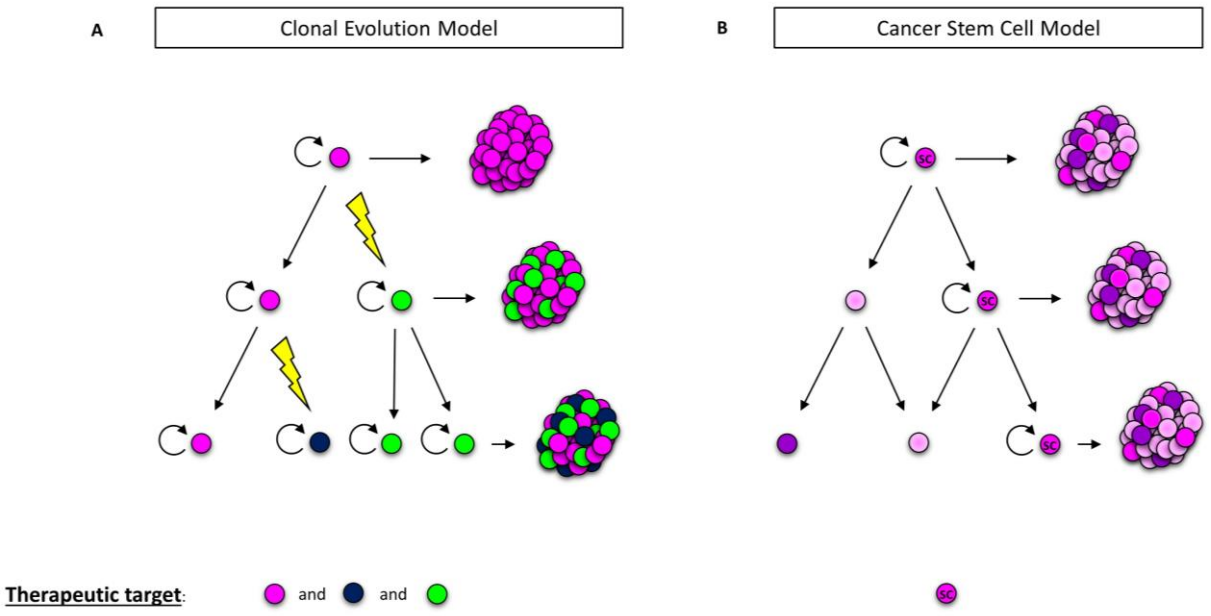


FIGURE 6. Evolution of models explaining the onset of malignant brain tumors. Two alternative models have been put forward to explain how tumors initiate and develop. According to the clonal evolution model (or stochastic model - A), tumor are heterogeneous since multiple cell populations are the consequence of several genetic mutations (yellow broken arrows). Every tumor cell is able to self-renew and initiate tumor formation, making them all targets for therapeutic interventions. Conversely, the cancer stem cell model (or hierarchical model - B) postulates that a restricted amount of cells possesses the ability to self-renew, form clonal spheres and initiate tumors. This forms a hierarchical lineage system where the primary therapeutic cells of target are CSC. (Illustration adapted from Vescovi et *al*., 2006). sc: stem cells

Although they differentially consider the weight of GSC in driving tumorigenesis and do not take into account micro-environmental cues, these two models are not mutually exclusive and the concept of a complex adaptive system actually unifies them into one model (Figure 7). Malignant brain tumors should indeed be viewed as aberrant entities characterized by a really complex network of cell to cell interactions more than distinct units residing in the normal environment. Cell plasticity also adds another level of complexity in the subclonal landscape of GBM since dedifferentiate GBM cells may enter back into the GSC pool and regain long-term tumor repopulation capacities [158]. These dedifferentiation abilities may either be

inherited (hierarchical theory) or acquired through mutations leading to a stem cell-like permissive epigenome (stochastic theory).

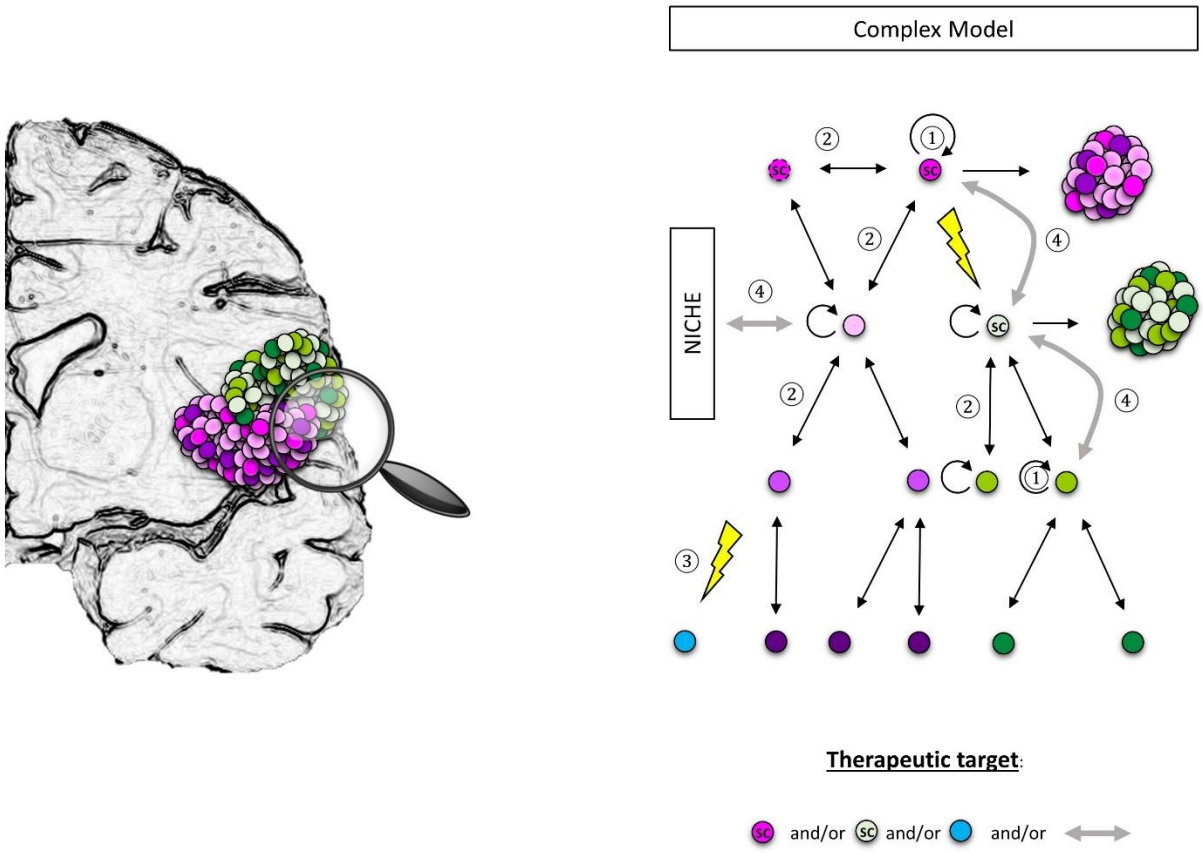


FIGURE 7. The complex adaptive system. In a complex adaptive system, both genetic and epigenetic changes occur within a single tumor, resulting in a multifaceted cell system where several GSC types may co-exist ①. This model postulates that genetic mutations ③ produce new types of tumor cells while epigenetic changes ② enable tumor cells to temporarily adopt different states (cell plasticity) characterized by the expression of different cell markers and potentially involved in therapeutic resistance. The other important feature of a complex system is that individual cells interact with one another, creating emergent properties ④. While all potential GSC have to be targeted for successful therapy, this models also suggests the interruption of the cell-cell/cell-niche interactions to weaken the tumor system as a whole (Illustration adapted from Lacks et *al*., 2010)

In a complex adaptive system, tumors are no longer regarded as an homogeneous population of cells possessing equal tumorigenic potential (which, I admit, was my opinion four years from now), but as a hierarchical organization in which different GSC subtypes may co-exist (maybe related to Verhaak's classifications - see Chapter I, section 5.5) and interact with

each other to raise complex emergent properties potentially involved in therapeutic resistance [159].

In this context, further research is desperately needed to determine whether treatment resistance is specifically due to GSC intrinsic characteristics, mutational evolution, redundant molecular pathways or to the adaptation of a complex system in which multiple genetically distinct GSC subtypes co-exist, and to what extent signaling between GSC, differentiated GBM cells and the niche could devise appropriate therapeutic approaches. From these questions, there is still much to be learned.

Chapter III

1. The Neurogenic Niche Concept

The discovery by Eriksson et al., in 1998, of neural progenitor cells (NPC) capable of becoming mature neurons in the human brain, thought for decades to be a quiescent organ, has brought the brain plasticity into sharp focus [160]. In the adult brain, newborn neurons are able to integrate the mature neuronal circuitry and take on various functions contributing to the structural and functional plasticity of the system [161]. The process of neurogenesis hinge on a complex cascade of molecular signaling pathways including Notch, Bone Morphogenetic Protein, Wnt, and sonic hedgehog signaling known to be crucial in regulating neuronal differentiation [162]. These newly pumped up neurons then migrate from their site of genesis to their final destination guided by physical, chemical and biological signals [163]. The process of cell migration particularly plays a key role in the structural organization of the brain. Indeed, even when the latter is completely built and structured, it maintains a high degree of complexity/plasticity throughout life, notably as a result of different migration processes, axonal remodeling and synaptogenesis [164]. NSC are regulated by the integration of intrinsic factors together with extrinsic cues emanating from the surrounding micro-environment or "niche" [165]. A niche can be defined as a restricted, functional and specialized anatomic and histologic compartment able to integrate local and systemic factors, support maintenance/survival and actively regulate cell proliferation [165]. In this context, NSC reside in the adult brain within two specific neurogenic niches: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus [166-168]. In these regions only, stem cells will be able to undergo asymmetrical cell divisions to ensure their own maintenance throughout years and years.

2. Neural Stem/Progenitor Cells Patterns of Migration

In the adult human SVZ, NSC are hierarchically organized. Quiescent type B cells notably give rise to highly proliferative cells or transit-amplifying progenitor cells (type C cells) which, in turn, differentiate in two different types of progenitor cells: neuroblasts (type A cells)

and OPC [105, 106, 169]. The ratio of cell types between rodents and humans differs with the particularity that type A cells are most abundant in rodents while type B cells, identified as the most quiescent primary progenitors in rodents, are predominant in humans (Figure 8A and C) [170, 171]. A similar hierarchical neurogenic system is found in the murine and human SGZ [172]. The SGZ is located between the hippocampal granular layer and the hilus. Similar to the SVZ, the SGZ operates as a source of NSC which give rise to transiently amplifying progenitors. These progenitors then generate neuronal precursors which finally migrate a few μm along radial fibers to functionally integrate the granular layer of the DG (Figure 8B and D) [173].

During neural development, two main modes of migration come into play: radial and tangential migration [102, 174]. NSC from the ventricular zone extend long processes from the ventricular wall to the pial surface of the cortex. These cells not only generate glutamatergic projection neurons but also provide tracks along which immature neurons migrate into the cortex. This type of migration is called radial migration because cells migrate perpendicularly to the ventricular surface. Conversely, most of cortical GABAergic interneurons arising from ganglionic eminences (ventral forebrain) migrate tangentially, parallel to white matter tracts, then turn and migrate radially into the cortex [174].

At birth, neurogenesis is largely complete but for some exceptions. In rodents for instance, the SVZ continues to generate large numbers of olfactory interneurons during the postnatal development. Neuronal progenitors born in the SVZ (type A-cells) migrate tangentially (a few mm) toward the olfactory bulbs (OB) in a well-defined pathway called the rostral migratory stream (RMS) (Figure 8A) [175-177]. Curtis and collaborators reported the first evidence that human neuroblasts also migrate from the SVZ to the OB trough the RMS [178, 179]. The human RMS has been further characterized as a neurogenic corridor for early-born neurons on their way to the OB [180]. Gliogenesis also persists in the SVZ during the early postnatal period, generating astrocytes and oligodendrocytes. Glial progenitors migrate from the SVZ into the overlaying white matter, the cortex and deep gray nuclei. Surprisingly, the SVZ largely exceeds the DG in the numbers of cells generated. In rodents for instance, from 30.000 to 80.000 SVZ-derived newborn cells are generated every day, representing 1% of the total OB granule cells. In contrast, only 9.000 progenitor cells are generated every day in the DG (around 0.03% of the total dentate neuronal population) [181-184]. As already mentioned, these newborn neurons integrate pre-existing neuronal networks and appear to be important for

the maintenance and renewal of OB interneurons as well as for the refinement of the DG circuitry involved in hippocampal-dependent memory [185-187].

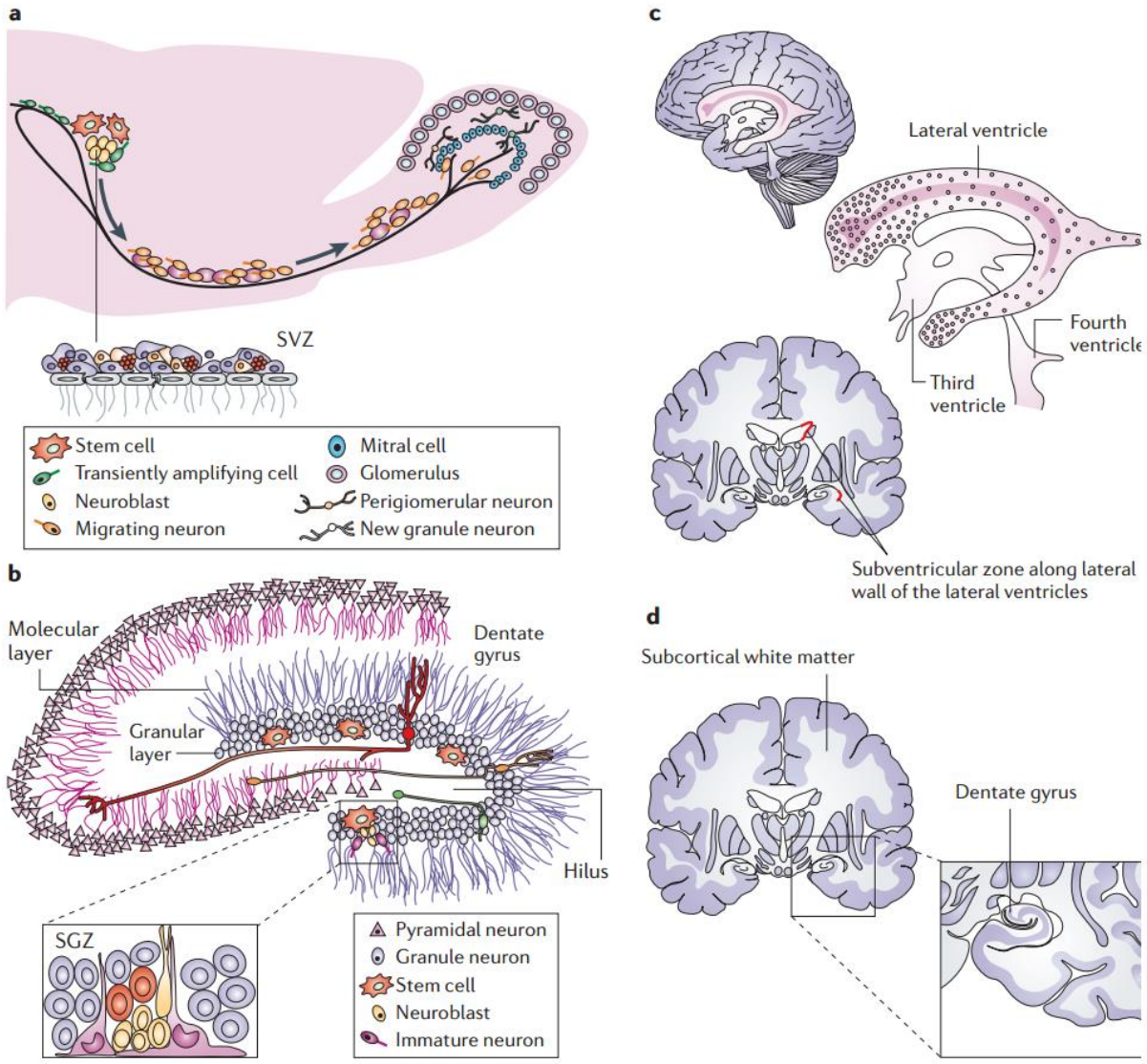


FIGURE 8. The anatomy and physiology of the SVZ and SGZ in rodents and humans. A) A sagittal section through the lateral ventricle shows the murine SVZ. This region lines the lateral ventricles of the forebrain and is made of three main cell types. Multipotent type B astrocytes-like cells have been identified as true SVZ stem cells and give rise to fast-cycling precursor cells (type C precursors) which, in turn, generate mitotically active type A neuroblasts. Type A cells, while dividing, migrate tangentially toward the OB where they integrate as new interneurons. B) An additional adult neurogenetic region is found in the SGZ which is located within the dentate gyrus of the hippocampus. A cellular hierarchy, somewhat similar to that of the SVZ, is also seen in the SGZ. True stem cells give rise to intermediate type D progenitors which finally generate granule neurons. These neurons integrate functionally into the granular layer of the DG. C) In the adult human brain, a population of SVZ astrocytes-like cells, organized as a periventricular ribbon, has also been identified/characterized as NSC. D) Location of the germinal zone of the adult human hippocampus within the *dentate gyrus* (Vescovi et al., 2006).

3. Involvement of Neural Progenitors in GBM

During pathological processes, such as a variety of neurological diseases, brain reactivity occurs in a spontaneous attempt to protect the injured region [188-190]. These pathologies result in further NPC activation and migration toward the altered region. This has notably been reported in rat models of Huntington's disease and stroke [191, 192]. Throughout the last decade, evidence have documented that GBM also interact with NPC in the micro-environment. In this context, SVZ endogenous NPC preferentially home to experimentally induced brain tumors [193-196] probably in a CXCR4 dependent-manner [197]. Those tumor-associated NPC are in fact deflected from their physiological migratory path in order to end up their journey in cellular layers surrounding the tumor mass [196]. Safe arrival guaranteed, they then display important anti-tumorigenic effects by releasing soluble factors interfering with GBM cell proliferation [198, 199] and causing GBM cell death [196] or promoting GSC differentiation [193]. Interestingly, significant survival improvements have been observed using experimental orthotopic co-injections of NPC and GBM cells [196]. This study also demonstrated that NPC tumor-suppressor effects were largely related to aging and neurogenic abilities since younger mice significantly outlived older ones. Strikingly, this survival default was sealed by inoculating GBM cells along with NPC in older mice, suggesting a close relationship between NPC anti-tumorigenic properties and neurogenic aptitudes [196]. Let's keep in mind that NPC anti-tumorigenic capacities have only been described in rodent models. It would therefore be interesting to check whether human NPC also display anti-tumorigenic properties similar to what has been described in rodents so far, especially since aging is considered as one of the most important prognostic factor in GBM disease. Moreover, the fact that neurogenesis declines with aging in humans [200] strengthens the link between GBM and this prognostic factor even more, possibly by decreasing the amount of NPC and their related anti-tumorigenic effects throughout lifespan.

4. Patterns of Migration in GBM

The study of developmental human biology suggests that GBM invasion patterns in the CNS finely recapitulates the behavior of normal NPC observed during development [201]. In the facts, GBM dissemination patterns were carefully studied in the 1930's by the Belgian

pathologist Hans-Joachim Scherer. He noted that GBM infiltration of the brain systematically followed specific paths including myelinated fiber tracts, subpial surfaces and blood vessels [202]. These infiltration patterns strikingly resemble the patterns of migration followed by glial progenitors during normal brain development [203, 204]. Furthermore, many infiltrating GBM cells display a specific morphology with a prominent leading process that, once again, resembles the morphology of migrating glial progenitors [205]. The idea that GBM infiltration recapitulates the migration of glial progenitors is strongly supported by studies focusing on migration in GBM animal models. In this context, Kakita and colleagues investigated the *in vivo* migration patterns of glial progenitors from the neonatal SVZ by labeling these cells with a green fluorescence protein (GFP). In this way, they showed that glial progenitors disperse widely through the subcortical white matter into the contralateral hemisphere [206]. In a very similar way, infecting SVZ glial progenitors with a retrovirus expressing both PDGF and GFP induced the formation of diffusely infiltrating brain tumors that closely resemble GBM conditions. This original study demonstrated that GFP-positive cells showed extensive infiltration into the cortex and the subcortical white matter, allowing the infiltration of the contralateral hemisphere as well [207].

As already mentioned, the migration of NPC from the murine SVZ to the OB is a well-established process. Interestingly, Kroonen and colleagues demonstrated that a sub-population of GBM cells specifically invade the adult SVZ after orthotopic transplantation [208]. The authors also described these infiltrative tumor cells to migrate across the *Corpus Callosum* (CC) and invade the contralateral hemisphere. In the SVZ, these GBM cells were shown to express NSC markers and mimicked the NPC behavior by migrating to the OB. Surprisingly, SVZ-nested GBM cells also retained the ability to give rise to secondary tumors when re-implanted in new animals. The authors finally concluded that the cancer cells located in the SVZ were enriched in GBM-initiating capacities and that migration of these GSC into the adult neurogenic regions may hypothetically contribute to GBM deadly relapses. More recently, Sadahiro and collaborators made similar observations. They compared the invasive properties of human GSC versus non-GSC in an orthotopic mouse model and reported that GSC were more likely to infiltrate the SVZ and the contralateral hemisphere than their non-GSC counterparts [209]. Adding a tremendous amount of credibility to the present work, Piccirillo (a name to remember for the rest of the manuscript) and colleagues lately confirmed these experimental data in a clinical study. They demonstrated for the first time the presence of GSC in the human SVZ of 42 patients. These GSC could notably recapitulate the original disease in orthotopic patient-

derived xenogeneic models [210]. The clinical implications of these findings will be further discussed in details in the second part of this work.

5. The Adult SVZ as an Independent Prognostic Factor

To date, many studies in rodents have supported the idea that the "cell of origin" in malignant brain tumors may derive from SVZ progenitors (see Chapter II, section 2). However the biology and precise contribution of neural progenitors to normal human brain functions remain to be addressed and the understanding of their roles in neurological diseases has just started. Beyond the hypothetic role of the SVZ in GBM initiation, it could be that GSC do not originate from NPC. Using bio-mathematical models, Bohnam and collaborators estimated that 50% of GBM are actually located away from the SVZ environment and suggested their SVZ origin to be doubtful [211]. On the contrary, evidence accumulating from clinical observations tend to corroborate a close relationship between the SVZ stem cell niche and malignant brain tumors.

In 2007, Lim and collaborators reported the very first association between lesions surrounding the neurogenic niche and the tumor phenotype [212]. Based on a MRI classification, they showed that GBM connecting the SVZ (almost 50%) were more frequently characterized as multi-focal tumors. At recurrence, tumors connecting the neurogenic niche also favored lesions at greater distance. In a similar fashion, Barami and colleagues also investigated the relationship between malignant brain tumors and the ventricular walls. Based on retrospective radiographic analyses, they showed that 93% of the cases displayed lesions contacting at least one region of the SVZ. Interestingly, contact with the SVZ was independent from GBM size or mass effect [213]. Last but not least, a very recent study on 607 patients demonstrated that GBM in close proximity to the SVZ were significantly associated with decreased survival and displayed higher risks of multifocal/distant progression [214]. Relying on these findings, it is enticing to speculate that SVZ-contacting tumors directly derived from NPC. However, large scale GBM genetic analyses failed to provide any evidence that SVZ-related tumors are more likely to derive from NPC [215]. As a matter of fact, Kappadakunnel and collaborators pinpointed a significant overexpression of immune system genes in SVZ-contacting tumors but failed to show any stem cell derived genetic signature specific for GBM

in contact with the SVZ. The small size of the cohort (47 patients) was pointed out as an explanation for this lack of reproducibility.

The SVZ contact has further been assessed as a potential independent prognostic factor for OS and progression free survival (PFS) in GBM patients [215-218]. Radiological observations of intimate contact with the SVZ allowed to highlight an association with poor survival rates. In 2008, Chaichana and colleagues reported a significant decrease of survival in patients bearing tumors connected to the ventricular walls [216]. Conversely to Chaichana's work, two independent study failed to highlight any significant correlation between SVZ-contacting tumors and survival [215] or tumor extension/recurrence patterns [219]. A trend toward shorter survival rates was nevertheless observed in both studies when GBM were in contact with the SVZ environment (median OS of 358 vs 644 days). Further down the road, Kaplan-Meier analyses on a cohort of 91 patients recently demonstrated shorter PFS at 6 months (47% vs 69% survivors) and shorter OS at 2 years (23% vs 48% survivors) in the group of patients whose tumors were connecting the SVZ [218]. Last year, new survival analyses on a cohort of 100 GBM patients corroborated the previously cited study. Age under 60 years ($p < 0,001$), total resection status ($p < 0,001$) and tumor localization without any SVZ contact ($p = 0,05$) were indeed reported as significant reliable factors for prolonged survival and fuelled the controversy surrounding the SVZ as a predictive factor for survival [220]. Piling evidence also suggest an association between the initial surgery and a SVZ contact in survival. A very recent univariate retrospective analysis with prognostic factors including age and Karnofsky Performance Status (KPS) scores confirmed survival after repeat surgery to decrease in patients with recurrent GBM originally contacting the SVZ ($p = 0.022$) [221].

As you have understood, conclusions reached by independent groups may differ and the precise meaning of a SVZ involvement as a prognostic factor is not clear yet. Most of the studies encounter several limitations due to their retrospective design. A lack of cohort homogeneity may also fuel the debate. In this line, tumor sizes were quiet different among tumors classified according to their SVZ contact [213], although the tumor volume was not shown as a criteria impacting on survival rates [222]. Furthermore patient's KPS scores have not consistently been documented in each study. That is unfortunate as poor KPS scores are described to correlate with reduced OS and may consequently introduce major bias in survival analysis.

The type of surgery performed, TMZ adjuvant chemotherapy protocols and KPS scores are well-established independent prognostic factors in GBM [223-225]. Whether the contact of

the initial lesion with the ventricular walls may join this closed group is yet to be fully demonstrated. But, even if we don't completely understand the main reasons why GBM patients usually display less favorable outcomes when the tumor contacts the SVZ, it might be worth to consider the latter as a strong and modern independent prognostic candidate that may potentially undertake therapeutic significance.

6. Irradiation of the Stem Cell Niche

Nowadays, a growing body of clinical evidence correlates delivered doses of radiotherapy to the SVZ with increased PFS and OS in newly diagnosed GBM patients. Although really appealing at first glance (who would argue against improving PFS and OS in GBM patients?), the alarm bell needs however to be rung. Indeed, while some reports suggest that increasing doses delivered to the SVZ positively influence outcomes, others have failed to confirm any significant correlation between SVZ doses and survival.

It all started in 2010 when a group from the University of California Los Angeles (UCLA) tested the hypothesis that targeting adult neurogenic niches could be of benefit for GBM patients. The study was conducted retrospectively on a cohort of 55 patients uniformly treated with surgery, focal radiotherapy and chemotherapy [226]. Apart from the small size of the study, the authors reported that a dose of 43Gy delivered to the ipsi- and contralateral SVZ significantly increased the median PFS (15.0 vs 7.2 months - p=0.028). Unfortunately, researchers from the Vrije Universiteit Medical Centre (VUMC) in The Netherlands could not entirely replicate these results (p=0.74) using 43Gy as a cut-off dose (as proposed by the UCLA team) [227]. However, in patients with gross total resection (n = 32), a cut-off dose of 30 Gy to bilateral SVZ showed a significant correlation with OS (p = 0.015) and a borderline significant correlation with PFS (p = 0.077).

In 2012, another retrospective study measured the SVZ dose-volume parameters and revealed a significant correlation with survival outcomes in 40 patients treated with maximal safe resection followed by post-operative focal radiotherapy and adjuvant TMZ chemotherapy [228]. Multivariate analyses proved that increasing mean doses to the ipsilateral SVZ was associated with improved OS and identified both KPS and mean ipsilateral SVZ doses as independent predictors of survival. Furthermore, higher contralateral SVZ doses were associated with poor PFS and OS. Likewise, another study demonstrated that tumors involving

the SVZ and tumor growth rate following radiation therapy were independent predictors of shorter PFS and OS [217]. These results suggest that GBM in close proximity to the ventricular walls usually convey a worse prognosis, corroborating with previously described findings (see Chapter III, section 5).

The cat was finally thrown among the pigeons when researchers from the UCLA team and from the VUMC decided to pool their data together to create more reliable evidence questioning the potential benefic impact of the stem cell niche irradiation in survival outcomes. Hence, they generated an extended cohort of 173 patients and confirmed the correlation between the ipsilateral SVZ irradiation and improved PFS and OS [229]. Multivariate analyses only confirmed this advantage for PFS. In the same line, a large single-institution report from Johns Hopkins Hospital recently confirmed these trends and specified that patients with GBM are more likely to benefit from SVZ irradiation when gross total resection (GTR) is performed [230]. In this case, both PFS and OS were significantly improved in patients receiving at least 40Gy to the ipsilateral SVZ (PFS: 15.1 vs 10.3 months, p=0.023 - OS: 17.5 vs 15.6 months, p=0.027). Such benefits could not be observed in patients treated with biopsy or subtotal surgery.

Muddying the waters further, the most recent study trying to correlate SVZ irradiation doses with survival outcomes reported somewhat contradictory results in a cohort of 60 patients [231]. Elicin and colleagues indeed reported a statistically significant correlation ($p = 0.009$) between a dose of 59.2Gy delivered to the contralateral SVZ and poor PFS (10.37 months vs 7.1 months - p=0.009). Contralateral SVZ doses greater than 59.2Gy were also associated with poor OS in the subgroup of patients who underwent subtotal resection/biopsy ($p = 0.004$). The authors finally reported a dose of 62.2Gy delivered to the ipsilateral SVZ to be associated with poor PFS in patients with good performance status.

Once again, this section presents a plethora of published results that are somewhat conflicting and at times contradictory. Maybe these observations are all true, but for reasons we don't yet completely understand. Furthermore several reports suffer from a lack of clarity. The UCLA/VUMC study has i.e. a few lacunae in its design: important covariates such as performance status and MGMT promoter methylation status are not included. Multivariate analyses carried out without such important prognostic covariates can lead to a wrong interpretations. Additionally, very little is known regarding the use of adjuvant chemotherapy. Whether or not patients received the standard of care consisting of adjuvant TMZ therapy

remains an open question. Finally, methodological limitations include variability in radiotherapy dose-prescription, cut-off values of doses and delineation of the SVZ. A lack of precision regarding the margins used for SVZ delineation (3-5 mm) can directly alter volumes, create confusion about the actual true dose response relationship and therefore, alter the mean dose received by the SVZ region. From another point of view, let's also mention that the literature is replete with reports associating brain radiation and the development of long-term neurocognitive impairments. Of direct relevance to this discussion are data suggesting that NPC residing in neurogenic regions are more sensitive to mutagenic exposure than differentiated cells (see Chapter II, section 2), further suggesting neurocognitive complications [232, 233]. Recently, a prospective study indeed drew a link between radiation dose to neuronal progenitor cell niches and neurocognitive deficits in children [234].

We all agree that current management strategies for high-grade primary brain tumors are unsatisfactory. GBM are probably one of the nastiest scourges faced in oncology. Therefore, any provocative study which generates innovative hypotheses and potential clinical benefits is a welcome contribution. Nevertheless, as with any brand new encouraging proposition which promises significant progress, we must ask ourselves: is it real, is it a trap or is it just an illusion?

> *"[The Cheshire Cat] vanished quite slowly, beginning with the end of the tail, and ending with the grin, which remained some time after the rest of it had gone" (Lewis Carroll, Alice in Wonderland).*

Irradiation of the SVZ in association with the tumor location remains a growing matter of controversy which is prone to speculation and errors of interpretation. Carefully designed prospective studies are mandatory to ensure SVZ dosimetry not to be compared to the fantastic fading feline within a few years, appearing at first to be a solid truth and later understood as a fantasy. Forthcoming studies correlating SVZ irradiation with survival outcomes and patterns of failure could definitely provide more robust and reliable data to guide therapeutic decision-making.

Chapter IV

1. Chemokines

Chemokines represent a family of small, chemotactic cytokines consisting of more than 50 closely related peptides (8-10 kDa) [235]. They are classified into highly conserved subfamilies (XC, CC, CXC and CX3C chemokines) based on the arrangement of four conserved N-terminal cysteine residues which are key to forming their tertiary structure [236, 237]. CXC and CX3C subfamilies respectively hold one and three amino acids in between the first two cysteine residues whereas the CC subfamily presents two adjacent cysteine residues. Surprisingly, chemokines from the XC subfamily only display two conserved cysteine residues instead of four. As mentioned, these residues are important for the chemokine tertiary structure as they form disulphide bonds between themselves (C1 to C3 and C2 to C4). This subsequently results in the characteristic three-dimensional folding of the chemokine, essential for the receptor recognition and its biological activity [238].

Chemokines can also be classified in two distinct groups (inflammatory and homeostatic chemokines) according to their expression patterns and functions [239, 240]. Homeostatic chemokines are constitutively secreted and play a key role in maintaining the immune system functions. In contrast, inflammatory chemokines are only expressed upon activation of immune cells, including leukocytes or related cells (epithelial cells, endothelial cells, fibroblasts and so on), in response to inflammatory or immunological stimuli [240]. Chemokines with both properties are called dual function-chemokines.

Chemokines were initially named by the laboratories which first identified them. Consequently, a single chemokine often has many names which results in much confusion. To avoid any misunderstanding, a new nomenclature system has been developed. The chemokine structural code (XC, CC, CXC or CX3C) was conserved and directly followed by the letter “L” (ligand) for each chemokine and by the letter “R” (receptor) for each receptor [241]. The list of chemokines identified so far in humans and mice as well as their official names is provided in table 1.

Table 1. Human and mouse chemokines and receptors.

Chemokine	Other Names (human)	Category	Gene symbol		Receptor(s)
			Human	**Mouse**	
XC family					
XCL1	Lymphotactin	D	*XCL1*	*Xcl1*	XCR1
XCL2	SCM-1β	D	*XCL2*	–	XCR1
CC family					
CCL1	I-309	I	*CCL1*	*Ccl1*	CCR8
CCL2	MCP-1	I	*CCL2*	*Ccl2*	CCR2
CCL3	MIP-1α, LD78α	I	*CCL3*	*Ccl3*	CCR1, CCR5
CCL4	MIP-1β	I	*CCL4*	*Ccl4*	CCR5
CCL5	RANTES	I, Pt	*CCL5*		CCR1, CCR3, CCR5
CCL7	MCP-3	I	*CCL7*	*Ccl7*	CCR1, CCR2, CCR3
CCL8	MCP-2	I	*CCL8*	–	CCR1, CCR2, CCR5
–	–	I		*Ccl8*	CCR8 (mouse)
CCL11	Eotaxin	D	*CCL11*	*Ccl11*	CCR3, CCR5
–	–	I	–	*Ccl12*	
CCL13	MCP-4	I	*CCL13*	–	CCR2, CCR3
CCL14	HCC-1	P	*CCL14*	–	CCR1, CCR3, CCR5
CCL15	HCC-2	P	*CCL15*	*Ccl9*	CCR1, CCR3
CCL16	Leukotactin-1 LEC, HCC-4	U	*CCL16*	–	CCR1, CCR2, CCR5 CCR8
CCL17	TARC	D	*CCL17*	*Ccl17*	CCR4
CCL18	PARC, DC-CK1	H	*CCL18*	–	PITPNM3
CCL19	MIP-3β, ELC	H	*CCL19*	*Ccl19*	CCR7
CCL20	MIP-3α, LARC	D	*CCL20*	*Ccl20*	CCR6
CCL21	SLC, 6Ckine	H	*CCL21*	*Ccl21a*	CCR7
–	–	H	–	*Ccl21b*	CCR7
–	–	H	–	*Ccl21c*	CCR7
CCL22	MDC	D	*CCL22*	*Ccl22*	CCR4
CCL23	MPIF-1	P	*CCL23*	*Ccl6*	CCR1, FPRL-1
CCL24	Eotaxin-2	H	*CCL24*	*Ccl24*	CCR3
CCL26	MPIF-2 Eotaxin-3	I	*CCL26*	*(Ccl26-ps)*	CCR3, CX3CR1
CCL27	CTACK, ILC	H	*CCL27*	*Ccl27a*	CCR10
–	–	H	–	*Ccl27b*	
CCL28	MEC	H	*CCL28*	*Ccl28*	CCR10, CCR3

CXC family

CXCL1	GROα, MGSA	I, ELR	*CXCL1*	*Cxcl3*	CXCR2
CXCL2	GROβ	I, ELR	*CXCL2*	*Cxcl2*	CXCR2
CXCL3	GROγ	I, ELR	*CXCL3*	*Cxcl1*	CXCR2
CXCL4	PF4	Pt, non-ELR	*PF4*	–	CXCR3
CXCL5	ENA78	I, ELR	*CXCL5*	–	CXCR2
CXCL6	GCP2	I, ELR	*CXCL6*	*Cxcl5*	CXCR1, CXCR2
CXCL7	NAP-2	Pt, I, ELR	*PPBP*	*Ppbp*	CXCR1, CXCR2
CXCL8	IL-8	I, ELR	*IL-8*	–	CXCR1, CXCR2
CXCL9	MIG	I, non-ELR	*CXCL9*	*Cxcl9*	CXCR3
CXCL10	IP-10	I, non-ELR	*CXCL10*	*Cxcl10*	CXCR3
CXCL11	I-TAC	I, non-ELR	*CXCL11*	*Cxcl11*	CXCR3, CXCR7
CXCL12	SDF-1	H, non-ELR	*CXCL12*	*Cxcl12*	CXCR4, CXCR7
CXCL13	BLC, BCA-1	H, non-ELR	*CXCL13*	*Cxcl13*	CXCR5, CXCR3
CXCL14	BRAK	H, non-ELR	*CXCL14*	*Cxcl14*	Unknown
–	–	U, non-ELR	–	*Cxcl15*	Unknown
CXCL16	SR-PSOX	I	*CXCL16*	*Cxcl16*	CXCR6
CXCL17	DMC	U	*CXCL17*	*Cxcl17*	Unknown

CX3C family

CX3CL1	Fractalkine	D	*CX3CL1*	*Cx3cl1*	CX3CR1

I: inflammatory chemokines, H: homeostatic chemokines, D: dual chemokines, P: plasma or platelet chemokines, U: unknown, ELR: presence of the motif associated with an angiogenic activity in tumors, non-ELR: absence of the ELR motif.

The XC chemokine subfamily is represented by only two members, XCL1 and XCL2 which specifically promote chemotaxis of T lymphocytes [242]. Similarly, CC chemokines are known to induce the migration of various cell types including monocytes, dendritic cells, basophils, eosinophils and natural killer cells [242]. CX3CL1 is the only member of the CX3C chemokine group. Unlike other chemokines, CX3CL1 exists as a membrane bound glycoprotein which undergoes a shedding before being released. It promotes chemotaxis and adhesion of monocytes, natural killer cells and T-lymphocytes to endothelial, epithelial and dendritic cells [243].

Chemokines from the CXC subfamily can further be divided structurally (and functionally) into two additional groups. One group displays a well-defined sequence of three amino acid (Glu-Leu-Arg), forming an ELR motif, found immediately before the first cysteine residue of the CXC motif. The second group of CXC chemokines lacks such an ELR signature.

ELR-positive CXC chemokines are chemoattractant for neutrophils and specifically promote angiogenesis in tumors [244]. Conversely, ELR-negative CXC chemokines are chemoattractant for T lymphocytes and monocytes and are known as potent angiogenic inhibitors [245]. One exception is to be made regarding CXCL12 which lacks the ELR domain but was shown to induce neovascularisation *in vivo* (see Chapter II, section 4) [246, 247]. Additionally, several chemokines also act as organ-specific angiogenic modulators [248]. ELR-positive CXC chemokines particularly play a crucial role in tumor growth including lung, colorectal, pancreatic, ovarian, prostate, melanoma and brain cancer [249].

Chemokine genes have only been described in vertebrates including their most primitive members (jawless fish and lamprey) [250]. The genomic organization of chemokine genes allows to classify them in two alternative groups: "major-cluster" chemokines (whose genes are located in clusters at specific chromosomal locations) and the "non-cluster" or "mini-cluster" chemokines (whose genes are located separately in unique chromosomal locations) [251, 252]. In the facts, large clusters of human CXC and CC genes are respectively found on chromosomes 4q13.3-q21 and 17q11.2 (Figure 9). An explanation is to be found in the evolutionary forces which have shaped the genome into gene super-families [253]. Over the course of evolution, gene duplications have been a common event, affecting most gene families [254]. Once a duplication occurred, the two copies evolve independently from each other and develop specialized functions. This could explain the origin of "major-cluster" chemokines which also show two additional features compared to "mini-cluster" chemokines: 1) the members of a given gene cluster usually bind to multiple receptors and vice versa and 2) cluster chemokines do not correspond well between species [255].

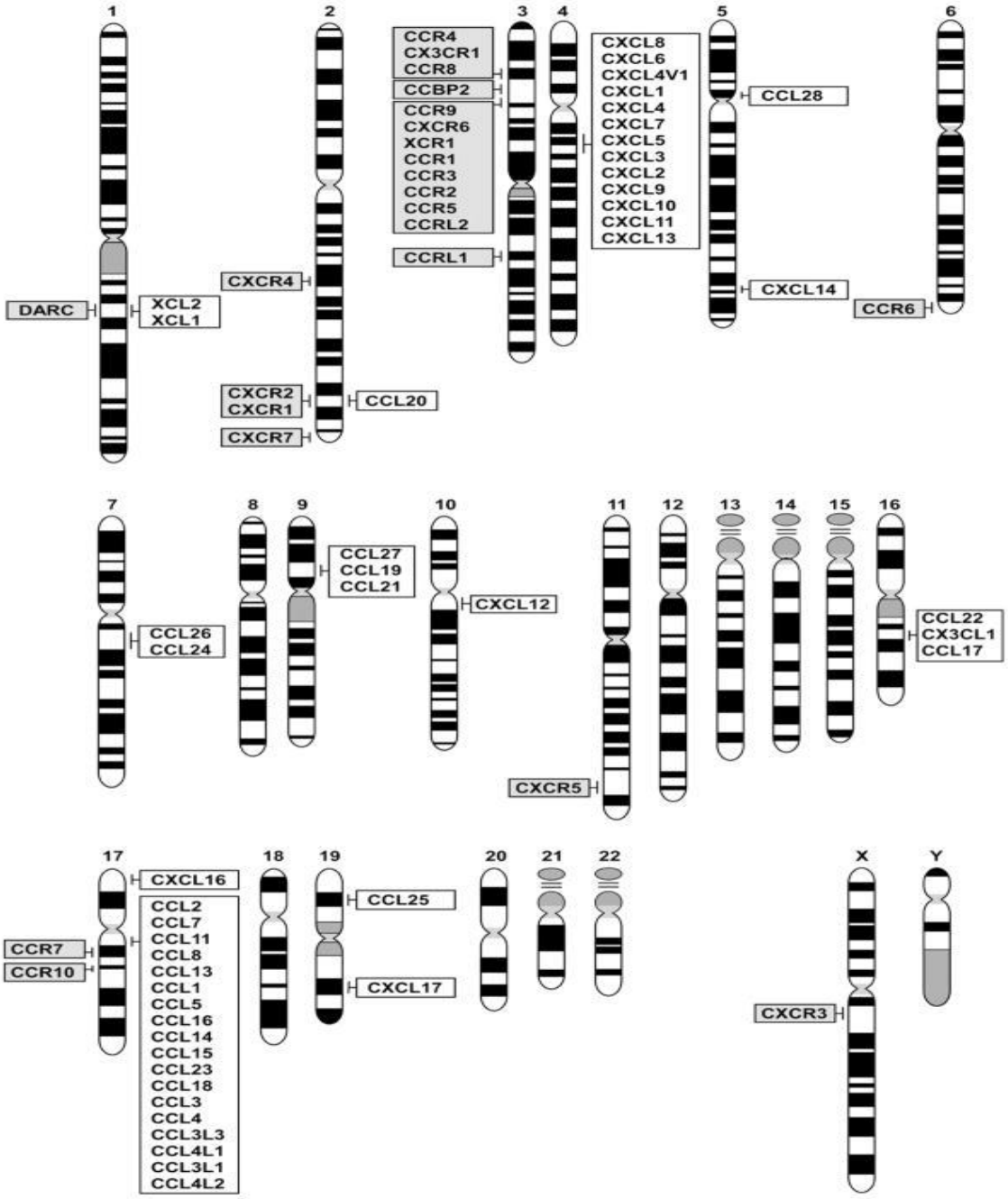


FIGURE 9: Chromosomal map of human chemokine and chemokine receptor genes. The full set of human chromosomes is shown with the location of each chemokine and chemokine receptor genes indicated as follows: chemokines (white) and chemokine receptors (gray) (Zlotnik et al., 2012).

2. Chemokine Receptors

Chemokines bind to receptors which all belong to the GPCR superfamily. This family also englobes receptors for hormones, neurotransmitters, paracrine substances and inflammatory mediators [256]. Chemokine receptors are classified into 4 groups according to the subfamily of chemokines they bind to. These seven transmembrane domains receptors are made of three extracellular and three intracellular loops, an acidic amino-terminal ligand binding domain and a serine/threonine-rich intracellular C-terminus. The first two extracellular loops present a conserved cysteine residue, allowing the formation of a disulfide bridge between the loops. The extracellular N-terminus contributes to the ligand recognition whereas transmembrane sequences, cytoplasmic loops and C-terminus are all involved in the receptor signaling mediated by the Gαi class of G proteins [257]. To date, 19 genes encoding chemokine receptors with chemotactic functions have been identified in humans [258]. Additionally, five other chemokine receptors have been identified including DARC, CCBP2 (or D6), CCRL1 and 2 and CXCR7 [259-263]. These atypical chemokine receptors do not couple to G proteins and therefore do not transduce the full spectrum of GPCR signals typically leading to chemotaxis and other cellular responses. As an explanation, these five receptors lack or display a modified DRY motif in their second intracellular loop (DRYLAIV) known to be essential for the efficient coupling with the Gαi class of G proteins. Their functions however include chemokine scavenging or act as decoy (interceptor) receptors or transporters.

Like their ligand counterparts, chemokine receptor genes are well conserved among vertebrates and are also found as clusters. A large cluster of genes is indeed located on human chromosome 3, suggesting a rapid evolution through repeated gene duplications [237]. Interestingly, most of these clustered genes have the ability to interact with a wide panel of inflammatory chemokines (Figure 10). The relationship between inflammatory chemokines and their receptors is therefore often qualified as promiscuous. Homeostatic chemokines usually display more specific interaction patterns [255]. The remaining chemokine receptor genes are found as individual genes on chromosomes 1, 6, 11 and X or to be present as “mini-clusters” on chromosomes 2 and 17.

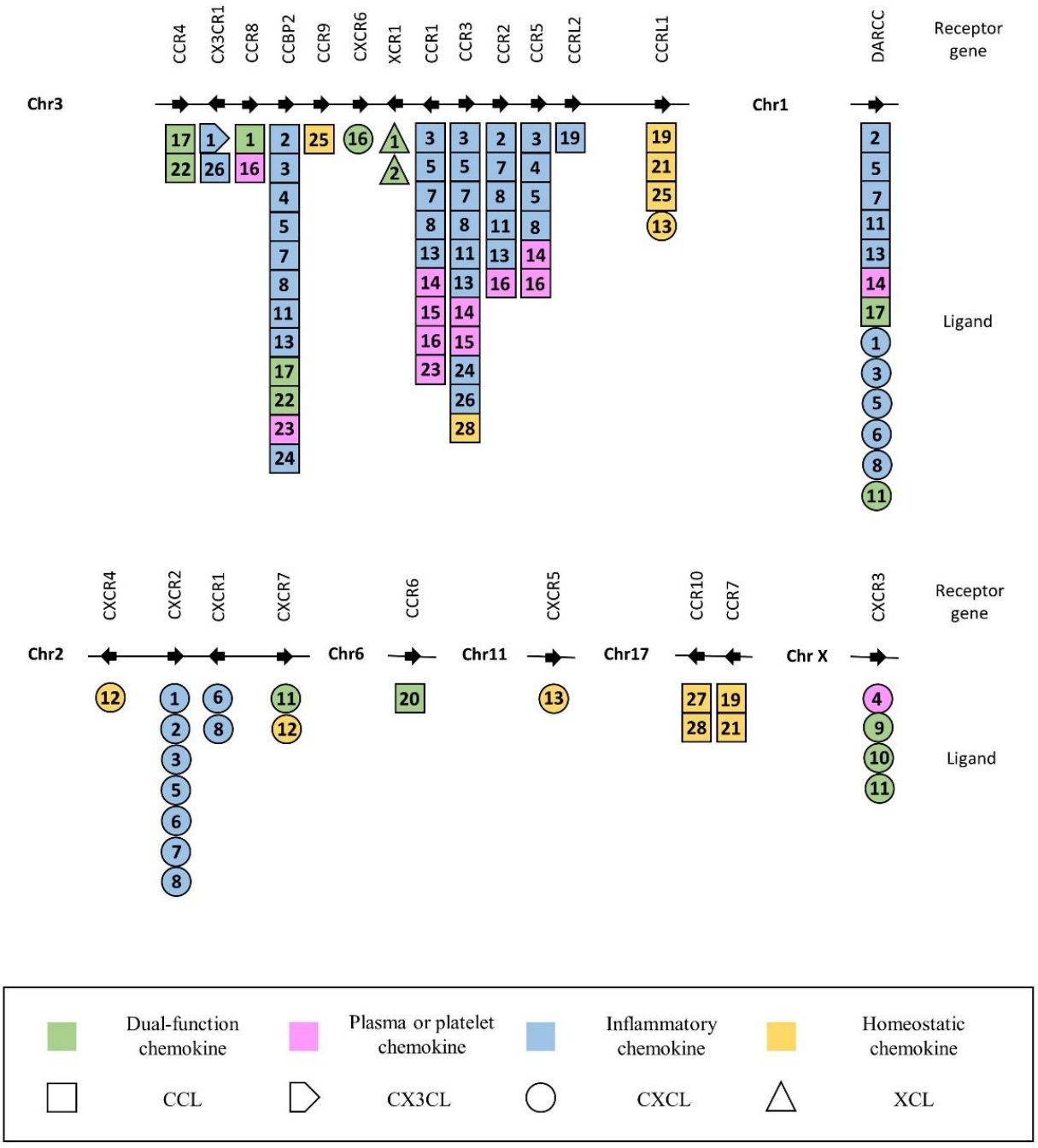


FIGURE 10. Gene clusters of human chemokine receptors. Receptors are represented according to their chromosomal locations and the type of chemokine they bind to (Illustration adapted from Zlotnik et *al*., 2006).

3. Chemokine CXCL12

CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is an homeostatic chemokine that was originally cloned from bone marrow (BM) derived stromal cell lines [264]. This chemokine has further been described as a key mediator in the homing of hematopoietic

stem cells (HSC) and the survival/proliferation of B-cell progenitors [265, 266]. CXCL12 is also involved in pathological processes affecting both brain and cardiovascular development. Mutant mice lacking CXCL12 die perinatally and present severe defects in heart morphogenesis, mainly suffering from cardiac septal defects [267, 268]. These mice also display severe cerebellar impairments as CXCL12 plays a central role in the development of the cerebellum [269-271]. Moreover, the number of B-cell progenitors was shown significantly impaired in the fetal liver and BM of CXCL12 knock-out embryos, whereas progenitors from the myeloid lineage were only reduced in the BM [272]. These findings particularly shed the light on the importance of CXCL12 in lymphopoiesis and BM myelopoiesis.

The human CXCL12 gene spans over 88 kilobase-pairs on chromosome 10q. To date six different CXCL12 isoforms have been identified (isoform α, ß, γ, δ, ε and φ) [273]. Interestingly, all isoforms are more than 90% homologous to each other. Furthermore, they share the 5'-coding sequences but differ in their 3'-coding sequences resulting in different C-terminal amino acid sequences further suggesting different physiological functions [274]. Chemotactic activity was demonstrated for all CXCL12 variants. CXCL12α is the predominant isoform found in all organs but rapidly undergoes proteolysis in the blood. Isoform β is, on the other hand, more resistant to blood-dependent degradation, stimulates angiogenesis and is consequently found in highly vascularized organs [275]. In contrast, CXCL12γ is usually found in less vascularized organs which are more susceptible to infarction [275]. The mouse homolog to the human CXCL12 is localized on chromosome 6. To date, the existence of two isoforms have been described (isoform α and ß), arising from alternative splicing. A third isoform (γ) has been identified in rat [276]. Overall homology between mouse and human CXCL12 is more than 90% with CXCL12α differing from only one amino acid between the two species [277].

The BM is a major source of CXCL12, mainly secreted by osteoblasts within the endosteum [278, 279]. This secretion is known to regulate the migration/retention of CXCR4 expressing CD34-positive HSC in distinct compartments. As a matter of fact, the administration of cyclophosphamide rapidly induces a MMP-dependent drop in CXCL12 concentrations inside the BM, further resulting in HSC mobilization into the bloodstream [280, 281]. Kollet and colleagues showed that mobilized HSC expressing low levels of CXCR4 were more likely to be kept into the bloodstream whereas HSC expressing high levels of CXCR4 were preferentially attracted toward CXCL12-enriched tissues [282]. In a similar fashion, administration of AMD3100, a specific CXCR4 antagonist, leads to the mobilization of CD34-positive HSC in the peripheral blood. This further underlines the importance of the

CXCL12/CXCR4 balance in maintaining HSC in the BM [283, 284]. At present, the major indication for a clinical use of AMD3100, also known as Plerixafor™ or Mozobil™, is precisely the mobilization of BM-residing HSC in order to transplant patients with hematological malignancies such as non-Hodgkin's lymphoma or multiple myeloma [285, 286].

Last but not least, CXCL12 also acts as an emergent salvage signal in tissue regeneration and repair. Of note, several tissues respond to chemical or physical insults such as toxic agents, hypoxia or irradiation by increasing their expression of CXCL12 [287]. This consequently favors the recruitment of CXCR4-positive stem/progenitor cells to the site where tissue regeneration is needed [288]. CXCL12 also increases perfusion of ischemic tissues through the recruitment of endothelial progenitor cells [289]. *In vivo* models of ischemia further demonstrated the expression of CXCL12 to be specifically upregulated by hypoxic gradients through HIF-1α [290, 291].

As a conclusion, and according to the light of scientific evidence, CXCL12 appears to undertake keynote functions in regulating the migration of progenitor cells during embryonic hematopoiesis and organogenesis as well as in organ homeostasis, vascularization and tissue regeneration.

4. Chemokine Receptor CXCR4

CXCR4, also known as Fusin, was first cloned by Loetscher and colleagues as an orphan chemokine receptor. Originally named LESTR, this receptor was first reported to be expressed on neutrophils, myeloid cells and T lymphocytes [292]. Two years later, LESTR was described as a necessary co-receptor for the entry of T-tropic HIV-1 and HIV-2 viruses into CD4+ cells [293]. LESTR turned into CXCR4 when CXCL12 was identified as its ligand in 1996 [294, 295]. CXCR4 is ubiquitously expressed by many types of cells including cells from the immune and central nervous system [296, 297]. In the immune system, CXCR4 is specifically expressed by monocytes, naive T-cells and B-cells [297]. CXCR4 expression also regulates CD34-positive HSC maintenance and trafficking respectively in and toward the BM [297, 298].

Knocking down the expression of CXCR4 in mice results in fetal lethality of homozygous mutant embryos [299]. The rarely born mutant mice die within hours after birth and display impaired B lymphopoiesis (absence of B220+/CD43+ progenitors), BM

myelopoiesis and hematopoiesis [268]. The absence of B-lineage in CXCR4-KO mice has been associated with a lack of proliferation and/or fate determination. Furthermore, mice lacking the expression of CXCR4 develop generalized edema, display cardiac/cerebellar defects and an incomplete formation of large vessels [300-302]. Interestingly, all these phenotypes are somewhat similar to ones encountered in CXCL12-KO mice [268].

The human gene encoding for CXCR4 is found on chromosome 2 (Figure 10). Two isoforms have been described so far: CXCR4-A (the unspliced variant) and CXCR4-B (the spliced variant) [303, 304]. CXCL12 exerts a similar chemotactic activity on both isoforms. The only difference comes from the N-terminus region of CXCR4-A which displays a supernumerary extension of 9 amino acids. Conversely to its human counterpart, the murine gene encoding for CXCR4 is located on chromosome 1 [305]. Two functional isoforms arising from alternative splicing have been identified (CXCR4a and CXCR4b) [306]. Despite differences between murine and human transcripts (alternative splicing versus one unspliced/spliced variant), the CXCR4 protein displays an overall homology of 91% between both species [307].

CXCR4 firing is involved in modulating different transduction pathways starting from the inhibition of cAMP production, the mobilization of intracellular Ca^{++} and the induction of ERK, MAPK, JNK and AKT effector molecules [308-310]. A detailed description of intracellular transduction pathways related to the activation of CXCR4 is further provided (see Chapter IV, section 6).

5. Chemokine Receptor CXCR7

Discrepancies between CXCR4 expression and CXCL12 binding affinity in several human cell lines challenged the dogma of a monogamous interaction between CXCR4 and CXCL12. This consequently led to the identification of CXCR7 (RDC1) as another CXCL12 receptor [311, 312]. CXCR7 has originally been cloned from a dog thyroid cDNA [313]. The human CXCR7 gene is found on chromosome 2 (Figure 10) whereas its murine homolog is mapped on chromosome 1. Interestingly, the gene encoding for CXCR7 displays 90% of homology between dogs, humans and mice [305]. In 2006, Burns and colleagues identified a CXCR7 binding activity with chemokines CXCL12 and CXCL11. Although CXCR7 binds to

both chemokines, this receptor displays a 10-fold stronger affinity for CXCL12 compared to CXCL11 [311].

Different from CXCR4, CXCR7 is considered as an atypical member of the chemokine receptor family. It shows indeed a DRYLSIT motive instead of the conserved DRYLAIV motive in its second intracellular loop. As previously mentioned, the DRYLAIV motive is necessary for the coupling to heterotrimeric G proteins and subsequent signaling events. CXCR7 expression has been described on a wide variety of cells including B lymphocytes, T cells and natural killer cells [314-316]. Interestingly, the role of CXCR7 appears to be different between cell types. CXCR7 can either act as a non-signaling and decoy receptor or as a scavenger undertaking an active role during developmental processes [260, 317]. As an example, interesting studies on zebrafish models have shown CXCR7 to be required for directional migration patterns during organ morphogenesis [318, 319]. In this line, Boldajipour and co-workers reported CXCR7 to mainly act as a decoy receptor by inducing the sequestration of CXCL12 in the stroma. This actually creates a chemokine gradient along which CXCR4 expressing cells migrate to their target organs [320]. Despite overlapping ligand specificities, the functions of both CXCR4 and CXCR7 do not appear completely redundant. Indeed, the *in vivo* perinatal mortality encountered in CXCR4-KO mice is not rescued by CXCR7 functions, suggesting these two receptors to mediate different pathways during development [311]. CXCR7 functionalities are precisely required during cardiac development and seem essential for perinatal survival [316, 321]. As a matter of fact, 95% of CXCR7-KO mice die as neonates and display severe defects in heart morphogenesis (chondrogenic transformation of the valves, thickening of the aortic valve, defects in ventricular and atrial septa as well as in pulmonary and tricuspid valves) [314, 321].

CXCR7 is also expressed in the developing and adult brain [315]. Its expression has been documented in human spinal ganglia and descending neurons as well as in the cerebellum of adult mice [322]. A recent study reported that CXCR7 is required for the tangential migration of cortical interneurons during the murine CNS development [323]. Although CXCR7 is widely expressed across various adult tissues at the mRNA level, its protein expression at the cell membrane is much more restricted, probably due to post-translational regulations [311]. In this line, Shimizu and collaborators highlighted CXCR4 as the main CXCL12 signaling receptor in differentiated neurons and suggested CXCR7 to interact with CXCR4 at the intracellular level, potentially affecting CXCR4 trafficking and/or coupling to other proteins [324].

6. Insights into the CXCL12–CXCR4/CXCR7 Signaling

Ligand binding changes the CXCR4 three-dimensional conformation which favors the heterotrimeric G protein GDP/GTP exchange and its dissociation into α- and βγ-subunits [325]. The α-subunit inhibits the production of cAMP by impacting on the adenylyl cyclase activity and activates the phospholipase C. This phospholipase then hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) in diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3) which control the release of intracellular Ca^{2+} from the endoplasmic reticulum (Figure 11). Additionally, the α-subunit also activates transcription factors including NF-κB, the Ca^{2+}-dependent tyrosine kinase PYK2, JAK/STAT and controls the activation of the phosphoinositide-3 kinase (PI3K) pathway, leading to cell survival and proliferation. The βγ dimer, acting as a functional subunit, is involved in the Ras activation of ERK1/2 MAPK cascade, leading to changes in gene expression and cell cycle progression. CXCR4 also regulates cell survival by the G protein-dependent activation of JNK and p38 MAPK. The βγ dimer finally interacts with ion channels and activates the PI3K, further modulating the CXCL12-dependent chemotaxis [326].

CXCL12 triggers CXCR4 desensitization and uncoupling from G proteins by GPCR kinase (GRK)-dependent phosphorylation. The subsequent interaction of CXCR4 with β-arrestin then mediates its internalization [327] and targets the desensitized receptor to clathrin-coated pits for endocytosis (Figure 11). Moreover, the interaction between CXCR4 and β-arrestin also underlies the activation of downstream MAPK mediators including p38 and ERK1/2 [328]. Cell migration is thought to be directed by CXCR4 and a CXCL12 gradient. This gradient is controlled by the CXCL12 internalization level potentially created by CXCR7 without generating any intracellular signaling [329]. Indeed, due to the lack of heterotrimeric G protein coupling, CXCR7 was initially described as a decoy receptor able to promote ligand internalization and degradation, therefore reducing the activity of CXCR4 [330]. However, this simplistic view only represents one of the possible mechanisms by which CXCR7 specifically modulates cellular functions [331]. Emerging evidence strongly suggest CXCR7 to activate intracellular signaling pathways, including the MAPK pathway, either by direct recruitment of β-arrestin [332, 333] or after heterodimerization with CXCR4 [334].

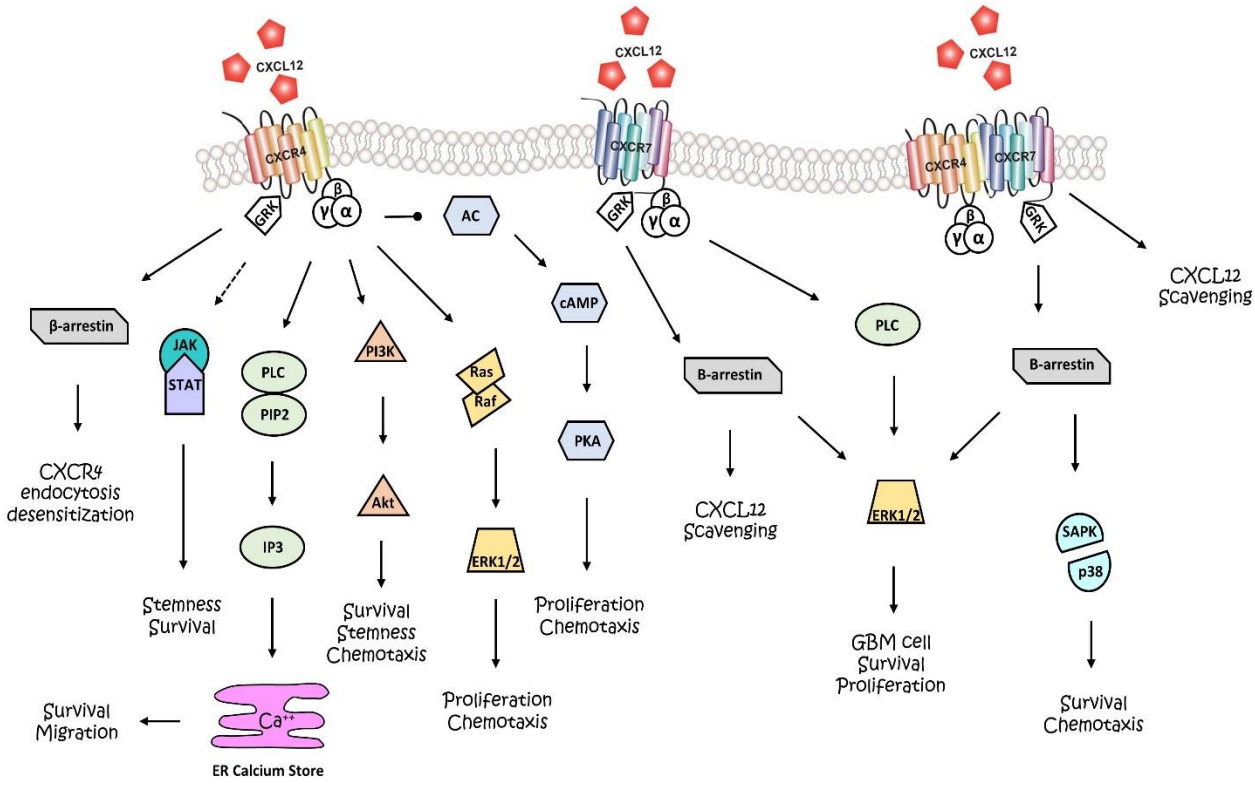


FIGURE 11. Schematic diagram of CXCR4/CXCR7 crosstalk affecting major signaling pathways related to cell survival, proliferation and migration. CXCL12 binds to CXCR4 and CXCR7, which can form homodimers or heterodimers. CXCR4 activation by CXCL12 triggers GPCR signaling through PI3K/Akt, PLC/IP3, ERK1/2 pathways and mobilization of Ca^{++} from the endoplasmic reticulum, further regulating cell survival, proliferation, and chemotaxis. Beta-arrestin pathway can be activated through GRK to internalize CXCR4. When CXCR7 binds to CXCL12, activation of β-arrestin may lead to scavenging of CXCL12. CXCL12/CXCR7 also controls cell survival through ERK1/2. AC: Adenylyl Cyclase, PLC: Phospholipase C, PIP2: Phosphatidylinositol 4,5-bisphosphate, IP3: Inositol 1,4,5 trisphosphate, PI3K: Phosphoinositide-3 Kinase, ERK1/2: Extracellular Regulated Kinase ½, GRK: GPCR Kinase, PKA: Protein Kinase A, JAK: Janus Kinase, STAT: Signal Transducer and Activator of Transcription (Illustration adapted from Würth et *al*., 2014)

In this line, the formation of CXCR4/CXCR7 heterodimers has been documented to modulate the CXCR4 signaling [335] and to enhance both the CXCL12-dependent intracellular Ca^{2+} mobilization and ERK1/2 phosphorylation [314]. The enhanced activity of CXCR4/CXCR7 heterodimers in recruiting β-arrestin specifically provides mechanistic insights into growth, survival and migratory advantages provided by both CXCR4 and CXCR7 in cancer cells. Indeed, β-arrestin recruitment to the CXCR4/CXCR7 complex enhances downstream β-arrestin-dependent cell signaling including ERK1/2, p38 and SAPK/JNK (Figure 11) [332].

7. Role of CXCL12–CXCR4/CXCR7 in GBM Stem Cells

The CXCL12-CXCR4/CXCR7 axis is known to play a central role in tumor development and tumor cell proliferation. It mainly contributes to dissemination and invasion mechanisms in a plethora of human malignancies including pancreatic, colon, ovarian, prostate and breast carcinomas as well as lymphoma, melanoma, neuroblastoma and GBM [249, 332, 336]. Furthermore, CXCR4 expression is usually associated with metastatic abilities favoring malignant cells to migrate toward CXCL12 expressing organs including lungs, liver, brain, lymph nodes and BM [337, 338]. Relying on these facts, CXCL12 may potentially represent a promising target in the fight against malignant tumors and metastases. Moreover, the identification of cancer stem cells (CSC) in different types of neoplasms potentially highlights new roles for the CXCL12-CXCR4/CXCR7 axis in tumor biology. CXCR4 (over)expression has indeed been detected in CSC deriving from pancreatic, colon, lung, breast, prostate, renal and brain cancer [92, 339-343]. The recent demonstration of CXCR7 as an active receptor for CXCL12 has, on its own, also increased the interest for this chemokinergic system in CSC-related research [334].

In the facts, the CXCL12/CXCR4 signaling is regarded as a key regulator in GSC biology, involved in self-renewal, proliferation, migration, angiogenesis and therapeutic resistance. CXCR4 is indeed found specifically overexpressed in GSC compared to differentiated tumor cells originating from the same culture [344]. Deeper in details, Liu and colleagues recently reported high levels of GSC heterogeneity in CXCR4 expression and functions [345]. This observation was further confirmed by another study demonstrating GSC to display different levels of CXCR4 expression among different human GBM primary cultures [346]. The role of CXCR4 in GSC survival and self-renewal has been assessed using a CXCR4 specific antagonist (AMD3100). Interfering with the CXCR4 signaling significantly impeded both parameters with greater efficacy in cultures releasing high amounts of endogenous CXCL12 [346] and decreased the expression of genes associated with self-renewal, including Oct4 and Nanog [347]. As a matter of fact, chemokine CXCL12 can be released by GSC, suggesting an autocrine/paracrine signaling mechanism (see Chapter II, section 4) [348]. In this line, *in vitro* experiments showed that both exogenous and GSC-secreted CXCL12 promote GSC proliferation [345, 349]. Once again, the use of AMD3100 allowed to drastically overturn this CXCL12-induced proliferation benefit [350] and to impair colony formation, further confirming the autocrine growth-stimulation effect of this chemokine [135].

Conversely to CXCR4, a definitive establishment of the CXCL12/CXCR7 functions in GSC cannot be provided in details since this axis has not been completely investigated yet. Nevertheless, a growing body of evidence already supports the involvement of CXCR7 in GSC maintenance and tumorigenicity. In this line, the pharmacological inhibition of CXCR7 post irradiation has been shown to induce tumor regression, block tumor recurrence and substantially prolong survival in xenotransplanted mice and rats [351]. In this study, CXCR7 expression in human GBM cells correlated with neurosphere-forming abilities. Additionally, the inhibition of this atypical chemokine receptor also interfered specifically with GSC self-renewal abilities *in vitro*.

As already mentioned, heterogeneous expression levels for both CXCR4 and CXCR7 have been reported in GBM primary cultures. Interestingly, GSC-enriched cultures display a higher percentage of CXCR4 and CXCR7 expression than differentiated cells, suggesting both receptors to be involved in regulating the stem phenotype [345]. On the other hand, Liu and collaborators showed that CXCL12-induced cell growth, migration, sphere formation and tube formation was either mediated by CXCR4 and/or CXCR7, depending on the level of expression found in primary patient-derived GBM cells [345]. Discordant findings have been recently reported regarding the expression of CXCR7 and its role in GSC. Two independent studies have indeed claimed that GSC do not express CXCR7 [346, 352]. Moreover, upon GSC differentiation, CXCR4 levels were shown to decrease whereas CXCR7 levels increased, suggesting a prevalent role of CXCR7 in differentiated GBM cells [352]. As a consequence, the specific role of CXCR7 in GSC remains nowadays unclear, some findings suggesting a key role in maintaining GSC properties whereas others have failed to demonstrate its expression so far. Further investigations are therefore mandatory to clarify the situation.

Aim of the Project

and

Results

Aim of the Project

Objectives

Glioblastoma relapses are one of the most puzzling situations for hospital staff and doctors. A tremendous amount of data appoints GSC as evident culprits for tumor initiation, progression, treatment resistance and ultimately tumor relapses. However, we have been stuck in a rut with GBM treatment for quite a while now. Indeed the mean survival of GBM patients does not exceed 3% at 5 years from diagnosis. In this context, a better understanding of GSC features and the underlying mechanisms leading to tumor recurrence could significantly help to design new innovative therapeutic avenues.

Tools We Developed

Our laboratory recently established an orthotopic model of GBM invasion. Kroonen and colleagues indeed demonstrated a sub-population of GBM cells to specifically invade the adult SVZ after orthotopic transplantation. In this neurogenic niche, GBM cells were shown to express NSC markers and retain the ability to form secondary tumors when re-implanted in other animals. Being away from the initial tumor mass, these GBM-initiating cells or GSC may therefore contribute to GBM deadly relapses and potentially represent a reliable model of clinical obstacle to therapy.

Goals

1. The *How* question - trying to shed the light on the underlying molecular mechanisms which drive the GSC specific invasion of the SVZ. Counteracting with these mechanisms could indeed help to better encompass GSC invasive properties.

2. The *Why* question - trying to investigate whether there is a particular reason why GSC specifically end up part of their journey in the ventricular walls. This neurogenic region may hypothetically represent a suitable environment for GSC implantation. In this context, subsequent tumor relapses would occur because the appropriate seed (GSC) has found its suitable soil (SVZ).

Results

PART 1: The "How" Question

a. The Context

Infiltrative patterns of GBM make tumor cells hard to target. Furthermore, recent experimental evidences have demonstrated that GBM cells are able to escape the tumor mass and specifically invade the SVZ in orthotopic GBM models (Figure 12) [208]. Interestingly, GBM cells located in that environment were shown to be highly tumorigenic and further characterized as GSC. The SVZ is known to be a major source of NSC in adults and functions as a supportive niche which promotes NSC self-renewal and inhibits differentiation [353]. This "seed-and-soil" relationship has also been adapted to cancer stem cell research as GSC also rely on a specific environment or "niche" to maintain their stem cell properties and their ability to drive tumor growth [154, 354].

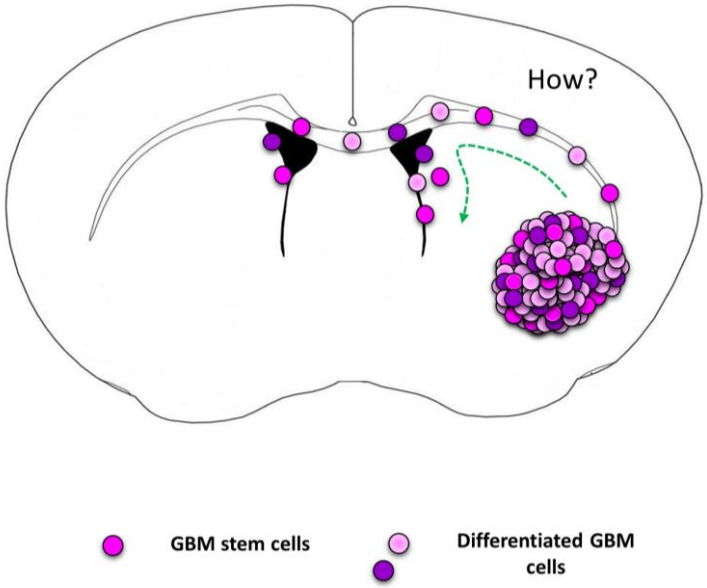
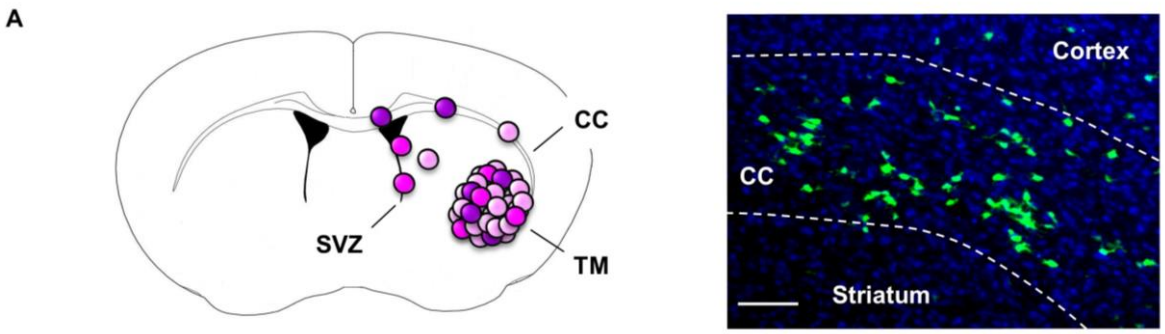


FIGURE 12. Glioblastoma stem cells specifically invade the adult SVZ in orthotopic GBM models.

b. Presentation of the GSC Invasion Model

We previously demonstrated human GSC to invade the SVZ environment once implanted in the striatum of immunocompromised mice [208]. Relying on this model of brain tumor invasion, we grafted human primary GBM cells (GB138 cells) expressing a green fluorescent protein (eGFP) in the striatum of mice tolerating xenografts. Twelve weeks after the injection, as the mice developed tumors, we specifically identified the presence of GB138 primary cells in the SVZ environment. As expected, those cells migrated along the CC, one of the main white matter structures connecting both left and right hemisphere together (Figure 13A).

We then validated this model of brain cancer cell invasion using human U87MG cells which we implanted in the striatum of immunocompromised mice as well. Three weeks after the injection, we identified U87MG cells in the CC of mice using a specific anti-human nuclei antibody (white arrows, Figure 13B). By the end of the 4th week after the graft, U87MG cells had colonized the SVZ environment, some of them still retaining the ability to proliferate as shown by the Ki67 staining (white arrows, Figure 13C and C'). Relying on this specific model of brain cancer invasion, we isolated U87MG cells and GB138 primary cells from the tumor mass (U87MG-TM and GB138-TM) and from the SVZ (U87MG-SVZ and GB138-SVZ) in order to further compare their biological properties. We specifically questioned the ability of these cells to give rise to self-renewable "gliomaspheres". Interestingly, both U87MG-SVZ cells and GB138-SVZ cells displayed stronger abilities to generate floating spheres compared to U87MG-TM cells (p=0.002) and GB138-TM cells (p=0.001), further confirming the enriched stem cell properties of SVZ-nested GBM cells (Figure 13D).



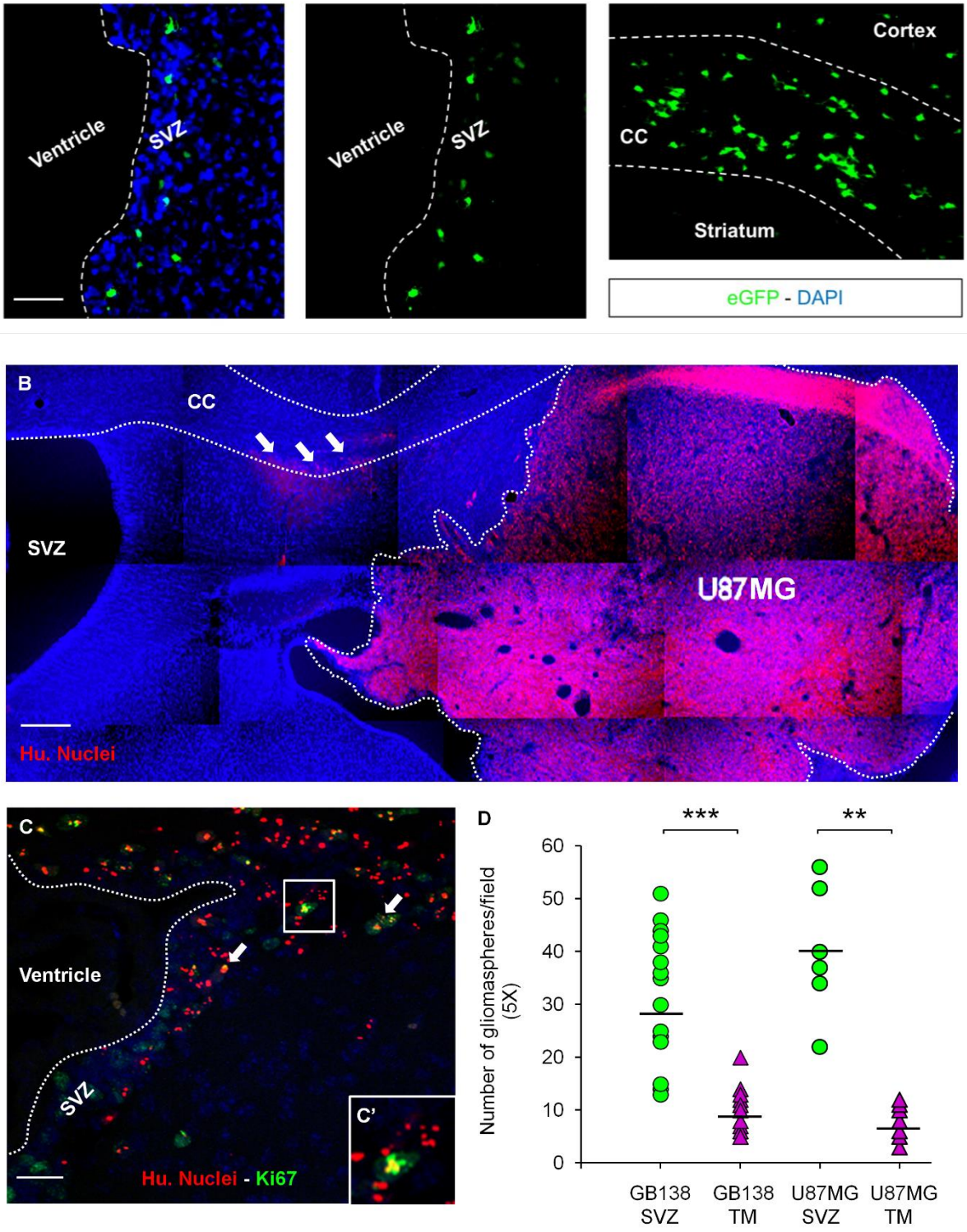


FIGURE 13. Invasion of the SVZ by U87MG cells and GB138 primary cells after intrastriatal implantation. A) GB138 primary cells specifically colonized the SVZ environment after migrating through the CC. B) Human U87MG cells invaded the largest part of the right striatum 3 weeks after the graft. At this point, U87MG cells already escaped the TM and started to migrate along the CC (white arrows). C) U87MG cells finally invaded the SVZ environment 4 weeks after the injection, some of them still being in a proliferation state (white arrows - magnified square). D) U87MG and GB138 primary cells isolated from the SVZ displayed stronger abilities to form gliomaspheres than U87MG or GB138 primary cells isolated from the TM.

Human U87MG cells were detected using an anti-human nuclei antibody (Hu. Nuclei - red). GB138 cells were engineered to express a green fluorescent protein (eGFP - green). Cell nuclei are counterstained with DAPI (blue). Scale bars = 500 µm for B, 40 µm for A and C. ** $p<0.01$, *** $p<0.001$

Of note, this original *in vivo* model of brain cancer invasion has successfully been reproduced/validated last year by Sadahiro and collaborators [209]. More importantly, Piccirillo and colleagues recently reported the very first evidence that the human SVZ endorses the role of a reservoir for brain tumor-initiating cells [210]. These findings actually reveal novel insights in GBM growth dynamics and may help to better understand the patterns of tumor recurrence.

c. Injection of Distant Invaders Triggers GBM Initiation

The operational definition of a CSC includes the ability to sustain tumor growth after orthotopic transplantation. We therefore questioned the ability of U87MG-SVZ cells to trigger GBM initiation after implantation in the striatum of immunocompromised mice. Prior to the injection, we made sure these cells derived from human material, stained by a specific anti-human nuclei antibody (red). U87MG-SVZ cells also expressed Nestin (green), a well-known NSC marker (Figure 14). Injection of 50.000 U87MG-SVZ cells systematically gave rise to large tumors within two weeks whereas injection of only 100 U87MG-SVZ initiated tumor development in 41.6% of the cases (5 mice bearing tumors out of 11 xenografted animals - Figure 14, adapted from Kroonen et *al.*). In comparison, the injection of 100 native U87MG cells never induced tumors whereas 100 U87MG-TM cells were enough to initiate tumors in 25% of the cases (3 mice bearing tumors out of 12 xenografted animals).

Chi-square test analysis between these two conditions (TM vs SVZ) was not statistically significant showing, the same amount of CSC enrichment in the different populations of U87MG cells. However, these findings demonstrated that U87MG-SVZ cells specifically retain tumor-initiating abilities and were therefore characterized as a population of cancer cells displaying enhanced GSC abilities [208]. Being away from the initial tumor mass and relying on GSC properties (see Chapter II, section 2 and 3), U87MG-SVZ cells could therefore represent a reliable model of clinical obstacle to therapy and subsequent tumor recurrence.

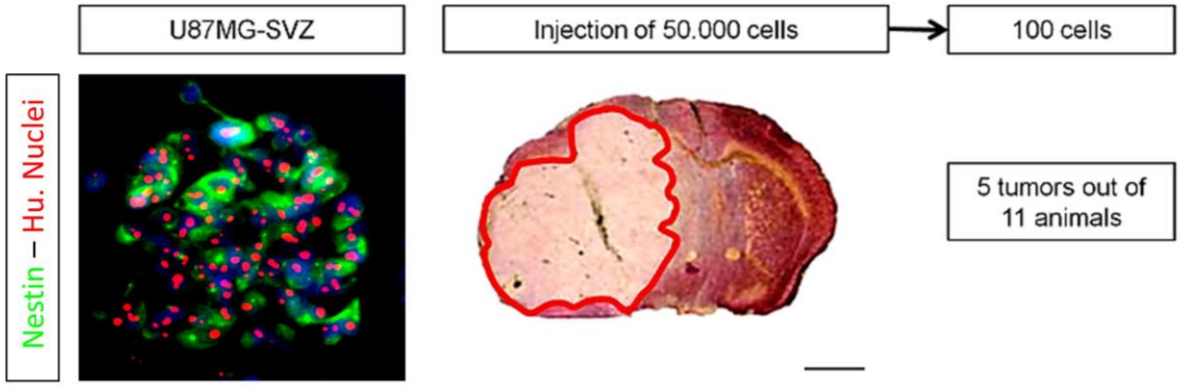


FIGURE 14. SVZ invaders trigger GBM initiation. Human U87MG cells isolated from the SVZ (U87MG-SVZ) are able to initiate tumor growth when implanted in immunocompromised mice. Human U87MG-SVZ cells specifically expressed Nestin (green), a well-known NSC marker, and were specifically detected using an anti-human nuclei antibody (Hu. Nuclei - red). Cell nuclei are counterstained with DAPI (blue). Scale bars = 1.8 mm.

d. Expression of Chemokines in the Subventricular Zone Environment

In order to identify potential targets involved in the GSC specific invasion of the SVZ, we looked for soluble factors secreted by the SVZ environment which could potentially play a role in this migration phenotype. To do so, total mRNA extractions from 12 independent SVZ whole-mounts and a wide scale RT-qPCR array were conducted. This analysis highlighted genes encoding for cytokines and chemokines which have been further classified according to their level of expression. High mRNA expression levels are found in the yellow rim of the graph and include *Ccl12, Ccl19, Cxcl12* and *Cx3cl1* among others. Basal mRNA levels are found in the purple rim of the graph and notably include *Ccl5, Ccl17, Cxcl10* and *Cxcl16.* Low mRNA levels of cytokines and chemokines expressed in the SVZ environment were finally classified in the white rim of the graph (Figure 15). This non-exhaustive database actually gives the "big picture" of different factors ultimately involved in our *in vivo* model of brain cancer invasion.

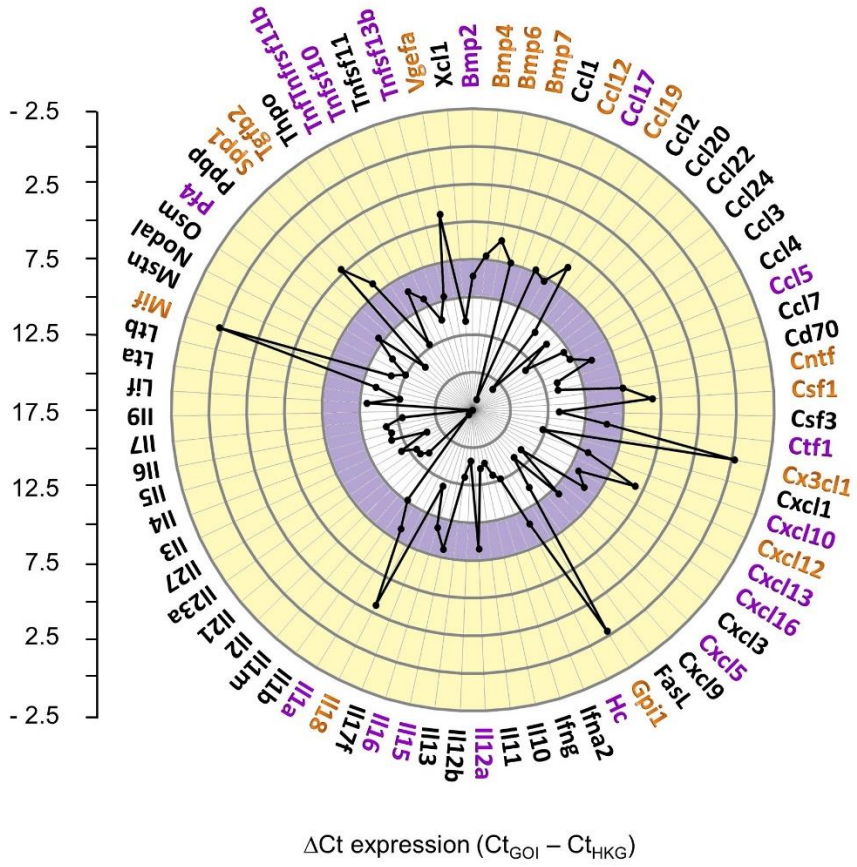


FIGURE 15. Mini-transcriptomic analysis of the SVZ environment. A RT-qPCR screening brought to light the high expression level of CXCL12 among many other genes encoding for cytokines and chemokines specifically related to the SVZ environment.

From those data, we made the hypothesis that CXCL12 could potentially explain the *in vivo* migration phenotype. As previously described, the CXCL12-CXCR4/CXCR7 axis plays a key role in GSC biology involved in self-renewal, proliferation, migration, angiogenesis and therapeutic resistance (see Chapter IV, section 7). Furthermore, CXCL12 has recently been the focus of very interesting studies highlighting its expression in the SVZ environment [355, 356]. Taken together, these findings underpin the hypothetical role played by the CXCL12-CXCR4/CXCR7 signaling in our *in vivo* model of brain cancer invasion.

To verify this theory, we first confirmed the CXCL12 mRNA expression by classic RT-PCR performed on SVZ whole-mounts, using mesenchymal stem cells (MSC) as a positive control (Figure 16A). We then highlighted the expression of CXCL12 (green) on brain coronal sections of mice previously grafted with human U87MG cells (red) and demonstrated a close

relationship between the expression of CXCL12 (green) and the presence of U87MG cells (red) in the SVZ (Figure 16B). We validated this observation on coronal sections of brains implanted with GB138 primary cells (green) as well (Figure 16C and D). Human U87MG cells and GB138 primary cells were respectively detected in brains using specific anti-human nuclei and anti-eGFP antibodies.

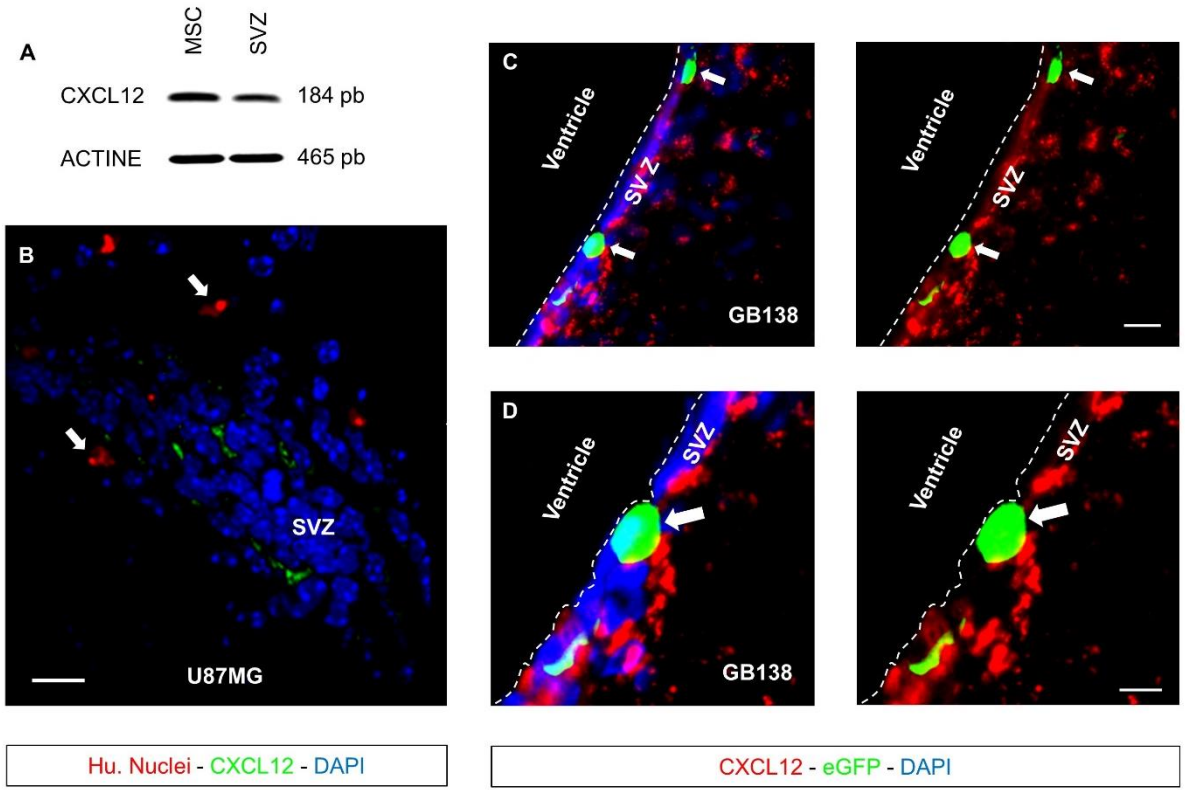


FIGURE 16. Close association between CXCL12 and GBM cells in the SVZ. A) Validation of the CXCL12 mRNA expression by RT-PCR using mesenchymal stem cells (MSC) as a positive control. B-D) The expression of CXCL12 within the SVZ was next demonstrated on brain coronal sections. This expression was consistent with the presence of U87MG cells (Hu. Nuclei) or eGFP-positive GB138 primary cells in the SVZ (arrows). Scale bars = 20µm for B and C and 10µm for D.

We managed to unravel the shape of a CXCL12 gradient within the SVZ (Figure 17A). To do so, we transformed each CXCL12 acquisition from the SVZ environment into binary images and quantified the CXCL12 expression in predefined areas (A: SVZ, B: transition SVZ-Striatum, C: Striatum). We systematically found a constant decrease in CXCL12 expression starting from area A to C, suggesting that CXCL12 is mostly secreted within the SVZ and diffuses toward the striatum along a decreasing concentration gradient. This observation corroborates with Erzebet Kokovay's hypothesis who postulated that different levels of CXCL12 may exist in the SVZ, creating in this way a gradient across this germinal zone [356].

To further explore the expression of CXCL12 in the SVZ, we then evaluated the amount of CXCL12 released by SVZ whole-mounts compared to other brain regions such as cerebellar and OB whole-mounts. Conducting ELISA, we found a significant increased amount of CXCL12 in SVZ media conditioned for 60 hours (SVZ-CM) compared to 24 hours ($p = 0.0006$). Moreover, significant higher levels of CXCL12 were found in media conditioned by SVZ whole-mounts for 60 hours compared to cerebellum or OB media conditioned for the same amount of time ($p = 0.005$ - Figure 17B). This actually shows how specific the CXCL12 expression is to the SVZ region. We finally demonstrated on SVZ whole-mounts that blood vessels overlaying the lateral wall of the ventricle (lectin staining) and GFAP-positive cells (astrocytes) were sources of CXCL12 in the adult SVZ environment (Figure 17C).

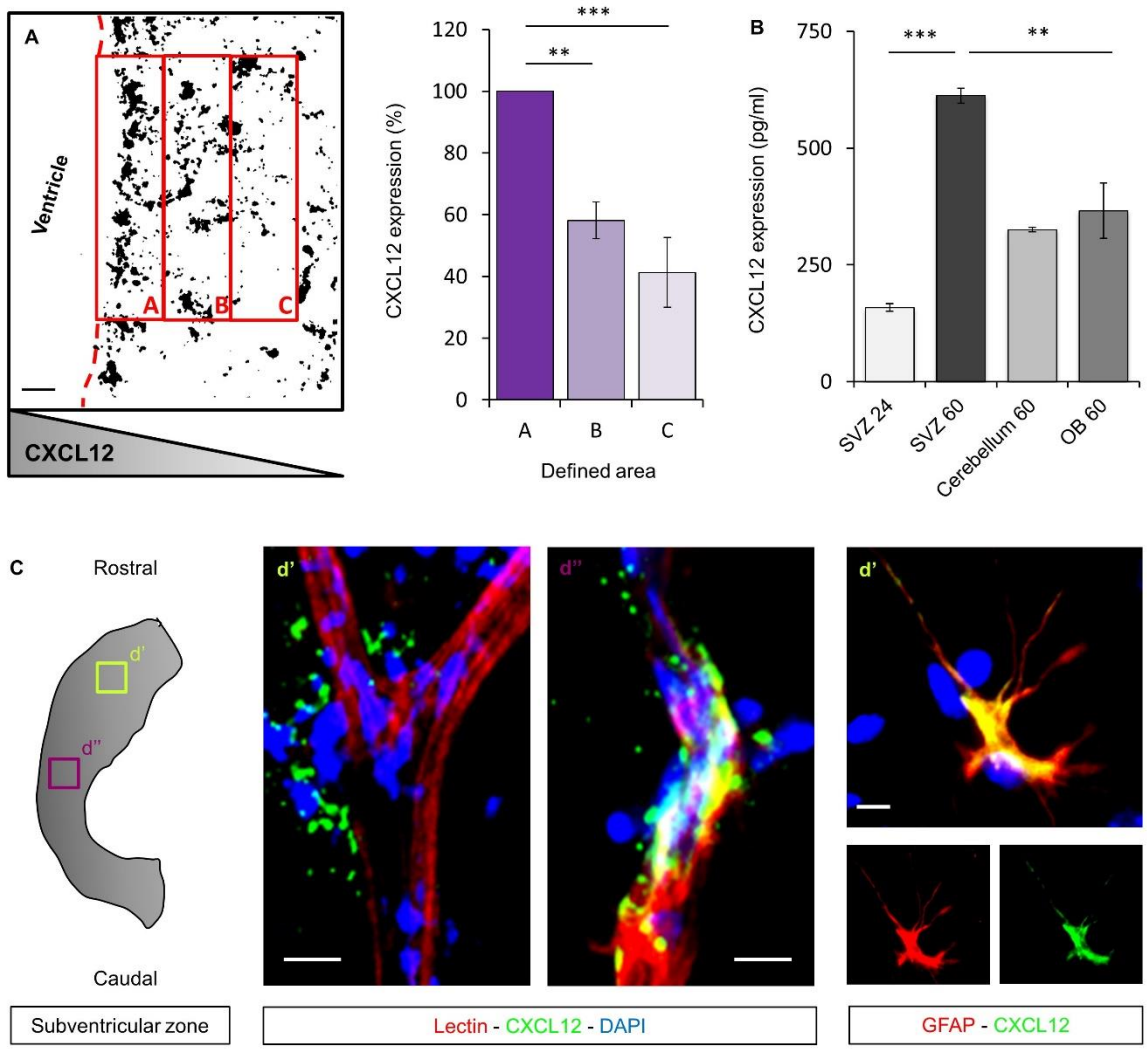


FIGURE 17. Expression of CXCL12 in the adult SVZ. A) CXCL12 acquisitions were processed as binary images and the mean intensity, with foreground 255 and background 0, in predefined areas of the SVZ environment (A, B and C) was calculated. A constant decrease of the CXCL12 expression was observed starting from area A to C, suggesting CXCL12 to be

secreted along a decreasing concentration gradient. B) CXCL12 levels were evaluated by ELISA in SVZ-conditioned media (SVZ-CM) as well as in cerebellum and OB media conditioned for 24 and 60 hours. C) CXCL12 is specifically expressed by GFAP-positive cells (astrocytes) and EC within the adult SVZ. Immunostainings on organotypic whole-mounts showed a CXCL12 expression pattern (green) closely related to the SVZ vasculature and astrocytes (red). SVZ blood vessels and astrocytes were respectively stained using a FITC-coupled lectin and a specific anti-GFAP antibody (red). Cell nuclei were counterstained with DAPI (blue). Scale bars = 40 µm for A and 10µm for C. Caption indicates where pictures and materials were taken.

e. Expression of CXCR4 and CXCR7 by Human GBM Cells

We then tackled the expression of CXCL12 receptors on GBM cells. While the expression and functions of CXCR7 remain controversial in GBM [357] (see Chapter IV, section 5 and 7), the expression of CXCR4 more evidently contributes to malignant dissemination and is usually associated with poor survival [358]. Although we tried, we never really succeed to unveil a convincing expression of CXCR7 *in vitro* (immunocytofluorescence or Western Blot analyses - data not presented), corroborating with previously described studies. Moreover, fluorescent-activated cell sorting (FACS) for both CXCR4 and CXCR7 revealed these receptors to be respectively expressed at 83.7% and 0.2% in U87MG cells (Figure 18), making CXCR7 unlikely to be involved in our model. Of note, FACS analyses also highlighted the expression of CXCR4 in 59.5% of GB138 primary cells (data not presented).

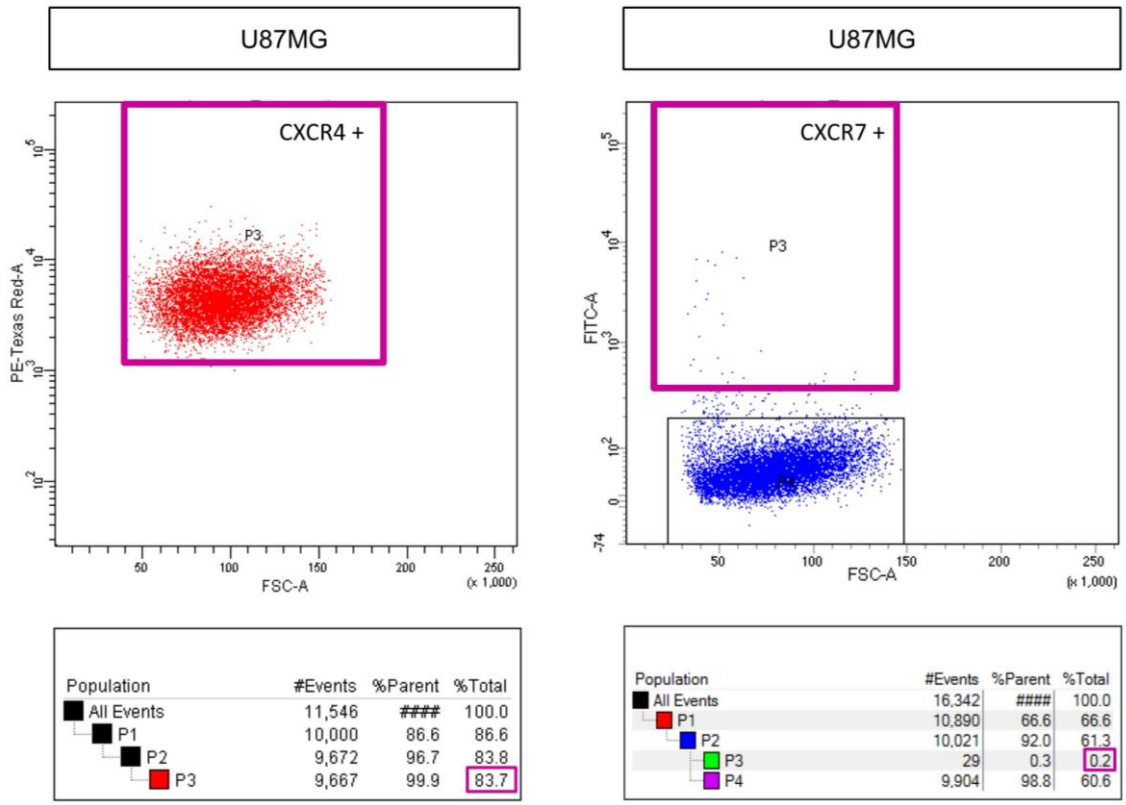


FIGURE 18. Expression of CXCR4 and CXCR7 on U87MG cells. FACS analyses demonstrated that CXCR4 is expressed in most U87MG cells (83.7%) whereas the expression of CXCR7 could barely be observed (0.2%). These data suggest that from those two receptors, CXCR4 is the one most likely to play a significant role in the *in vivo* model of brain cancer invasion.

Using RT-PCR and Western-Blot approaches, we confirmed the expression of CXCR4 on three GBM cell lines (U87MG-U373-LN18) and two GBM primary cell populations other than GB138 primary cells (GBM1-GBM2) (Figure 19A-B). We also investigated the CXCR4 expression profile on U87MG cells, U87MG cells isolated from the TM (U87MG-TM) or the SVZ (U87MG-SVZ) and U87MG cells cultured as floating spheres (U87MG NS). We noticed a comparable expression of CXCR4 in each of these cell populations (Figure 19C).

We then characterized the U87MG spheres for the expression of CXCR4 together with immature markers (Nestin and Sox2) and differentiated cell markers ("GFAP" and βIII-Tubulin). Immunostainings revealed the expression CXCR4 to be mainly associated with these four types of proteins, further highlighting the heterogenic nature of the cells composing the spheres (Figure 19D).

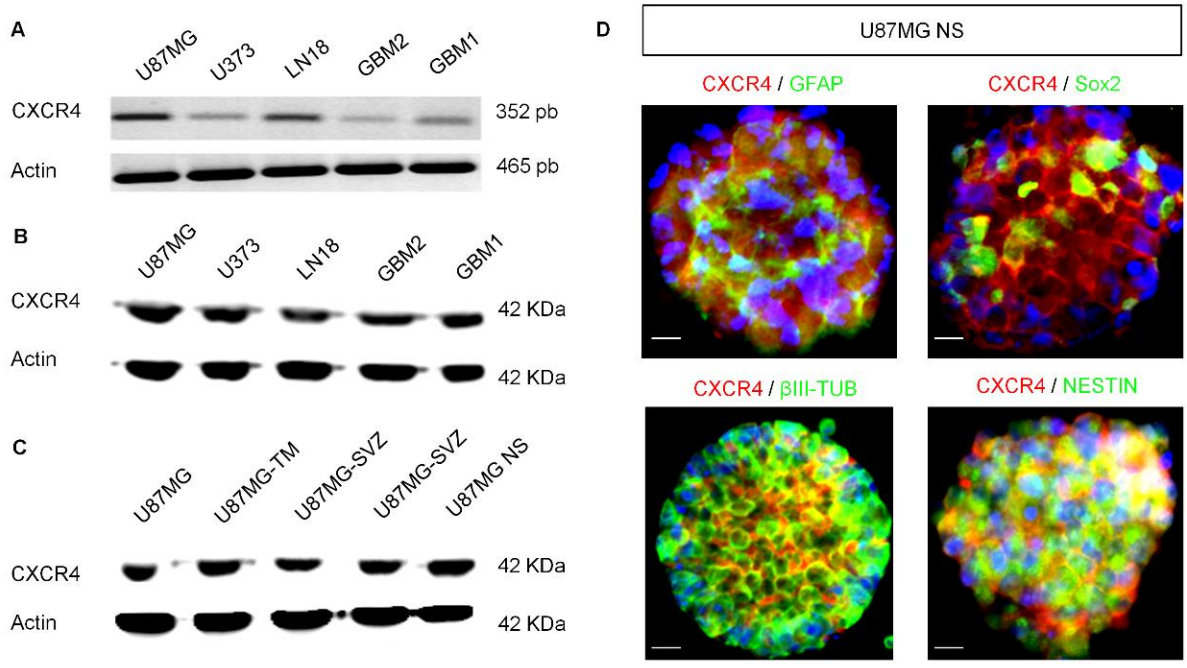


FIGURE 19. Expression of CXCR4 by various GBM cell types. A) *CXCR4* mRNA expression was found in three human GBM cell lines (U87MG, U373 and LN18) and two human primary cultures (GBM 1 and 2). B-C) Western-Blot analyses revealed a strong CXCR4 expression pattern in GBM cell lines and primary cultures as well as on U87MG cells isolated from the tumor mass (U87MG-TM) or the SVZ (U87MG-SVZ) and U87MG floating gliomaspheres (U87MG NS). D) Human U87MG NS showed a combined expression of CXCR4 (red) with GFAP, Sox2, Nestin and βIII-tubulin positive cells (green) within gliomaspheres. Scale bars = 15 µm.

We finally characterized gliomaspheres arisen from U87MG-TM and U87MG-SVZ cells and labeled them with different GSC markers such as Nestin, Prominin-1 (CD133) and Integrin α6 (ITGA6) [99]. Here we showed that GBM spheres express important levels of CXCR4, Nestin, CD133 and ITGA6 without any particular discrepancy between sub-populations (Figure 20).

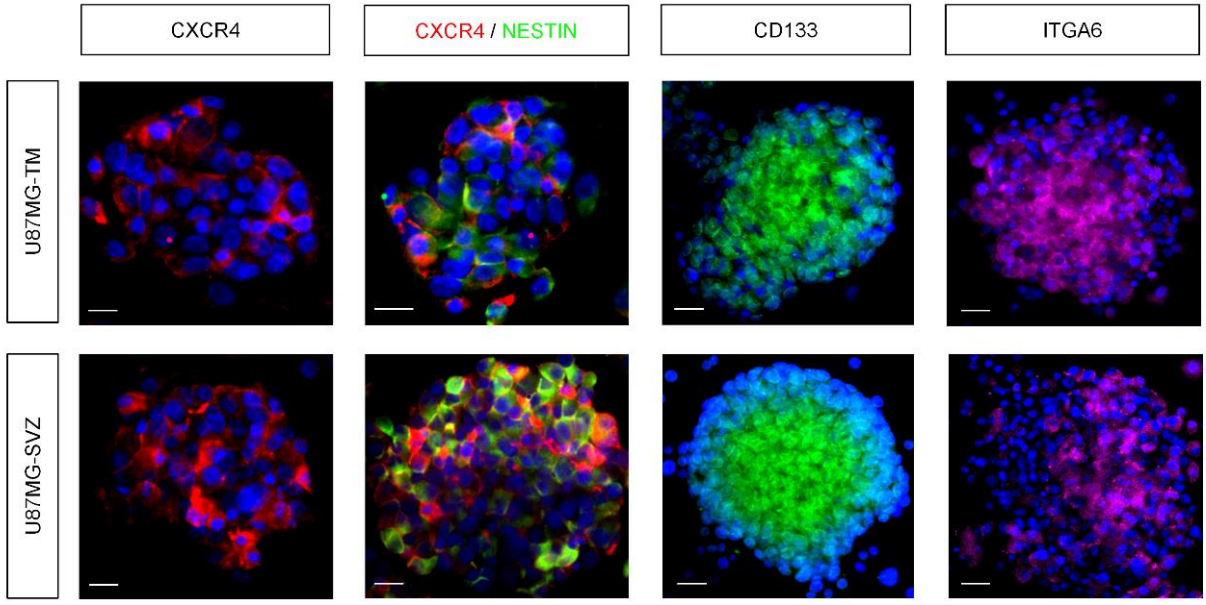


FIGURE 20. Characterization and expression of CXCR4 on GBM spheres isolated from the tumor mass and the SVZ. U87MG-TM and U87MG-SVZ gliomaspheres specifically expressed CXCR4 (red) as well as GSC markers such as CD133 (green), Nestin (green) and Integrin α6 (ITGA6 - pink). Nuclei are counterstained with DAPI (blue). Scale bars = 15 µm.

f. AMD3100 Disrupts the Chemotactic Effect of the SVZ-conditioned Medium

To investigate whether human GBM cells home to the SVZ through secreted factors in a direct manner, we first developed an *in vitro* modified Boyden chamber assay. Human GBM cells were suspended in serum-free media and seeded on top of a porous membrane which separates them from a bottom chamber filled with test or control media. The cells then move into the bottom part of the chamber in response to the chemotactic agent of interest. We first tested the ability of U87MG cells to migrate in response to various concentrations of CXCL12 and serial dilutions of SVZ-CM (conditioned for 60 hours). Interestingly, U87MG cells displayed a dose-dependent migration behavior in response to both stimulations (Figure 21A-B).

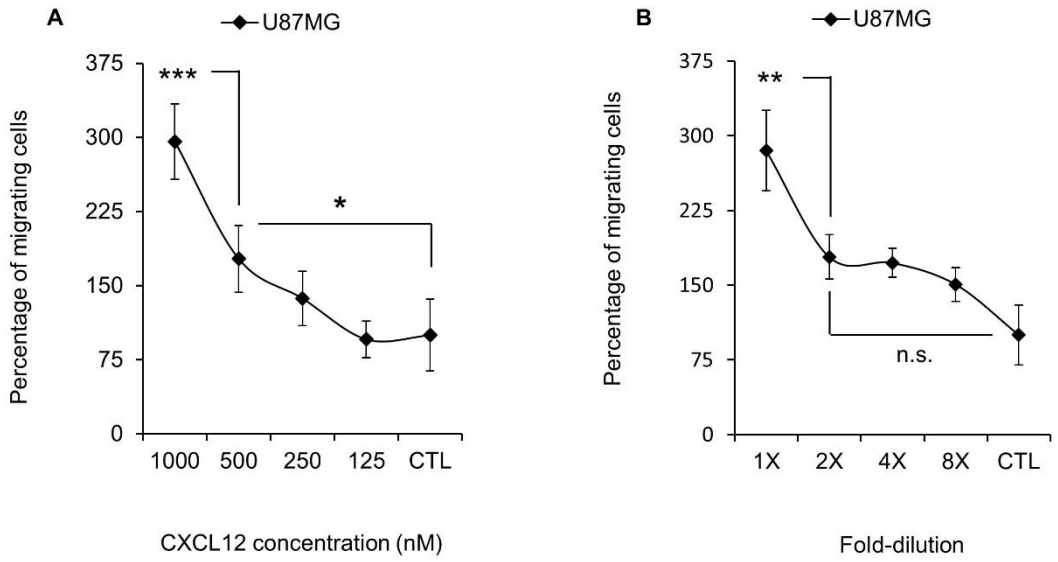


FIGURE 21. *In vitro* migration of GBM cells in response to recombinant CXCL12 and subventricular zone conditioned medium (SVZ-CM). A) Recombinant CXCL12 triggers migration of human U87MG cells. B) SVZ-CM also triggers migration of human U87MG cells. * p<0.05, ** p<0.01, *** p<0.001

To confirm the U87MG tropism toward a gradient of CXCL12 and SVZ-CM, we seeded U87MG cells on chemotaxis µ-slides. We recorded and analyzed the U87MG cell tracks over a period of 20 hours in response to both stimulations using time-course analyses. The overall distribution of migration was evaluated and revealed significant clusters of migration angles in response to the CXCL12 gradient ($p < 0.001$) (Figure 22A) and the SVZ-CM gradient ($p = 0.0006$) (Figure 22B).

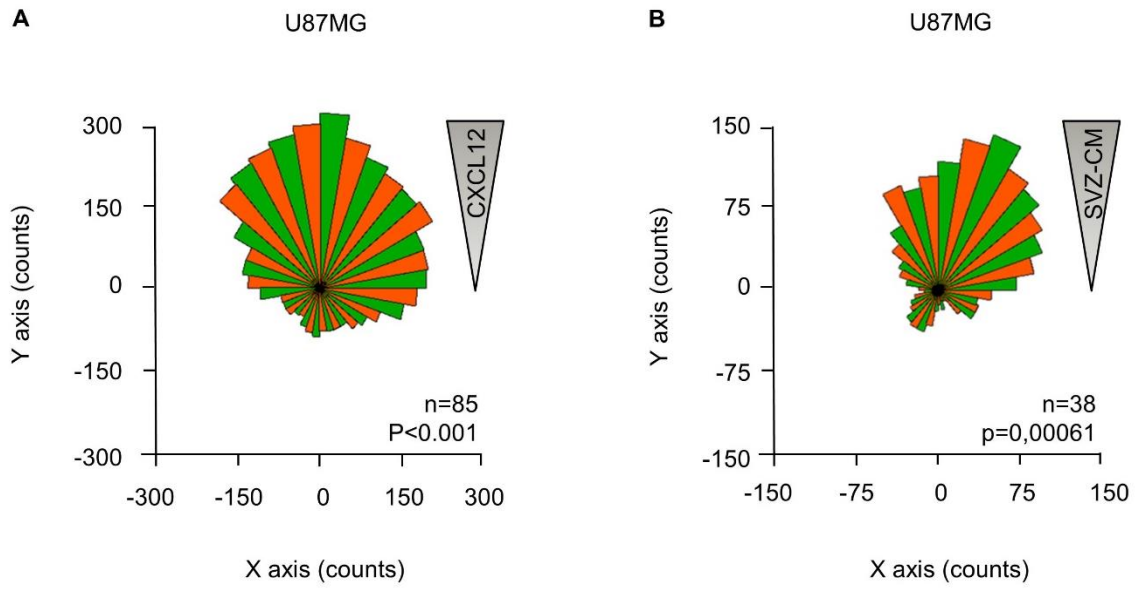


FIGURE 22. *In vitro* chemotaxis of GBM cells in response to recombinant CXCL12 and SVZ-CM. A-B) Both recombinant CXCL12 and SVZ-CM trigger migration and chemotaxis of human U87MG cells. * p<0.05, ** p<0.01, *** p<0.001

We then evaluated the impact of AMD3100 on the U87MG cells migration using the *in vitro* modified Boyden chamber assay. The CXCL12/CXCR4 signaling can indeed be blocked using AMD3100 [359], a bicyclam noncompetitive antagonist of CXCR4 [360]. Adding AMD3100 (25nM) to the SVZ-CM was followed by a significant reduction (-28.2%) of the number of U87MG cells (p=0.005) migrating through the filter. AMD3100 also inhibited the migration abilities of GBM2 primary cells in response to the SVZ-CM (-38.6% - p=0.001 - Figure 23A).

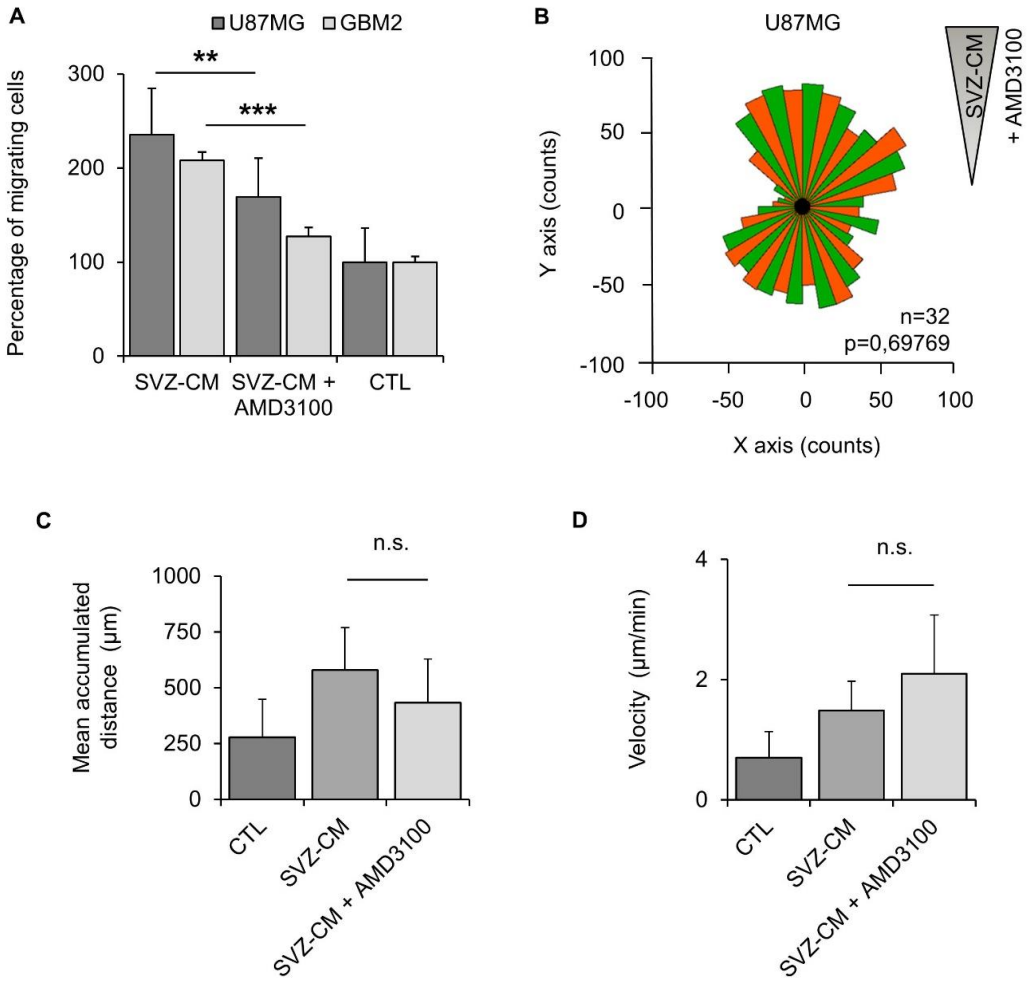


FIGURE 23. AMD3100 interferes with the *in vitro* migration abilities of GBM cells in response to the SVZ-CM. A) The migration of U87MG cells and GBM2 primary cells in response to the SVZ-CM was significantly reduced using AMD3100, a specific CXCR4 antagonist. B) AMD3100 disrupted chemotaxis of U87MG cells in response to the SVZ-CM. C-D) AMD3100 did not impact on parameters such as the mean accumulated distance (µm) and velocity (µm/min) of U87MG cells.

The impact of AMD3100 was further confirmed on U87MG cells using cell track recordings and time-lapse analyses. AMD3100 clearly disrupted the U87MG distribution of migration angles in response to the SVZ-CM gradient (p=0.697 - Figure 23B). However, AMD3100 did not impact on the mean accumulated distance or velocity of U87MG cells, suggesting the role of other chemokines in the *in vitro* U87MG migration behavior in response to the SVZ-CM (Figure 23C-D).

We finally compared how U87MG cells, U87MG-TM cells and U87MG-SVZ cells behave in response to recombinant CXCL12, SVZ-CM and SVZ-CM supplemented with AMD3100 (Figure 24). Surprisingly, U87MG-SVZ cells displayed superior migration abilities in response to recombinant CXCL12 than U87MG cells or U87MG-TM cells (p<0.001). Similarly, U87MG-SVZ cells were also significantly more attracted by the SVZ-CM in comparison to the other cell populations (p<0.001).

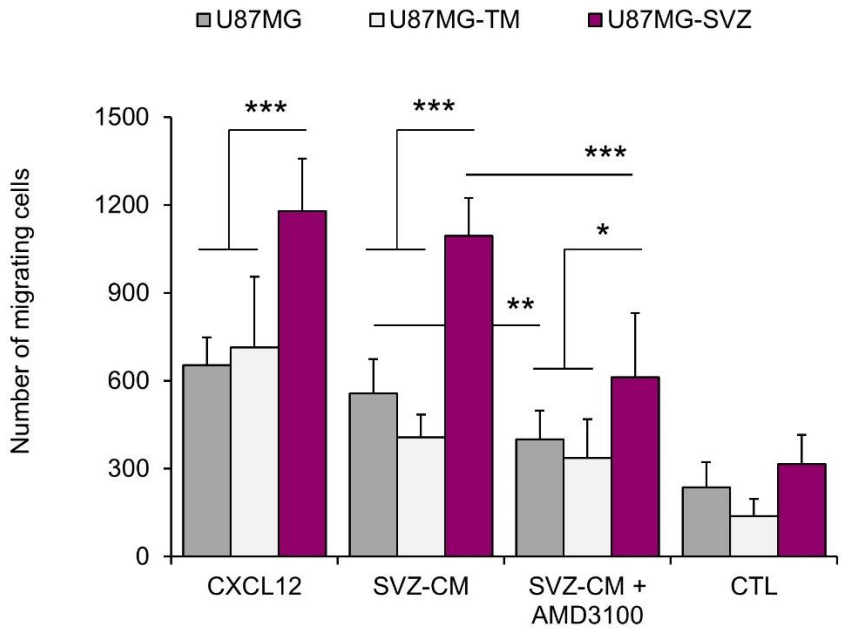


FIGURE 24. *In vitro* migration abilities of GBM cells isolated from the tumor mass and the subventricular zone. U87MG-SVZ cells showed stronger migration abilities in response to recombinant CXCL12 and SVZ-CM compared to U87MG-TM cells or U87MG cells. Moreover, AMD3100 more specifically inhibited the migration of U87MG-SVZ cells in response to SVZ-CM compared to U87MG-TM cells. * p<0.05, ** p<0.01, *** p<0.001

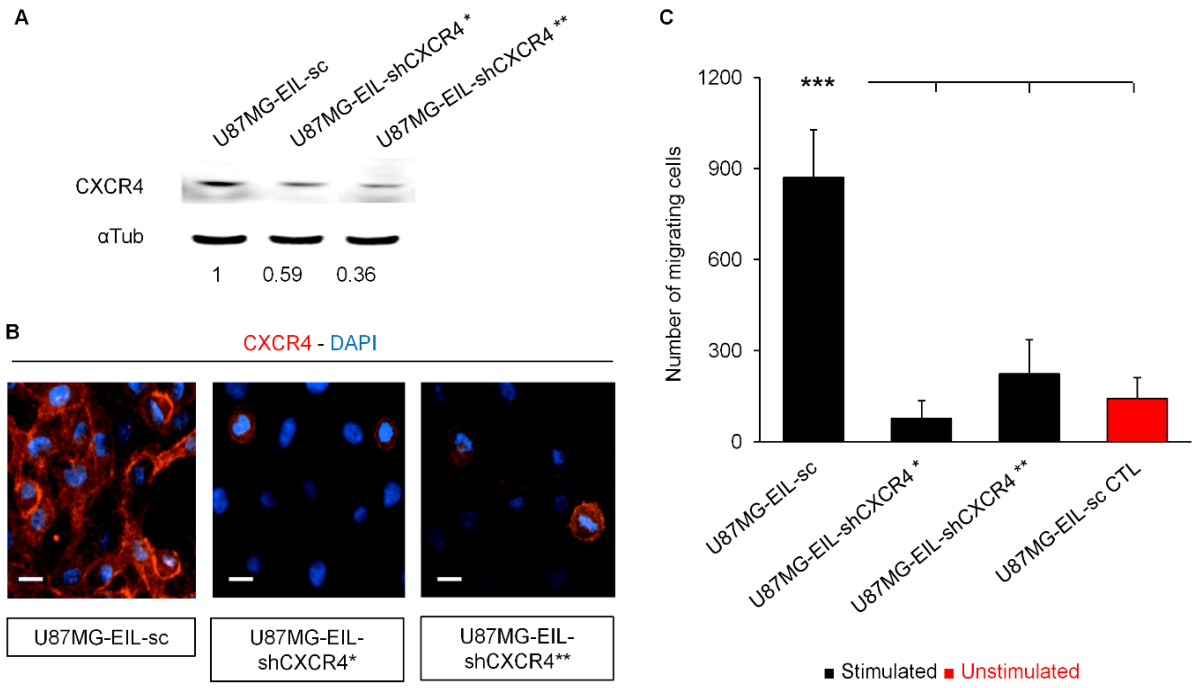
Interestingly, the *in vitro* inhibition of migration using AMD3100 was more important on U87MG-SVZ cells compared to their counterparts isolated from the tumor mass. AMD3100 (25nM) indeed allowed to decrease the U87MG-TM and U87MG-SVZ cells migration up to

17.7% (not statistically significant) and 44.2% respectively ($p<0.001$), suggesting a stronger impact of the drug on U87MG cells isolated from the SVZ. Since these cells have been characterized as GSC [208], forthcoming analyses on the molecular signaling triggered by both CXCL12 and the SVZ-CM in U87MG-SVZ cells should help to better define molecular mechanisms potentially leading to GBM relapses.

g. Depletion of CXCR4 Inhibits the Invasion of the SVZ

We generated stable CXCR4-invalidated U87MG cells. Those cells were in the same time engineered to express a fusion protein of firefly luciferase and eGFP (U87MG-EIL-shCXCR4* and U87MG-EIL-shCXCR4**). Control cells for this experiment were built through the expression of a scrambled shRNA (U87MG-EIL-sc).

We first confirmed the depletion of CXCR4 by Western-Blot analyses and validated those data by immunocytofluorescence (Figure 25A-B). The expression of CXCR4 was down-regulated by 41% and 64% respectively in the U87MG-EIL-shCXCR4* and U87MG-EIL-shCXCR4** cell populations (Figure 25A). We tested the functionality of those two CXCR4-depleted cell populations using the modified Boyden chamber assay to evaluate chemotaxis in response to recombinant CXCL12 (1000nM). The migration of both CXCR4-depleted U87MG populations was significantly decreased no matter the remaining level of CXCR4 (Figure 25C).



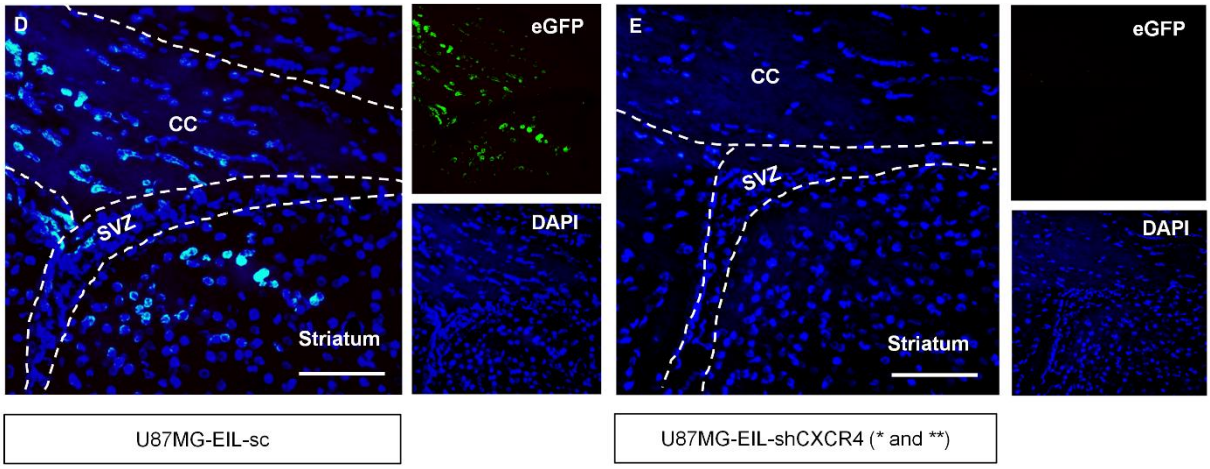


FIGURE 25. CXCR4-depleted U87MG cells do not invade the SVZ environment. A-B) Western-Blot and cytological analyses of the CXCR4 expression in U87MG-eGFP-Ires-Luc cells infected with a lentivirus encoding either a scrambled shRNA (U87MG-EIL-sc) or two short hairpins RNA directed against CXCR4 (U87MG-EIL-shCXCR4* and U87MG-EIL-shCXCR4**). CXCR4 expression was declined up to 41% in the U87MG-EIL-shCXCR4* cells and up to 64% in the U87MG-EIL-shCXCR4** cells. C) Invalidation of CXCR4 also decreased the *in vitro* migration abilities of U87MG cells in response to recombinant CXCL12 (1000nM). D-E) Mice were implanted either with control U87MG cells (U87MG-EIL-sc, n = 5 mice) or with CXCR4-depleted U87MG cells (U87MG-EIL-shCXCR4* and U87MG-EIL-shCXCR4**, n = 5 mice per group). As expected, human U87MG cells invaded the CC and the SVZ of mice grafted with U87MG-EIL-sc. No eGFP signal could be detected in the CC or the SVZ of both shRNA conditions. Scale bars = 10 µm and 100 µm respectively for B and D-E. *** $p<0.001$

We finally grafted the two populations of CXCR4-depleted U87MG cells and U87MG-control cells in the striatum of immunocompromised mice. As expected, eGFP-positive U87MG cells were found in the CC and the SVZ region of control mice after four weeks (Figure 25D). Surprisingly, this invasion phenotype was totally hampered in the CXCR4-KO conditions where no eGFP signal could be detected in the CC and SVZ environment (Figure 25E). In 2012, Rao and colleagues demonstrated the CXCR4 depletion in U87MG cells to significantly suppress intracranial growth, suggesting the key role undertaken by CXCR4 in GBM development [361]. We have been able to confirm Rao's findings since tumors arising from CXCR4-depleted U87MG cells were on average 90% smaller compared to control (data not shown). This observation nevertheless shed the light on the limitations of our CXCR4 *in vivo* knock-down model. Indeed, could the lack of eGFP signal in the SVZ of mice grafted with CXCR4-depleted U87MG cells be incriminated to the inhibition of CXCR4, therefore directly impacting on migration, or just be related to a lack of tumor growth? To answer this question, two alternatives have been put forward: (1) to work with CXCR4-inducible shRNA's or (2) to

pharmacologically inhibit the CXCR4 signaling. From those two options we chose the second one.

b. AMD3100 Specifically Interferes with the *in vivo* Invasion of the SVZ

We injected luciferase expressing U87MG cells in the right striatum of immunocompromised nude mice. Animals bearing xenografts were separated into two homogeneous groups at day 25 post-implantation according to *in vivo* bioluminescence data. Indeed, U87MG cells expressing luciferase allow to monitor non-invasive imaging of tumor-associated bioluminescence and to quantify tumor growth over time [362]. Half of the cohort was treated twice a day with intra-peritoneal injections of AMD3100 at a concentration of 1.25 mg/kg whereas the other group was treated with PBS (control). The treatment started at day 25 for two reasons. The first one was to minimize the already-known impact of the compound on tumor growth. Systemic administration of AMD3100 has indeed been reported to inhibit the growth of intracranial GBM xenografts by increasing apoptosis and decreasing tumor cell proliferation [363]. Second, this pharmacological procedure also allowed to treat the animals during the exact window of time when human U87MG cells start to invade the SVZ [208].

Growth curves established from serial measurements of bioluminescence revealed a significant anti-tumor effect of AMD3100 in U87MG xenografted mice ($p < 0.01$ - Figure 26A).

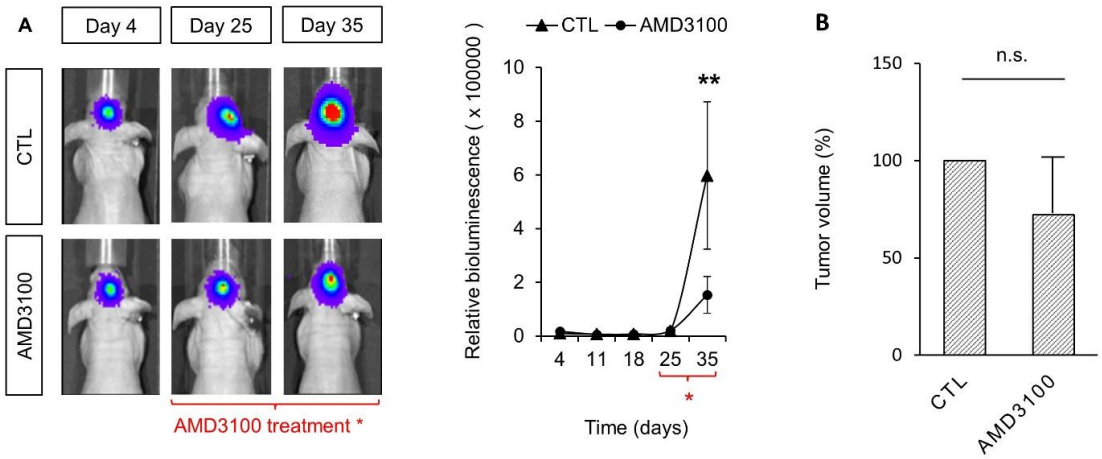
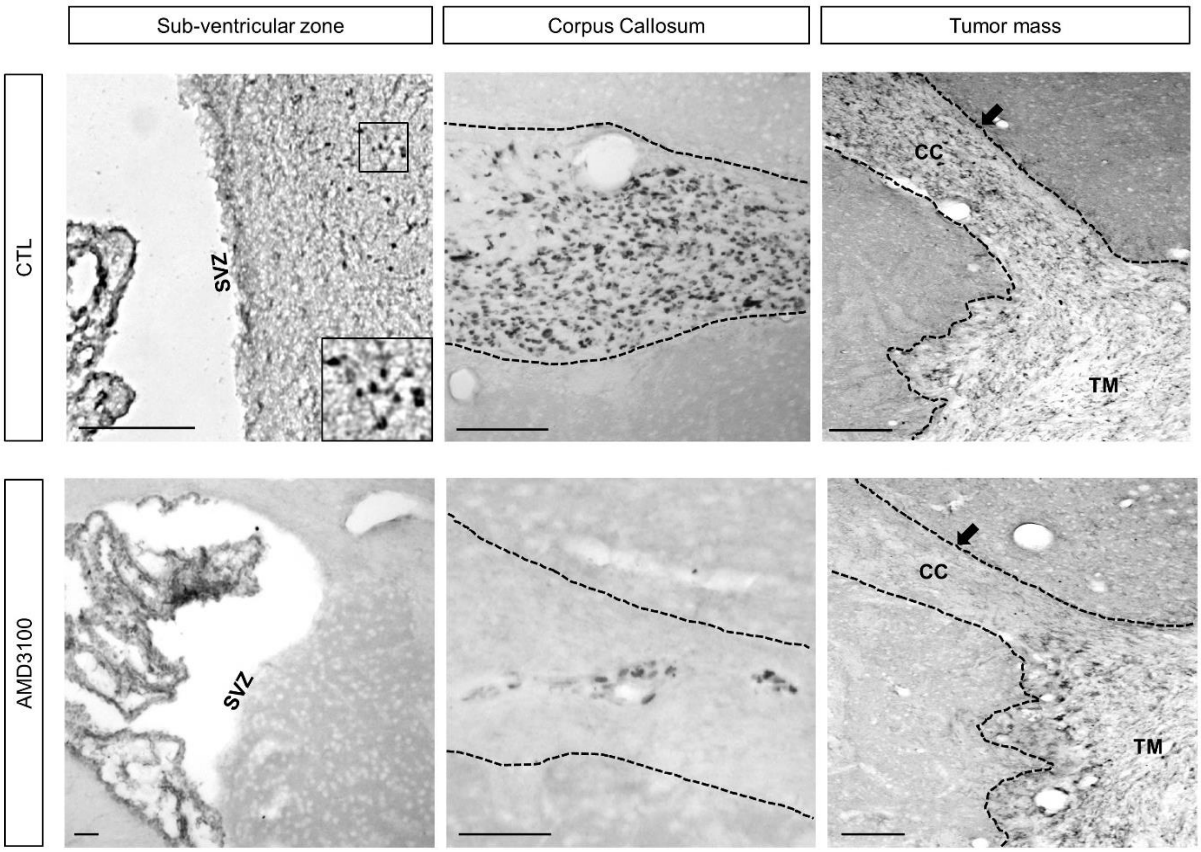


FIGURE 26. AMD3100 displays an anti-tumor impact in U87MG xenografted mice. A) AMD3100-treated animals showed significantly smaller amounts of relative bioluminescence compared to control animals ($p<0.01$) at the end of the treatment period (day35). B) Tumor volumes were on average 28% smaller in AMD3100-treated animals but this difference did not reach any statistically significant threshold. ** $p<0.01$

This observation was consistent with the fact that tumor volumes were on average 28% smaller in the AMD3100-treated group after histological examination. This reduction of tumor volume was nevertheless not statistically significant probably due to high variability between animals bearing xenografts ($p = 0.65$) (Figure 26B).

Regarding the invasion abilities of U87MG cells, the number of GBM cells in the CC of AMD3100-treated animals was found significantly reduced compared to control animals ($p<0.001$ - Figure 27). More importantly, we have not been able to detect the presence of any U87MG cells in the SVZ environment of AMD3100-treated animals ($p<0.01$ – Figure 27), confirming in this way the data we previously collected from the CXCR4 knock-down experiments. In control mice, U87MG cells migrated along the CC and finally invaded the SVZ environment as expected (Figure 27). As a last control, we made sure that AMD3100 did not induce U87MG cells death in xenografted animals after treatment (data not presented). In this way, we demonstrated the invasion of the SVZ by U87MG-initiating cells to be CXCR4 dependent and definitely not a consequence of a loss of cell viability.



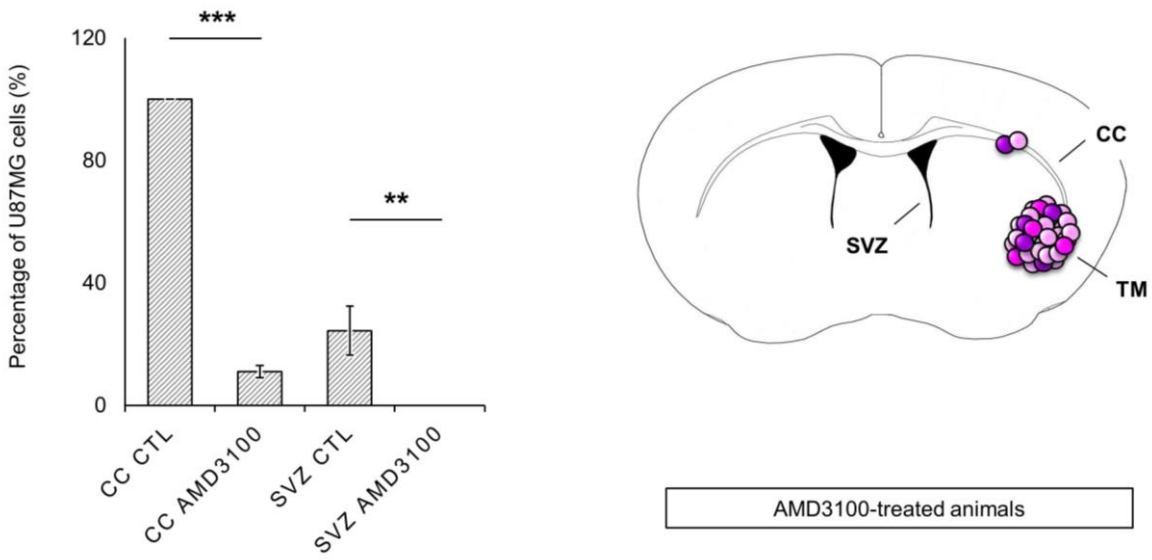


FIGURE 27. AMD3100 inhibits the U87MG invasion of the SVZ. A) AMD3100-treated animals showed significantly smaller amounts of relative bioluminescence compared to the control group ($p<0.01$) at the end of the treatment period. B - C) Only a few U87MG cells were found in the CC of AMD3100 treated-mice whereas U87MG cells fully invaded the CC of PBS-treated mice (control). U87MG cells could not be found in the SVZ area in AMD3100-treated animals whereas U87MG cells from the control group invaded the SVZ as expected. U87MG cells were labeled with a specific anti-human nuclei antibody. Scale bar = 100µm. Graphs are mean values ± SEM. ** $p<0.01$, *** $p<0.001$

A similar experimental procedure was conducted with eGFP-positive GB138 primary cells. Because of the slow growth rate of GB138 cells, the AMD3100 treatment started at week 10 post-implantation. Half of the cohort was treated twice a day for 10 days with intra-peritoneal injections of AMD3100 at a concentration of 1.25 mg/kg whereas the other group was treated with PBS (control). Similarly to U87MG cells, we observed a significant decrease of the CC invasion in AMD3100-treated animals compared to controls ($p<0.001$). Conversely to U87MG cells, GB138 primary cells still invaded the SVZ environment in AMD3100-treated animals but the invasion extent was significantly reduced compared to PBS-treated animals ($p<0.01$ - Figure 28). This observation further confirms the benefic impact of AMD3100 in reducing the GSC specific invasion of the SVZ.

From a clinical point of view, the safety of AMD3100 has been evaluated in clinical trials and raised great hope for new potential clinical implications [364]. This FDA approved drug has successfully been tested in patients with non-Hodgkin's lymphoma and multiple myeloma in order to support optimal stem cell mobilization for autologous stem cell transplantation [365]. AMD3100, in combination with Avastin (an anti-VEGF antibody), is

now under intense investigations in a phase I clinical trial and carries hope to prevent the growth of recurrent high-grade gliomas (NCT01339039).

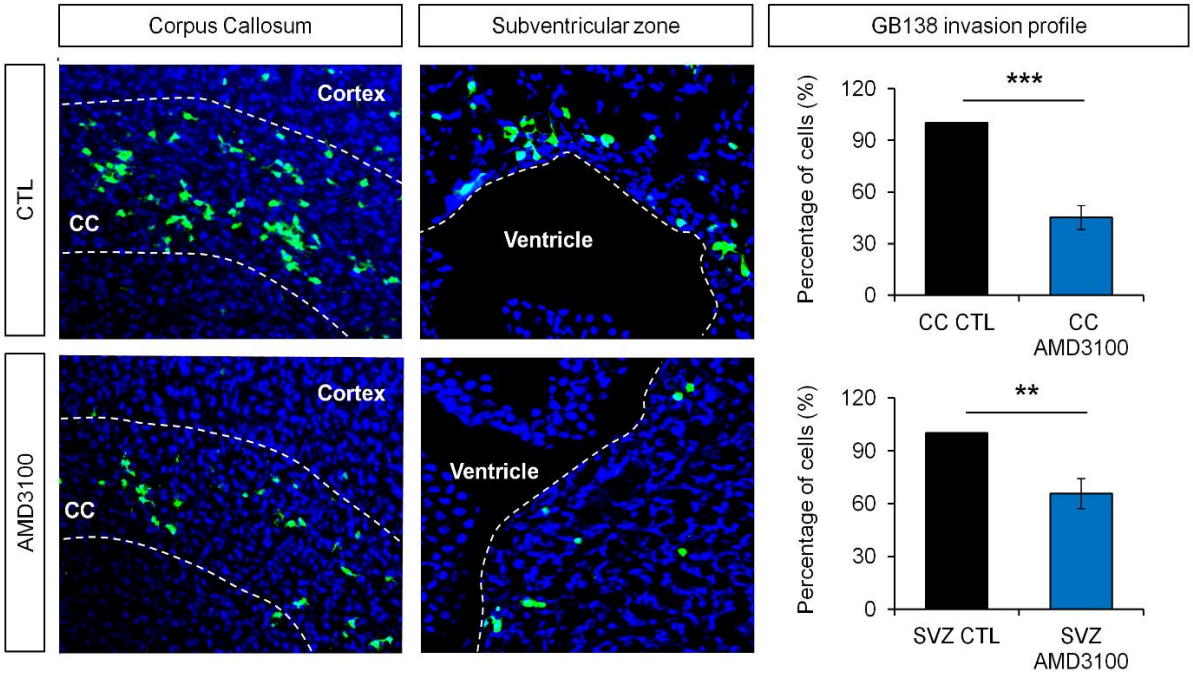


FIGURE 28. AMD3100 reduces the GB138 primary cells invasion of the SVZ. A) AMD3100-treated animals showed significantly smaller amounts of GB138 primary cells in the CC compared to PBS-treated mice (control). The same observation was made regarding the SVZ. GB138 cells were found in the SVZ of AMD3100-treated animals but the invasion extent was significantly reduced compared to control animals. GB138 cells were labeled with a specific anti-eGFP antibody. ** $p<0.01$, *** $p<0.001$

PART 2: The "Why" Question

a. The Context

GBM are made of a heterogeneous bunch of cells which do not only catch external signals from the local environment but also respond to the latter in order to take control. It is commonly accepted that tumor-associated parenchymal cells such as vascular cells, microglia, peripheral immune cells and NPC directly interact with GBM cells within the environment and play a crucial role in regulating the course of the disease. Therefore, targeting the win-win relationship between malignant brain tumors and their micro-environment seems like a smart strategy to exhaust the beast.

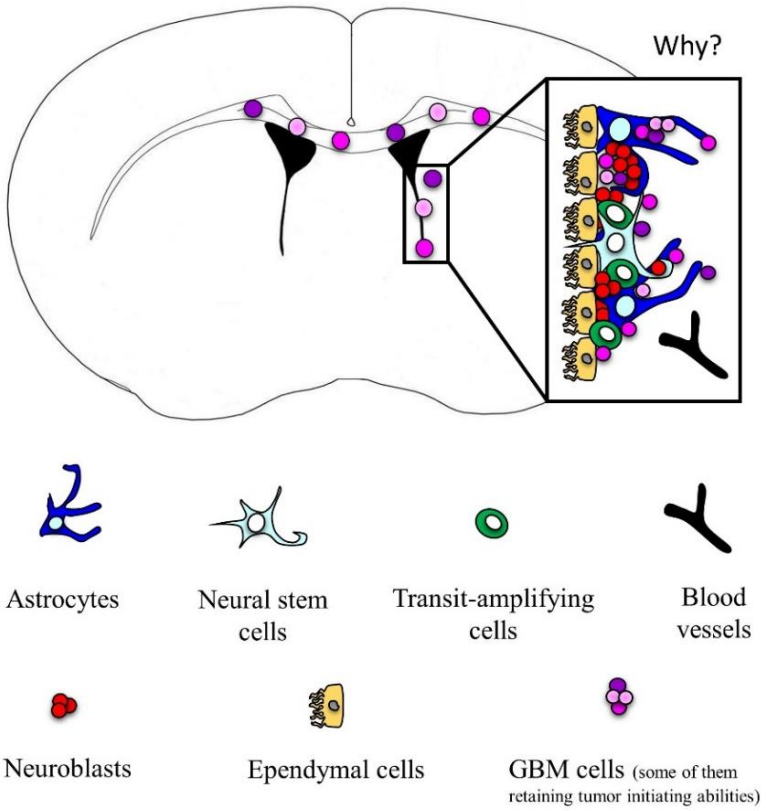


FIGURE 29. The SVZ as a supportive niche for GSC? Within the SVZ environment, GSC have the ability to interact with a variety of different cell types including astrocytes, NSC, ependymal cells and EC. This environment is suggested to sustain GSC critical properties and may therefore enter the line of sight for future therapeutic strategies.

In this context, we have just demonstrated that GBM cells enriched in tumor-initiating capacities (GSC) preferentially home to the adult SVZ after striatal implantation. As a brief reminder, the SVZ is one of the two mitotically active cell layers which retains the ability to produce neurons and glia throughout life, functioning as a source of stem cells and progenitors in adults [102, 103]. The SVZ is described as a supportive niche which maintains critical NSC properties [353]. Soluble factors released from the SVZ vascular plexus have notably been shown to promote NSC self-renewal and inhibit their differentiation, suggesting a critical vascular regulation within the neurogenic niche [366, 367]. This "seed-and-soil" relationship has been adapted to CSC research as GSC also rely on interactions with a vascular niche to maintain their stem-like properties and their ability to drive tumor growth (Figure 29) [154, 368].

As a matter of fact, GSC preferentially associate with endothelial cells which, in turn, supply tumor cells with secreted factors, maintaining these GSC in a self-renewing and undifferentiated state [154]. Interestingly, we made a similar observation as GB138 primary cells closely associated with the vasculature in the SVZ nearby environment (Figure 30A).

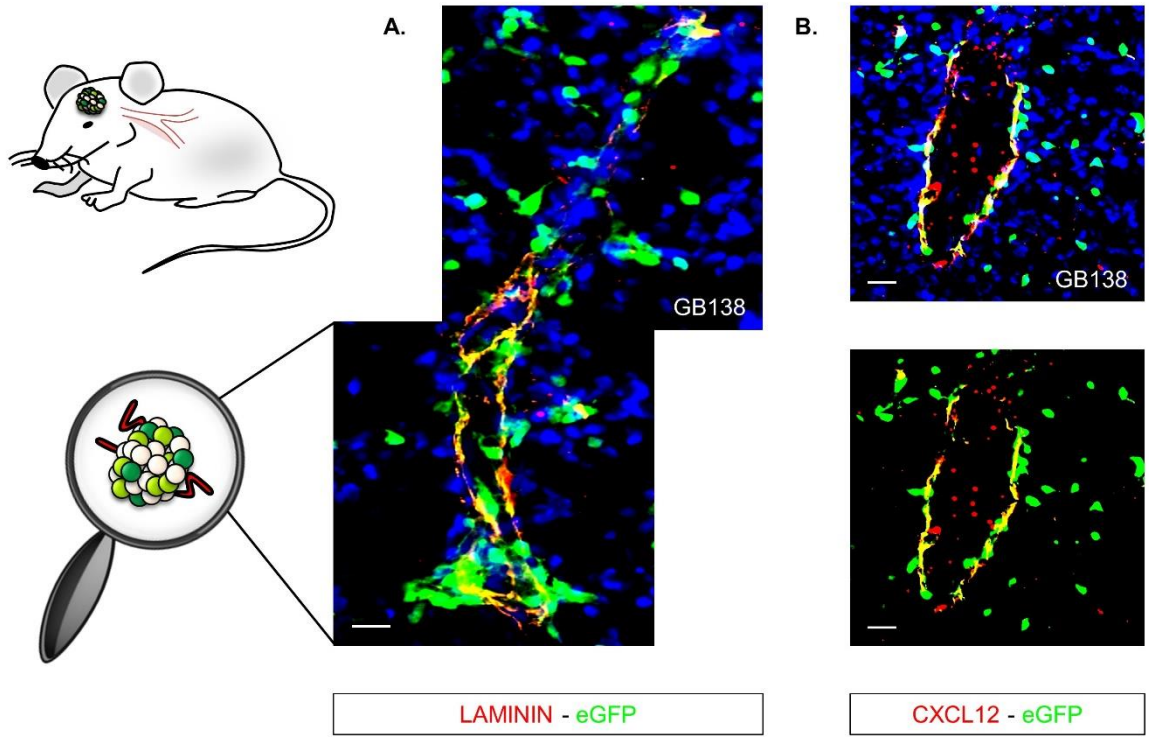


FIGURE 30. Human GB138 primary cells preferentially associate with blood vessels *in vivo*. A) GB138 primary cells were found close to murine blood vessels in the striatum and the SVZ, notably providing nutrients and lines of migration within

the brain. B) Murine EC actively release CXCL12 (red) in the SVZ environment. Interestingly, this secretion of CXCL12 was further characterized as the missing piece of the puzzle in the interaction between EC and GBM cells [361]. GB138 primary cells were specifically engineered to express eGFP whereas murine blood vessels were detected using an anti-laminin antibody (red). Cell nuclei were counterstained with DAPI (blue). Scale bars = 30 µm for A and 40 µm for B.

Protein ligands found within the vascular niche have been shown to regulate both stem cell self-renewal and angiogenesis, putting forward the idea that these two processes could be related. In the facts, Rao and colleagues described EC-derived CXCL12 to create a specialized niche within the perivascular space which mediates trophic interactions between endothelial and tumor cells [361] We confirmed this observation and showed that GBM cells closely associated with EC in regions where CXCL12 is present (Figure 30B). Interestingly, the inhibition of the CXCL12/CXCR4 pathway has later been reported to disrupt the viability of human GSC by affecting their self-renewal abilities [346, 347]. Since blood vessels are sources of CXCL12 within the SVZ stem cell niche [356, 369], it is tempting to suggest the molecular crosstalks between GSC and the SVZ vascular network to play a key role in the biology of malignant brain tumors. Further down the road, Piccirillo and co-workers not only demonstrated the existence of GSC within the human SVZ but also showed that GSC are specifically resistant to chemotherapy when located in the SVZ stem cell niche. This observation definitely enhances the need for a better characterization/understanding of the communication lines between the seed (GSC) and the soil (SVZ).

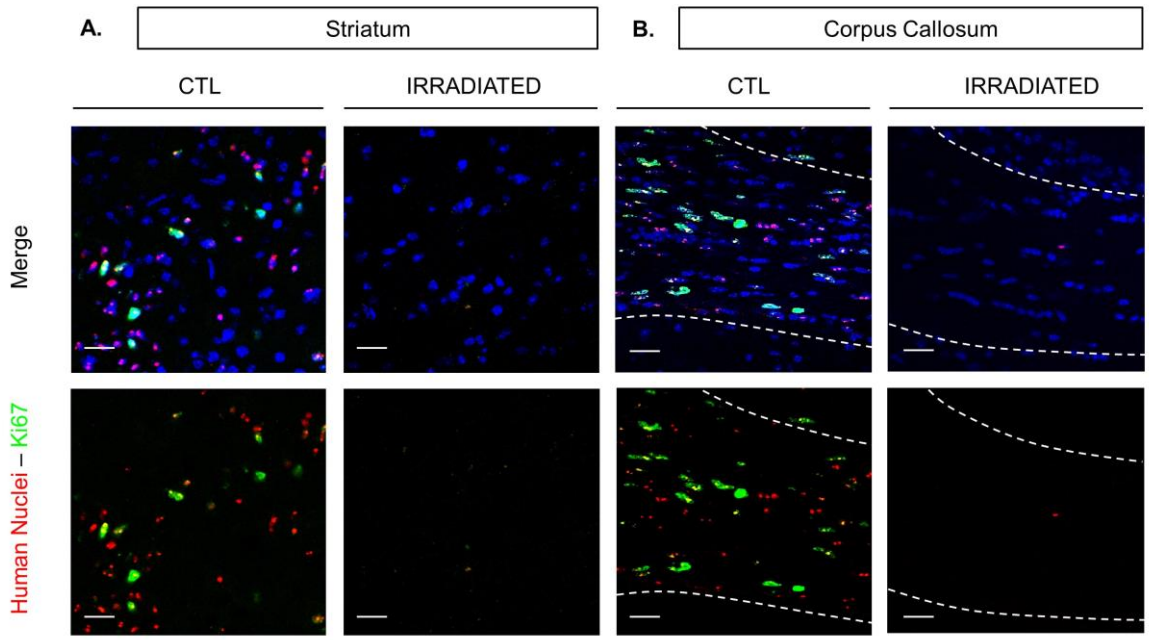
As a consequence, we decided to question the role of the SVZ-secreted CXCL12 in GSC extrinsic resistance to treatment and more specifically to radiotherapy. Chemokine receptor CXCR4 has indeed been recently proposed as a potential biomarker for radio-resistant CSC [370]. In the same line, the CXCL12/CXCR4 axis is also suspected to tightly control the epithelial to mesenchymal transition (EMT), a process potentially involved in GBM resistance to radiation [371, 372]. The implication of EMT in GBM resistance to radiotherapy is further described in details (see Discussion, section 3b).

b. GBM Resistance to Irradiation in the Adult SVZ

Using our *in vivo* model of brain cancer invasion, we grafted GB138 primary cells in the striatum of immunocompromised mice. Ten weeks after the implantation, 8 mice were submitted to daily doses of radiation (6Gy) for 5 days. By the end of the 11th week, animals from both control and irradiated groups were sacrificed. The impact of irradiation was assessed

by histological techniques using an anti-human nuclei antibody to specifically detect human GBM cells in brains. As expected, control animals displayed massive infiltration of the CC and SVZ (Figure 31B and C). Surprisingly, a very limited amount of cells was found in the CC of irradiated mice (0.68% of the initial population) whereas no tumor cells could be spotted in the vicinity of the injection site after histological examination of irradiated mice (Figure 31A and B). Proportionally to the number of GB138 primary cells which invaded the SVZ of control mice, we detected 12% of remaining GB138 primary cells in the SVZ of irradiated mice (Figure 31C).

These results strongly suggest the SVZ microenvironment to openhandedly host GSC and to protect a bunch of these cells from irradiation, therefore playing a key role in GBM resistance to treatment and corroborating with late periventricular patterns of recurrence usually observed in patients. A very recent retrospective study indeed evaluated the influence of tumor location, with respect to the SVZ, on recurrence behavior, PFS and OS after radiotherapy. GBM with SVZ infiltration specifically showed PFS and OS decreased rates and presented higher risks of multifocal/distant progression [214]. These findings could potentially be explained by the persistence of GSC within the SVZ environment after radiotherapy.



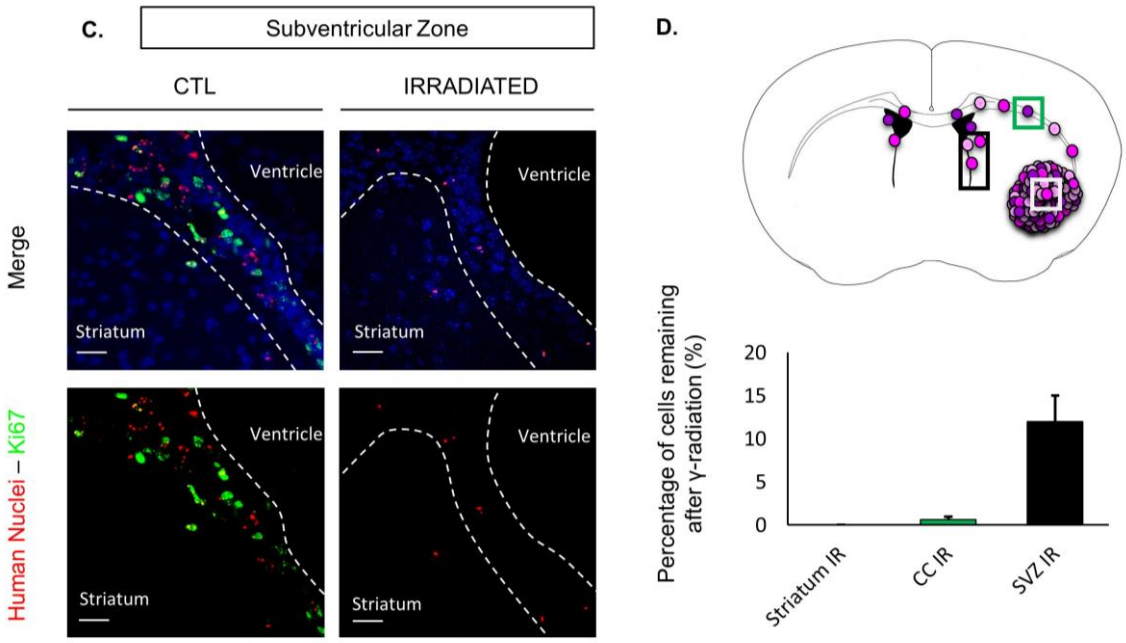


FIGURE 31. A substantial fraction of GB138 primary cells remains in the SVZ after irradiation. A) GB138 primary cells vanish from the striatum after irradiation. B) A similar observation was made with regard to the CC. C and D) Surprisingly, 12% of the initial amount of GB138 primary cells located in the SVZ persisted in this environment after radiotherapy. These results underscore the eventual role of the SVZ environment in GBM resistance to radiotherapy. GB138 primary cells were detected using an anti-human nuclei antibody (red). Cell nuclei were counterstained with DAPI (blue). Caption shows where pictures were taken. Scale bars = 40 µm for A, B and C.

c. GBM Resistance to Radiation is Mediated by the SVZ-CM *in vitro*

In order to confirm our *in vivo* findings, we decided to test whether the soluble environment of the SVZ could play a role in GBM resistance to radiotherapy. To do so, we serum starved U87MG cells and GBM2/3 primary cells overnight. We then added a 7.5 fold dilution of SVZ-CM to these 3 cell types prior to γ-radiation. After an hour of incubation, GBM cells were irradiated (10Gy) and the γH2AX response, a marker of DNA damage response, was assessed one hour following the end of the irradiation protocol. Regardless the nature of the GBM populations we used, addition of SVZ-CM prior to irradiation significantly decreased the radio-sensitivity of all three GBM cell types compared to control medium (Figure 32A to C). Consistent with prior studies in human and mouse epithelial cells [373], radio-desensitization was correlated with a significant decrease of γH2AX-positive cells (Figure 32D). These data indicate that the SVZ-CM abrogates the response to DNA damage in GBM cells therefore decreasing radiosensitivity.

53BP1 is a DNA damage checkpoint protein that is recruited to DNA double strand breaks to mediate the damage response. It binds to the central domain of p53 and usually interacts with histone H2AX when phosphorylated on serine 139 (γH2AX). We therefore gaged the 53BP1 response following irradiation (10Gy) in the presence of SVZ-CM or control medium. 53BP1 colocalized with γH2AX at the break sites but no discrepancy was found in terms of 53BP1 expression regarding control versus SVZ-CM conditions (Figure 32D).

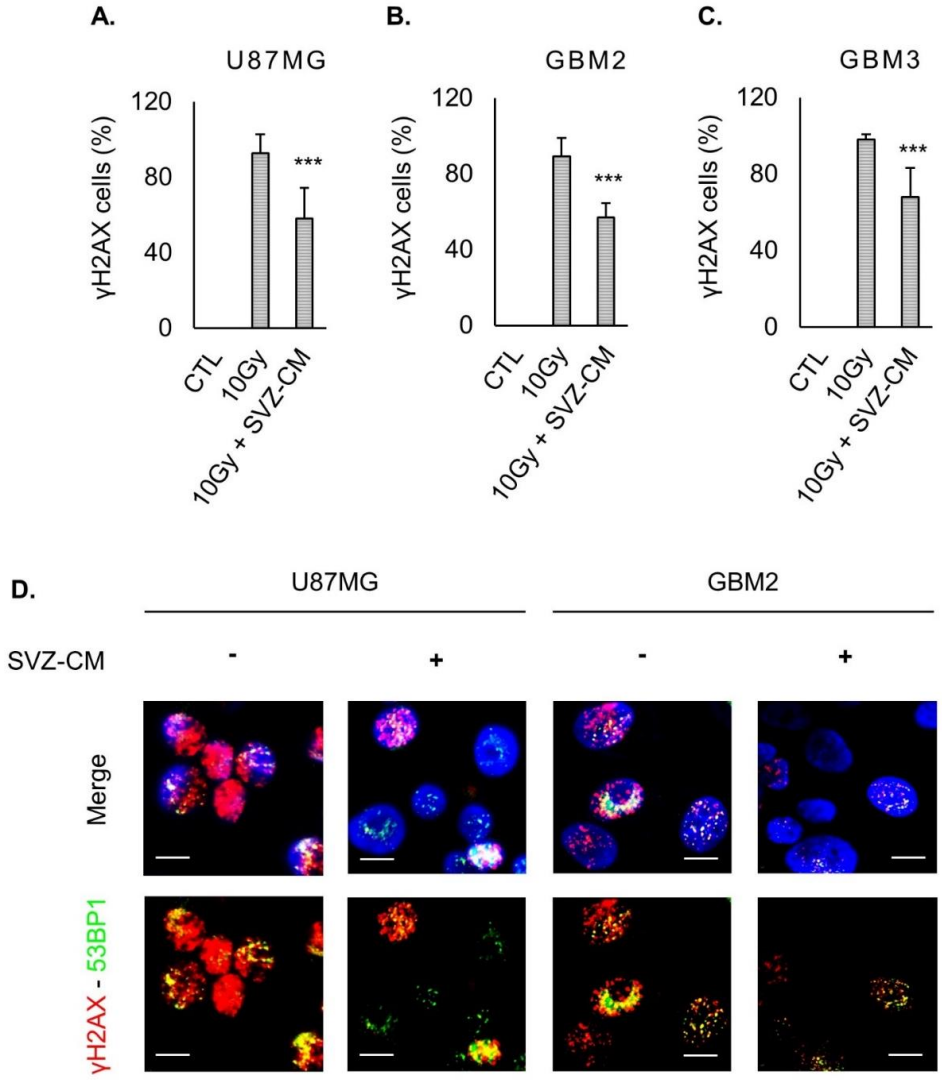


FIGURE 32. SVZ-CM mediates radio-resistance of GBM cells *in vitro*. A-C) Media were conditioned by SVZ whole-mounts for 60 hours and then added to GBM cells prior to irradiation. This significantly decreased the sensitivity of U87MG cells and GBM2/GBM3 primary cells to radiation by reducing the DNA damage response. D) The latter was specifically assessed by measuring the γH2AX (red) and 53BP1 (green) responses in U87MG cells as well as in GBM2 primary cells. Radio-desensitization was correlated with a significant decrease of γH2AX-positive cells. Cell nuclei were counterstained with DAPI (blue). Scale bars = 10 µm for D. *** $p<0.001$

We then checked whether the radio-protective impact of the SVZ-CM was to be incriminated to the SVZ itself or to other brain regions as well. We therefore irradiated human U87MG cells and GBM3 primary cells in the presence of a 7.5 fold dilution of SVZ, OB or cerebellum (CRBL) conditioned media. As previously observed, we demonstrated a significant decrease of γH2AX-positive cells in response irradiation and SVZ-CM in both U87MG cells and GBM3 primary cells. On the other hand, irradiation of U87MG cells and GBM3 primary cells in both OB- and CRBL-conditioned media did not impact on the γH2AX response (Figure 33A-B). Similar numbers of γH2AX-positive cells either supplemented with OB- or CRBL-conditioned media were found in comparison with a dose of 10Gy in control media. These data underlie that GBM resistance to irradiation is specifically mediated by the soluble environment of the SVZ and therefore not incriminated to random brain regions.

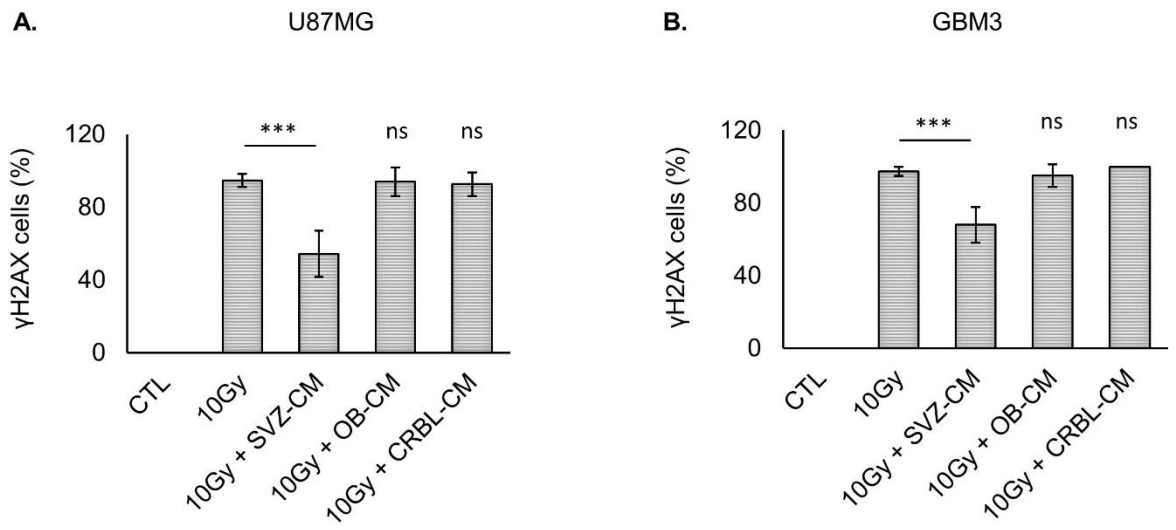


FIGURE 33. The radio-protective impact of the SVZ-CM is specific to the SVZ environment. A) Irradiation of U87MG cells supplemented with OB-conditioned media (OB-CM) or cerebellum-conditioned media (CRBL-CM) did not impact the DNA damage response as similar levels of γH2AX-positive cells were found in these conditions compared to a dose of 10Gy in control medium. B) A similar trend was observed in GBM3 primary cells. These data demonstrate that GBM resistance to irradiation is specific to the SVZ soluble environment. *** $p<0.001$

We finally compared the γH2AX response in U87MG-TM and U87MG-SVZ cells in the presence of SVZ-CM or control medium. These GBM sub-populations were pre-incubated for an hour in a 7.5 fold dilution of SVZ-CM or control medium and then received a dose of

10Gy. U87MG-TM cells displayed a γH2AX pattern of response similar to U87MG cells. Indeed, adding SVZ-CM prior to radiotherapy allowed to significantly decrease the number of γH2AX-positive cells in this sub-population (p=0.0002). U87MG-SVZ cells also displayed a significantly lesser extend of γH2AX-positive cells when supplemented with SVZ-CM prior to irradiation (p=0.012) (Figure 34A). Of note, SVZ-isolated U87MG cells have been previously characterized as a sub-population of cancer cells enriched in GSC properties [208]. In this line, GSC are known to be intrinsically radio-resistant as they preferentially activate DNA damage checkpoints and display stronger DNA repair capacities than differentiated GBM cells [124]. Interestingly, we here confirmed that U87MG-SVZ cells are innately more resistant to radiation than U87MG-TM cells as the basal level of γH2AX-positive cells in both GBM sub-populations was significantly lower in GBM cells isolated from the SVZ environment (p=0.009) (Figure 34A). A similar conclusion was drawn following clonogenic assays on GB138 primary cells isolated from the TM and from the SVZ. A significant higher number of colonies was indeed raised from GB138-SVZ cells compared to GB138-TM cells after a treatment of 4Gy (p<0.001 - Figure 34B). These data strongly support GBM cells located in the SVZ region to represent a sub-population of cancer cells with intrinsic radio-resistance abilities similar to what has been described in the literature so far and could therefore be a source of tumor recurrence after radiotherapy.

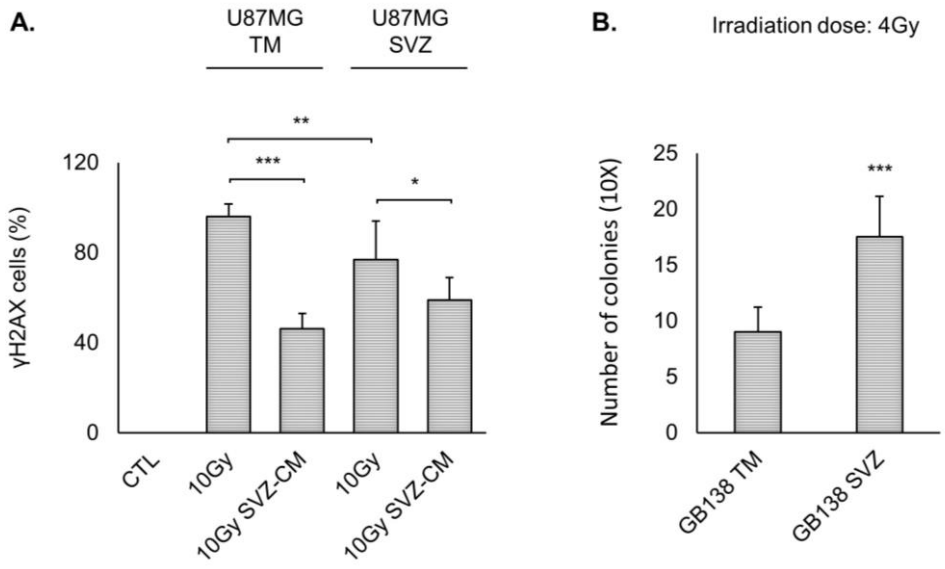
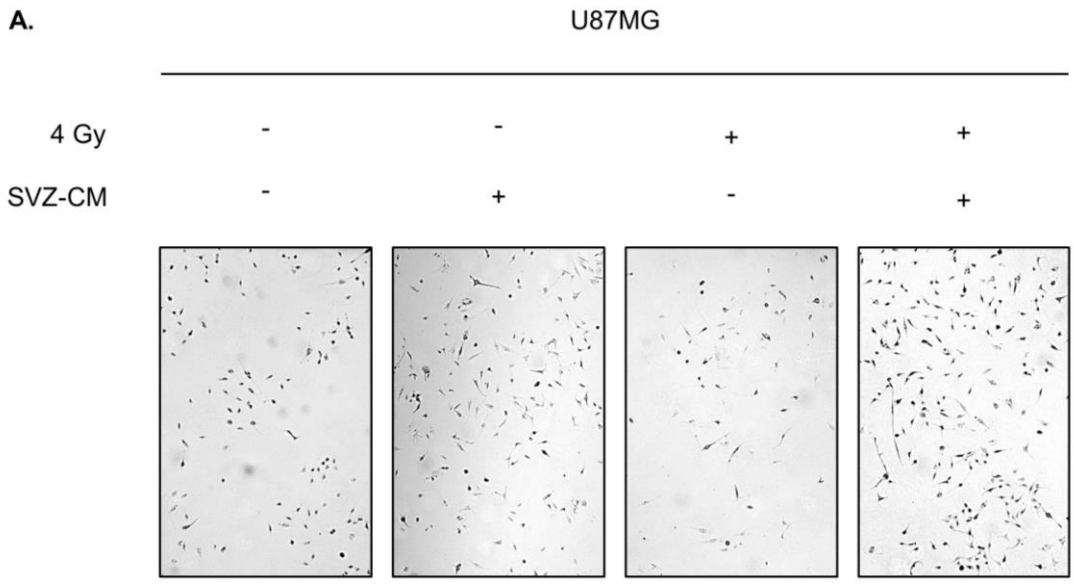


FIGURE 34. GBM cells nested in the SVZ are intrinsically resistant to radiotherapy. A) Irradiation of U87MG-TM and U87MG-SVZ subpopulations supplemented with SVZ-CM highlighted a significant decrease of the number of γH2AX-positive cells found in these subpopulations, further supporting the extrinsic role of the soluble environment of the SVZ in GBM radio-

resistance. Interestingly, U87MG-SVZ cells were intrinsically more resistant to radiation than their counterparts isolated from the tumor mass. B) Clonogenic assays on GB138 primary cells isolated from the TM and SVZ revealed that GB138-SVZ cells more efficiently give rise to colonies compared to GB138-TM cells after irradiation (4Gy). These findings strengthen even more the assertion that GBM cells located in the SVZ are enriched in GSC properties. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

d. The SVZ-CM Stimulates GBM Cell Proliferation and is Associated with Increased Survival after Radiotherapy.

We next investigated the SVZ-CM impact on U87MG cells and GBM1 primary cells growing abilities. We seeded 7.500 GBM cells in each well of a six well plate and serum-starved them for 12 hours. A 7.5 fold dilution of SVZ-CM was added to the different cellular monolayers prior to irradiation (4Gy). Six hours after irradiation, U87MG cells and GBM1 primary cells were finally placed in DMEM culture medium supplemented with fetal bovine serum (10%) and respectively cultured at an exponential growth phase for 7 and 9 days. Putting irradiation aside, we first noticed that media conditioned by SVZ whole-mounts had a significant positive impact on the proliferation of both U87MG cells and GBM1 primary cells. U87MG cells supplemented with SVZ-CM proliferated twice as much (2.03 times more) compared to U87MG cells in control medium ($p<0.001$). GBM1 primary cells proliferated on average 1.6 times more with a SVZ-CM supplementation compared to control medium ($p<0.001$) (Figure 35C). We then estimated the impact of a dose of 4Gy on the survival of both GBM populations. Irradiation of U87MG and GBM1 primary monolayers respectively led to a 43.5% and a 45.5% reduction of cell survival ($p<0.001$) (Figure 35B-C).



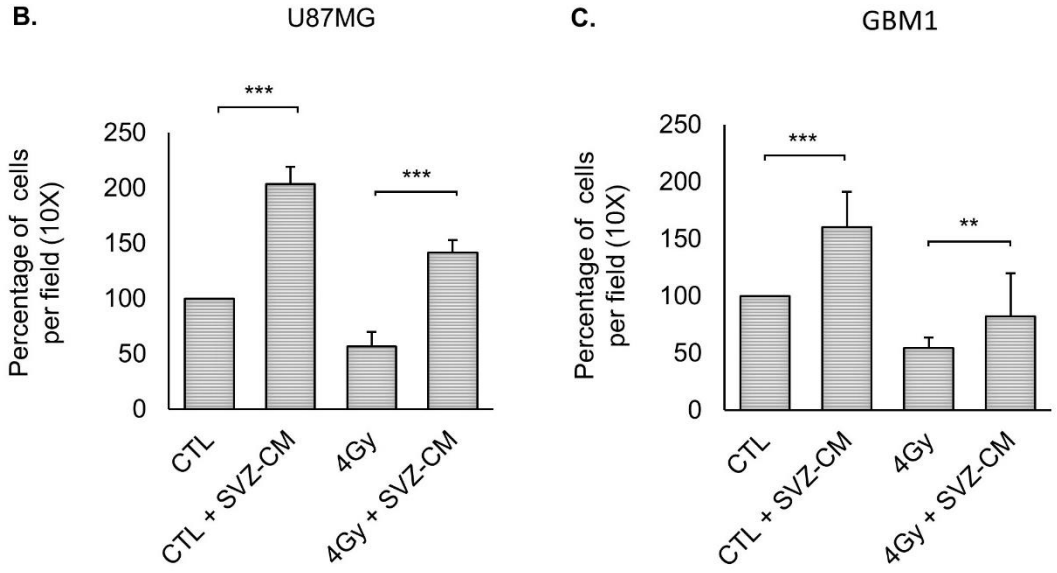


FIGURE 35. SVZ-CM stimulates GBM cell proliferation and increases survival after irradiation. A-B) Adding SVZ-CM to U87MG cells displayed a beneficial impact on their proliferation. The same conclusion was reached following a dose of 4Gy. C) The results collected with U87MG cells have been validated with GBM1 primary cells in the exact same experimental conditions. ** $p<0.01$, *** $p<0.001$

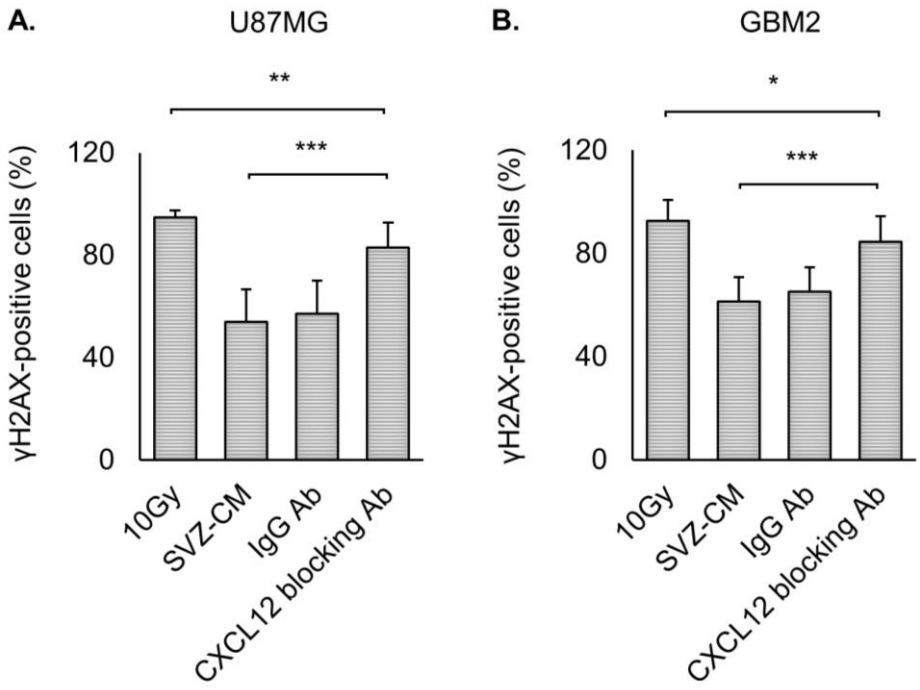
Surprisingly, the addition of SVZ-CM to U87MG cells prior to irradiation significantly propelled their proliferation since U87MG cells proliferated 2.5 times more compared to the 4Gy condition in control medium ($p<0.001$). Similarly, irradiated GBM1 primary cells proliferated 1.5 times more when supplemented with SVZ-CM ($p=0.07$) (Figure 35C). These data provide evidence that the SVZ-CM actively promotes GBM cell proliferation and is associated with increased cell survival after radiotherapy.

e. Inhibition of SVZ-released CXCL12 Sensitizes GBM Cells to Irradiation *in vitro*

CXCL12 is involved in cell cycle progression and has recently been suggested to regulate GBM *in vitro* EMT, especially through Snail and N-cadherin regulation [372]. On the other hand, GBM mesenchymal "differentiation" or activation (I'd rather use the term "mesenchymal activation" since GBM may already display mesenchymal properties - see Chapter I, section 5.5) has elegantly been reported to enhance radio-resistance in a NF-κB-dependent manner [374]. Relying on these considerations, we decided to investigate whether

SVZ-released CXCL12 undertakes a role in GBM radio-resistance. To do so, we implemented a 7.5 fold dilution of SVZ-CM with a specific CXCL12 blocking antibody or a non-relevant immunoglobulin (IgG) for 45 minutes. We then added these media to U87MG cells and GBM2 primary cells prior to irradiation (10Gy). We finally quantified the impact of blocking CXCL12 in the SVZ-CM by assessing the γH2AX response in both GBM populations. As expected, similar smaller amounts of γH2AX-positive cells were found in both SVZ-CM and SVZ-CM/IgG conditions. Interrestingly, the blockade of CXCL12 significantly allowed to sensitize both U87MG cells and GBM2 primary cells to γ-radiation ($p<0.001$) (Figure 36A and B).

However, attention has to be drawn on the fact that the CXCL12 inhibition did not completely restore the number of γH2AX-positive cells observed without any SVZ-CM supplementation (94.8 ± 2.6% vs 82.9 ± 9.8% ($p=0.005$) and 92.6 ± 8.1% vs 84.6 ± 9.9% ($p=0.02$), respecively for U87MG cells and GBM2 primary cells) (Figure 36A and B), suggesting the role of other SVZ components in GBM *in vitro* resistance to radiation.



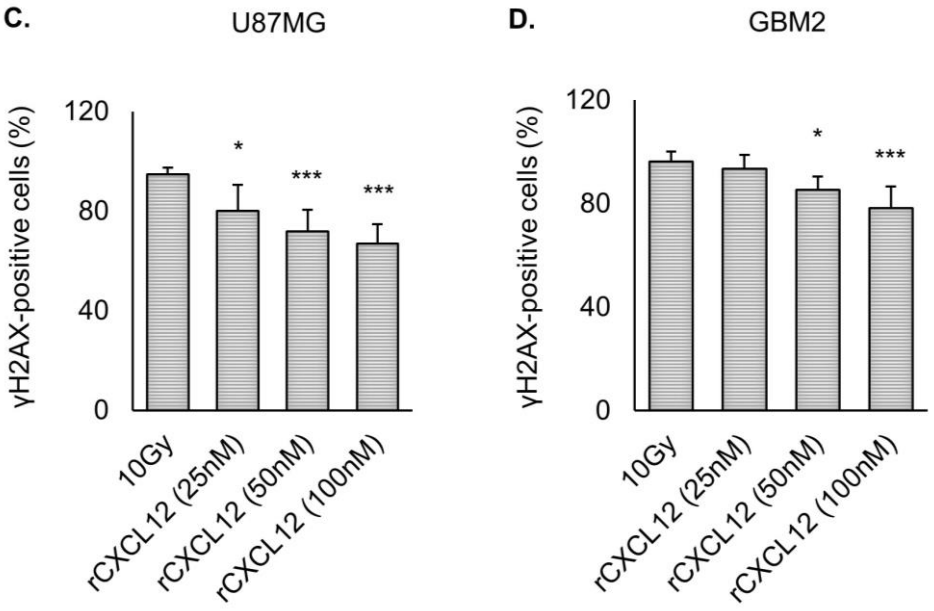


FIGURE 36. Identification of chemokine CXCL12 as a key mediator in GBM *in vitro* resistance to radiation. A and B) Inhibition of CXCL12 in the SVZ-CM led to a significant radio-sensitization of human U87MG cells and GBM2 primary cells. The number of γH2AX-positive cells was indeed found higher after the CXCL12 blockade compared to the SVZ-CM condition. C and D) Growing concentrations of recombinant CXCL12 significantly radio-protected U87MG cells and GBM2 primary cells in a dose-dependent manner. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Conversely to the CXCL12 blockade, we conducted rescue experiments to prove the potential of CXCL12 as a radio-protective chemokine for GBM cells. We serum-starved U87MG cells and GBM2 primary cells for 12 hours. We then stimulated these cells for one hour with growing concentrations of recombinant CXCL12 (rCXCL12) (25nM, 50nM and 100nM) prior to a γ-radiation dose of 10Gy and finally assessed the γH2AX response within these conditions. We noticed a significant decrease of γH2AX-positive U87MG cells starting at 25nM ($p=0.04$ - Figure 36C). A similar observation was made with GBM2 primary cells at 50nM ($p=0.02$) of rCXCL12 (Figure 36D). This slight but constant radio-desensitization was observed up to 100nM in both GBM cell populations ($p<0.001$). Importantly, rCXCL12 never allowed to reach the levels of radio-protection observed with a 7.5 fold dilution of SVZ-CM, suggesting once again the role of other SVZ soluble components in GBM resistance to radiotherapy.

To further confirm the potential implication of the CXCL12/CXCR4 axis in GBM resistance to irradiation, we seeded 7.500 GB138 primary cells previously sorted for the

expression of CXCR4 in a six well plate. CXCR4-positive and CXCR4-negative GB138 primary cells then received a dose of 4Gy and were finally cultured at an exponential growth phase for 7 days. In this way, we demonstrated that CXCR4-positive GB138 primary cells proliferated 1.6 times more than CXCR4-negative GB138 primary cells (p=0.03), suggesting the role of chemokine receptor CXCR4 as a potential biomarker for radio-resistant GBM cells (Figure 37).

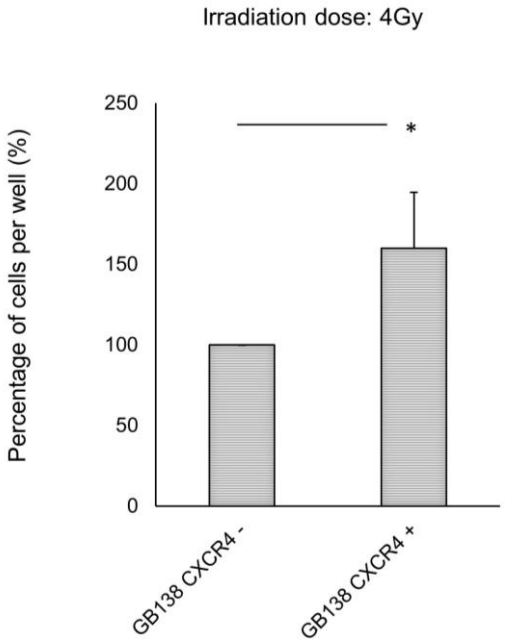


FIGURE 37. Identification of CXCR4 as a potential biomarker for radio-resistant GBM cells. The expression of CXCR4 allowed GB138 primary cells to better proliferate after a dose of 4Gy compared to CXCR4-negative GB138 primary cells. * $p<0.05$.

f. CXCL12 Promotes the Mesenchymal Activation of GBM cells *in vitro*

We have so far demonstrated the CXCL12/CXCR4 signaling to endorse a key role in GBM resistance to radiation but we still don't known the mechanisms which specifically work behind the scene. In this context, CXCL12 has recently been suggested to regulate GBM *in vitro* EMT or "mesenchymal activation" [372]. As previously described, the acquisition of mesenchymal traits is usually in line with poor survival outcomes and corresponds to the most rebellious type of GBM to therapy (see Chapter I, section 5.5) [68]. Many studies (reviewed in [375]) have documented a role for EMT and resistance to chemotherapy in several human

tumors. In contrast, very less is known about the involvement of EMT in radio-resistance. Two years ago, a very elegant study came out and reported that GBM mesenchymal differentiation actually promotes resistance to radiation in a NF-κB-dependent manner [374]. Relying on these findings, we questioned the role of CXCL12 in promoting the mesenchymal activation of GBM cells.

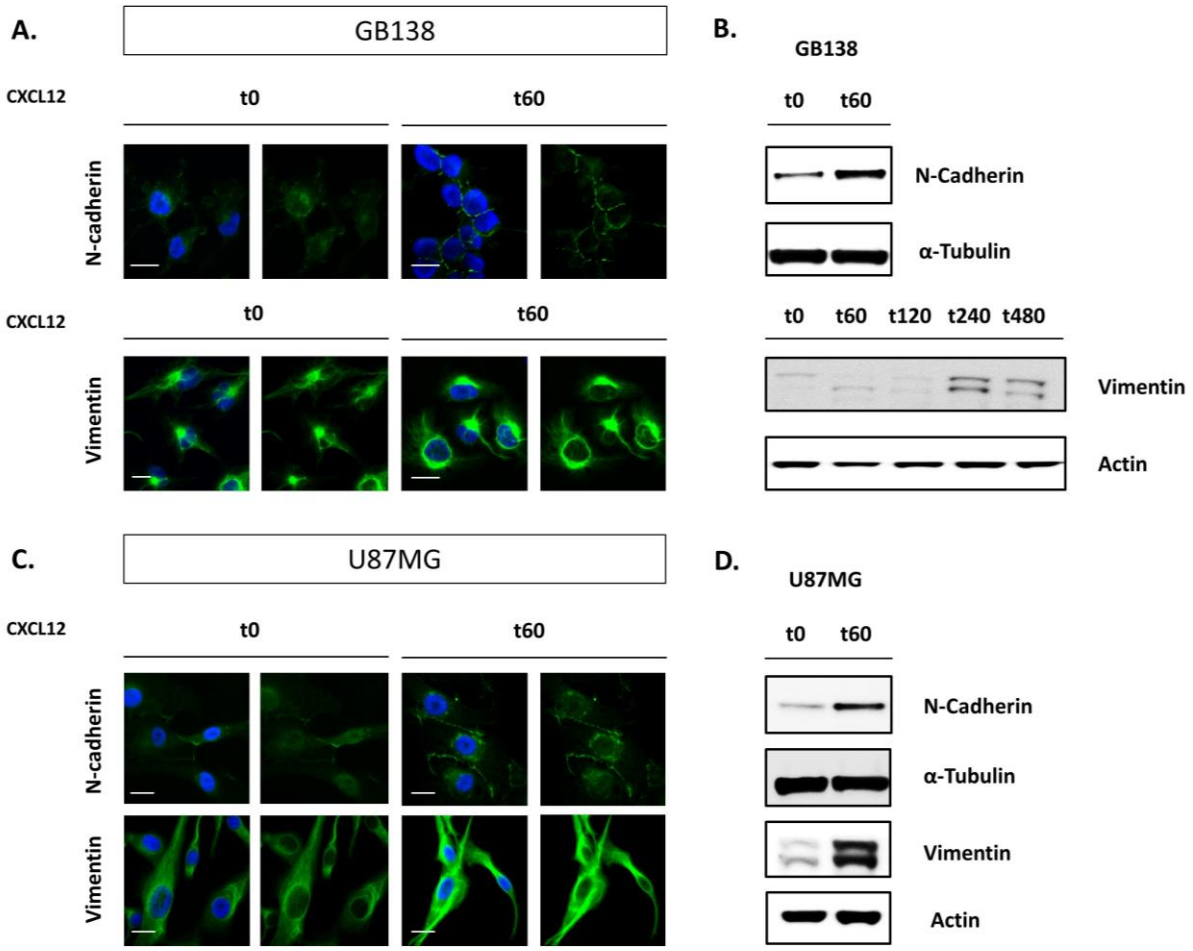


FIGURE 38. CXCL12 sustains the mesenchymal activation of GBM cells *in vitro*. A) Immunocytostainings (ICS) revealed an up-regulation of N-cadherin in GB138 primary cells upon an hour of CXCL12 stimulation. No change was observed regarding the expression of Vimentin. B) Wetsern Blot (WB) analyses confirmed these data and showed an increased expression of Vimentin upon 4 hours of stimulation. C-D) ICS and WB analysis highlighted an overexpression of both N-Cadherin and Vimentin in U87MG cells upon an hour of CXCL12 stimulation. Scale bars = 10µm for A and C.

Upon an hour of stimulation with rCXCL12 (20nM), both U87MG cells and GB138 primary cells expressed higher levels of N-cadherin, a protein characteristically involved in the mesenchymal activation (Figure 38). The expression of Vimentin, a type III intermediate filament protein specifically expressed in mesenchymal cells, was also upregulated within an hour of stimulation in U87MG cells (Figure 38C and D). No significant change in Vimentin expression was observed in GB138 primary cells within an hour of stimulation (Figure 38A). As a matter of fact, Vimentin expression started to increase after 4 hours of stimulation in this GBM population, suggesting different post-translational regulations in U87MG cells and GB138 primary cells. Anyway, these findings allowed to highlight the acquisition of a reinforced *in vitro* mesenchymal phenotype upon CXCL12 stimulation in both U87MG cells and GB138 primary cells.

g. GBM Cells Located in the SVZ Maintain Strong Mesenchymal Properties

Now that we demonstrated SVZ-released CXCL12 to be involved in GBM resistance to irradiation *in vitro* and CXCL12 to underlie an enhanced GBM mesenchymal phenotype, we looked for a potential mesenchymal signature in GBM cells nested in the SVZ. Acquisition of mesenchymal properties is indeed considered as a hallmark of therapeutic resistance in cancer [375]. To tackle the issue, we first performed immunohistostainings on coronal sections of brains previously grafted with GB138 primary cells (back to the *in vivo* model we developed). These stainings allowed to confirm the expression of mesenchymal proteins including N-cadherin and Vimentin (red) in GB138 primary cells (green) located in the ventricular walls (Figure 39A). These cancer cells were detected using a specific anti-human nuclei antibody (Hu. Nu. - green). Western Blot analyses on GB138 primary cells isolated from the TM and the SVZ were then performed. This approach allowed to shed the light on a higher expression level of N-cadherin and Vimentin in GB138 primary cells initially located in the SVZ compared to GB138 primary cells isolated from the TM (Figure 39B). This further suggests a selection of cancer cells with stronger mesenchymal properties from the TM or a SVZ-dependent enhancement of mesenchymal traits in GBM cells probably through the release of CXCL12. Anyway, in both cases, SVZ-nested GBM cells specifically display stronger mesenchymal traits, a characteristic often correlated with therapeutic resistance and may therefore potentially be involved in tumor recurrence.

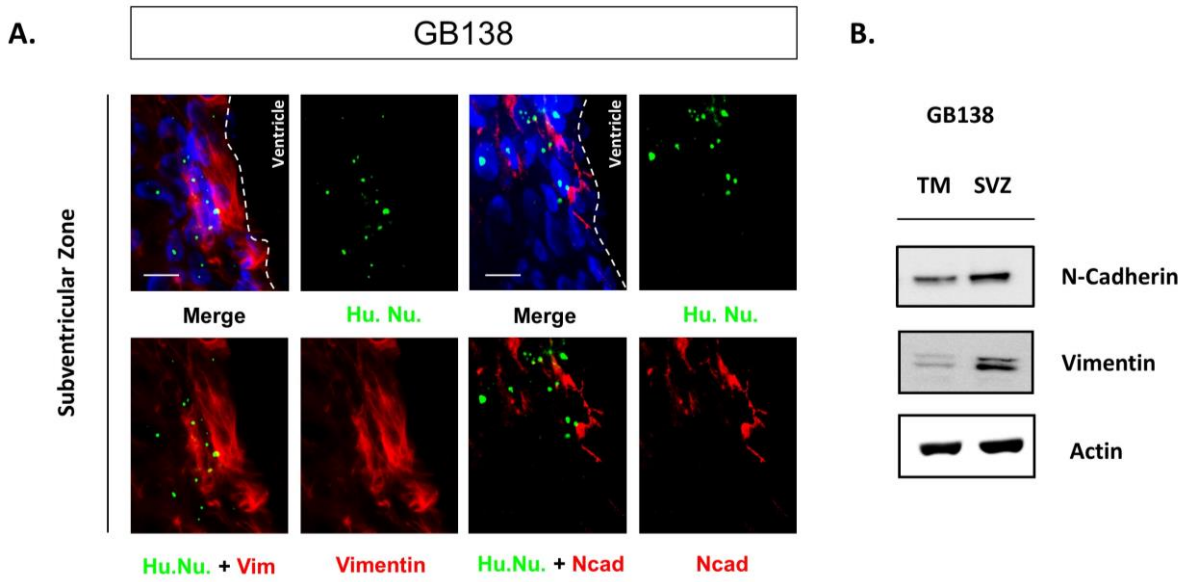


FIGURE 39. GBM cells nested in the SVZ display enhanced mesenchymal properties. A) Immunohistostainings on brain coronal sections revealed the expression of N-cadherin and Vimentin (red) in GB138 primary cells (green) located in the SVZ. B) By Western Blot analyses, the expression of these two mesenchymal proteins was shown up-regulated in GB138 primary cells isolated from the SVZ compared to G138 primary cells from the tumor mass. This further suggests the critical role of the SVZ in regulating these mesenchymal traits. Scale bars = 25µm.

Discussion and Perspectives

Discussion and Perspectives

Glioblastoma multiforme is a WHO grade IV brain tumor characterized by behavioral aggressiveness, specific histological features and a dismal prognosis despite multimodal therapy. Factors such as inter/intra-tumor heterogeneity, mutational evolution and the tumor microenvironment are pointed out as key mediators in GBM resistance to therapy. One of the key goals in GBM research is to identify genotypic and/or phenotypic hallmarks potentially involved in tumor heterogeneity and therapeutic resistance. In this context, chemotactic cytokines are known to mediate a variety of functional events in cancer including tumor growth, angiogenesis, metastasis and the recruitment of immune cells to the tumor. In this work, we addressed the functions and therapeutic potentials of a specific chemokinergic pathway using an *in vivo* model of brain cancer invasion we developed. More specifically, we questioned the potential role of the CXCL12/CXCR4 signaling axis in the oriented invasion of GSC toward the adult SVZ and further evaluated the impact of SVZ-released CXCL12 in GBM resistance to radiotherapy.

1. Establishing and Characterizing the *in vivo* Model of Brain Tumor Invasion

Several experimental models of GBM invasion have been developed over the last decade to investigate the issue. Unfortunately, many of these models yielded different results because they usually bear little physiological relevance to the structures found in the brain. A reliable experimental model of a human disease is indeed the one which resembles the human condition as close as possible. In this context, *in vitro* models are very popular as they are fairly cheap and easy to work with, allowing to keep under control multiple conditions and variables. They particularly offer great insights into cellular pathways and mechanisms involved in cancer growth. In addition, they are usually the first step in identifying and testing potential new anti-cancer drugs. As a major weak point, *in vitro* models are distant from true clinical conditions. On the other hand, *in vivo* models are more backbreaking but offer critical insights into tumor/host interactions. They are also regarded as a valuable platform for testing potentially new therapeutic targets. However, the lack of robust and reliable GBM experimental models

has been quite an issue so far. As the *sine qua non* condition is to bear as much resemblance with the human disease, one can easily realize it is sometimes a far cry from reality. For instance, GBM culture methods are known to trigger a selection bias over time. Moreover, traditional serum-containing *in vitro* culturing methods are shown to result in loss of important GBM hallmarks. Cells bearing *EGFR* mutations (the second most common genetic aberration encountered in GBM) are notably diluted and/or lost after few passages in serum-containing media, diverging in this way from the clinical GBM tissue they originate [71, 376, 377]. Interestingly, serum supplementation also changes both CXCR4 and CXCR7 expression levels in GBM cultures [352]. As an alternative, scientists could have recourse to commercially available GBM cell lines specifically engineered to express different GBM genetic hallmarks, including the amplified version of EGFR or its variant III mutant (see Chapter I, section 5.2.) [378]. These culture models, although closer from clinical reality, are likewise grown in serum-containing media and, therefore, do not perfectly resemble the patient tumor tissue [379]. In recent years, it rapidly became evident that GBM subtyping actually covers something much more complex than the basic primary versus secondary classification (see Chapter I, section 4). Of importance, based on gene expression profiling, specifically highlighting intermingled levels of inter/intra-tumor heterogeneity (see Chapter I, section 5.1-5), the actual GBM classification has reached a higher level of complexity and could potentially influence both prognosis and outcomes [10, 68, 75]. With all these considerations in mind, it would therefore be wrong to believe that commercially available cell lines, including the U87MG cells we used to validate our *in vivo* approach, entirely recapitulate the broad genetic and phenotypic diversity found within malignant brain tumors.

As a consequence, more reliable *in vitro* culturing methods have been developed over the past decade, including the culture of neuro/gliomaspheres in serum-free conditions. These methods have particularly gained increasing acceptance based on the original findings that a sub-population of cancer "stem-like" cells actively sustains leukemia malignant features [84]. This observation consequently led to the first identification/characterization of a similar cell population in malignant brain tumors in 2002 [380]. Ever since, a plethora of reports have demonstrated the existence of a tumorigenic brain cancer "stem-like" cell population and its significant implication in the course of the disease (see Chapter II) [95, 381, 382]. Interestingly, serum-free GBM culturing methods display the advantage to better reflect the parental tumor cytogenetic [376], exhibit characteristics similar to that of NSC (see Chapter II, section 2) and are highly tumorigenic *in vivo.* [95, 379]. As a consequence, almost every laboratory working

on establishing *in vitro* cultures from GBM tumors have more or less developed their own serum-free culturing formula [383].

As previously mentioned, one of the most important hallmarks of malignant brain tumors is their stunning invasive abilities, representing one of the major clinical obstacle to therapy [384]. In this context, tumors recur predominantly within 1 or 2 cm of the resection cavity after surgery [385]. In this line, GTR has been reported to significantly prolong survival and is suggested to always be attempted when possible [386]. This observation might nevertheless not be end in itself since GBM relapses are also documented to occur at great distance from the primary outbreak [219, 387]. GBM recurrence is to be incriminated to the fact that cells from the tumor bulk have already invaded the normal brain tissue at time of surgery. In 1925, Dandy already recognized the highly invasive characteristics of this type of tumor while performing hemispherectomy in patients who urged every effort to save their lives [388]. In a similar context, Matsukado reported in 1961 that more than 50% of untreated brain tumors had already reached the contralateral hemisphere at diagnosis [389]. As you have understood, diffuse gliomas are a long-standing issue and remain nowadays particularly challenging in terms of clinical management. In this context, we developed an *in vivo* model of brain tumor invasion which takes into account relevant brain territories. After striatal implantation, we demonstrated that a restricted fraction of tumor cells specifically invade the adult SVZ from both U87MG cells and GB138 primary cultures [208]. These cells were shown to reach the SVZ environment through invasion of the CC, also known as the callosal commissure. This white matter structure connects the left and right cerebral hemispheres and facilitates inter-hemispheric communications. In this matter, the *in vivo* model of brain cancer invasion we developed particularly resembles the human condition since infiltrative GBM are known to preferentially infiltrate white matter structures [390, 391]. This type of behavior has notably been reported to sustain contralateral lesions in patients [392]. Furthermore, a very recent retrospective study demonstrated that most GBM frequently arise from periventricular white matter regions adjacent to the SVZ (n=507 patients), suggesting the potential role of the SVZ in the onset of malignant brain tumors [29]. If the *in vivo* model we set up appears to share important features with the clinic, it also shows significant limitations, including the nature of the cells we implanted (GBM cell lines vs GBM primary cells) and the conditions in which these cells have been cultured prior to engraftment (as already discussed).

Our lab previously demonstrated that GBM cells isolated from the adult SVZ express NSC markers (including Sox2, Nestin, “GFAP” and CD133) and display high tumorigenicity

when secondary injected in new mice brains. These SVZ-nested GBM cells have consequently been characterized as GBM-initiating cells also known as GSC. In this line, U87MG cells are documented to contain a very small population of multipotent and self-renewable CSC [393]. In 2014, Sadahiro and colleagues validated our *in vivo* model of brain cancer invasion, using three different human GBM cell lines [209]. More importantly, Piccirillo's preliminary work, using 5-aminolevulinic acid (5-ALA) in patients, reported for the first time in 2012 the presence of fluorescent malignant material in the human SVZ. She further demonstrated the existence of GSC within this SVZ-isolated fluorescent material and characterized the fraction of cells, possessing all the "cancer-stem cell" cardinal features, to preferentially reside in the pro-angiogenic environment provided by the SVZ. Part of this brilliant work was finally released in January 2015 after the final demonstration that SVZ-nested GSC actively contribute to chemotherapeutic resistance [210]. Taken together, these findings highlighted the SVZ to harbor GSC in both animal models and humans. It is consequently enticing to speculate on the eventual role of the SVZ environment as a GSC reservoir involved in tumor recurrence. Notwithstanding the possible influence of this specific neurogenic niche on GBM resistance to therapy [394], the potential clinical implication of these findings may be of major importance for the future definition of new therapeutic avenues.

2. The CXCL12–CXCR4 Axis: Key Mediator in GBM Invasion

The migration of brain cancer cells is complex process involving interactions with the extracellular matrix (ECM) and chemo-attractants that either diffuse from blood vessels and/or are secreted by neighboring cells [395, 396]. Over the last decade, critical growth factors have been the topic of intense research for their role as regulators of tumor biology and chemotaxis [397]. It is widely believed that secreted factors diffuse over time in the environment and generate concentration gradients that are sensed by cancer cells to finally trigger the detachment and migration of these cells away from the primary tumor [398, 399]. Malignant gliomas are no exception to the rule as their invasion abilities are believed to be awakened by soluble cytokines stimulating directional and/or random tumor cell motility [400]. In this line, we specifically demonstrated SVZ-EC and astrocytes to express and release CXCL12 in the nearby environment along a decreasing gradient. Interestingly, cancer cells may alternatively communicate with specific distant targets through secreted micro-vesicles which either contain growth factors, receptors, functional mRNA's or miRNA's [401]. Such micro-vesicles are shed

by most cell types, including cancer cells, and have been found in numerous cancer patients sera [402]. In this context, the recent discovery of a new organelle, known as "migrasome", has been described to mediate the release of cytoplasmic contents (including vesicles and cytosolic proteins) into the extracellular space during cell migration [403]. The authors presented this organelle as a key regulator of cell migration since both spatial and biochemical information from outgoing cells can directly be taken up by incoming cells, consequently able to quickly adapt to any kind of conditions. Deeper investigations are needed to check whether migrating GBM cells release migrasomes during the CC invasion step. The existence of such organelles could potentially have a tremendous, unsuspected-before impact on GBM invasion abilities and, more specifically, give new insights in the way GSC take possession of the SVZ.

While the effects of mitogens on the *in vitro* motility of GBM cells have been well documented, the ability of soluble cytokines to drive different cellular functions (migration and/or proliferation) remains unclear and is thought to rely on several determinant factors. Some of these factors have been addressed in the literature such as dosage-dependence [404], contact inhibition [405] as well as autocrine/paracrine signaling-driven tumor growth [406]. In this context, malignant brain tumors are known to secrete a residual amount of CXCL12 suggested to act as an autocrine growth factor sustaining tumor development (see Chapter IV, section 7) [407]. Interestingly, GBM release of CXCL12 was shown to be significantly higher when GBM cells were cultured upon low serum supplementation [407], demonstrating one more time how serum culture conditions may influence the behavior of GBM cells *in vitro*. Additionally, inhibition of the CXCL12/CXCR4 autocrine/paracrine loop has recently been described to impede GSC viability by affecting their self-renewal abilities [346] and proliferation [344]. As a consequence, CXCL12 not only regulates crucial GBM migration processes [408, 409] but may as well contribute to the overall well-being of GSC within the SVZ niche. Insights on how CXCL12 potentially regulates the fate of SVZ-nested GSC are further discussed.

Cancer cell locomotion is highly sensitive to external stimuli from the ECM and from the surrounding environment. In this line, different cell types present in the tumor microenvironment may also contribute to the regulation of GBM tumor growth and diffusion through the release of CXCL12. This chemokine is indeed abundantly and selectively expressed both in the developing and mature brain [410]. Precisely, brain endothelial cells, neurons, microglia and/or reactive astrocytes are known to release CXCL12 in the nearby environment and are therefore suggested to play a role in GBM infiltrative patterns [411]. Using SVZ micro-dissected whole-mounts, we confirmed that EC and astrocytes express/release CXCL12 along

a decreasing gradient within the NSC niche. We also demonstrated the SVZ-CM to contain a significant higher amount of CXCL12 compared to OB/CRBL-CM. Interestingly, the SVZ expression of CXCL12 has specifically been highlighted in cells adjacent to proliferating neural progenitors [356]. This pattern of expression suggests neural stem/progenitor cells to develop and function under the influence of CXCL12. With these considerations in mind and relying on the *in vivo* model of brain cancer invasion we developed, we strongly suspect the SVZ stem cell niche to regulate the fate of GSC through the release of CXCL12. As already mentioned, GBM and GSC are totally capable of secreting CXCL12, but in a very limited manner. Indeed, the CXCL12 gene is found on chromosome 10q (see Chapter IV, section 3) which is, most of the time, lost in both primary and secondary GBM (see Chapter I, section 5.1). This observation actually enhances the idea that GBM, and more specifically GSC, migrate toward a CXCL12-enriched region in the brain in order to fulfil their needs.

Of importance, the physiological role of CXCL12 in the adult SVZ has recently been reported. CXCL12 specifically regulates the lineage progression of SVZ progenitors by transporting them from their relatively quiescent ependymal niche to the basal vasculature niche where these cells undergo active amplification [356]. In this scenario, CXCL12 fulfils its function by regulating NPC migration as a chemoattractant. It should however be noted that most CXCR4-expressing NPC reside in the SVZ environment, a region where neurogenesis takes place on site. This actually suggests CXCL12 to regulate other aspects of these progenitor cells, conceivably proliferation and survival. Based on parallelisms between NSC and GSC, one could easily imagine that CXCL12 could influence GSC in a similar way. So far, several studies reported CXCL12 to actively stimulate the *in vitro* proliferation of multipotent NPC as well as CXCR4-positive GSC [326, 344, 347, 412]. Contradictory results showed that high levels of CXCL12 derived from ependymal cells within the SVZ stem cell niche maintain human NSC in a quiescent state [356, 413]. Whether CXCL12 regulates the quiescence of GSC is yet to be determined. However, growing evidence suggest it could be the case. In this line, mesenchymal stem cells (MSC) have been described to promote cancer stemness through the CXCL12-dependent activation of NF-kB [414]. Interestingly enough, MSC are specifically recruited in GBM and actively contribute to the tumor progression [415]. Whether MSC are recruited to the SVZ in order to sustain GSC intrinsic properties is not known. Piling evidence also indict CXCL12 to promote survival of both NPC and GSC *in vitro* [346, 416]. However, beyond the light of these *in vitro* studies, the effect of CXCL12 on proliferation and survival of NPC in the SVZ of CXCR4 or CXCL12-deficient mice hasn't been reported yet. One possible

explanation for this lack of data could be redundancy resulting from the presence of a large number of growth promoting factors operating in this environment. As a matter of fact, Li and colleagues suggested CXCL12 to strictly stimulate NPC proliferation in synergy with epidermal growth factor (EGF) and fibroblast growth factor (FGF) [412]. Curiously, EGF and CXCL12 are able to stimulate GBM cell chemotaxis in a very similar manner as they both induce calcium mobilization and calcium-activated K^+ channel activation [417]. Another explanation comes from the fact that NPC and GSC share the expression of several chemokine receptors besides CXCR4 [418], raising the potential involvement of other chemokines in regulating the proliferation/survival of both cell populations. As an example, we demonstrated SVZ whole-mounts to express high mRNA levels of different cytokines and chemokines including CX3CL1. Interestingly, CX3CR1 is known to play a key role in the behavior of malignant human brain tumors notably through the recruitment of GBM-associated macrophages [419, 420] but, on the other hand, plays a negative role in GBM invasion [421]. Regarding the large panel of chemokines and cytokines expressed in the SVZ as well as their related properties (see Chapter IV, section 1), it is reasonably foreseeable that these proteins may act as individuals, cooperate or even compensate an eventual defective signaling in order to regulate NSC and GSC specific features including migration, proliferation and survival.

Regarding the present manuscript, we demonstrated the important role undertaken by the CXCL12/CXCR4 axis in GBM migration/invasion. We reported that CXCL12 triggers the *in vitro* migration and chemotaxis of GBM cells using modified Boyden chamber assays and time-course analyses. We made a similar observation conducting *in vitro* migration experiments with the SVZ-CM. This *in vitro* phenotype was partially inhibited using AMD3100, a CXCR4 specific inhibitor, suggesting one more time the role of other SVZ-related molecules in GBM migration abilities. In this line, the *in vivo* use of AMD3100 allowed to prevent the migration of U87MG cells toward the SVZ but failed to completely inhibit the migration of GB138 primary cells toward the same region. Two explanations are to put forward. The first one states it is easier to work with the U87MG model. U87MG cells indeed invade the SVZ in a specific window of time (between the third and fourth week post-implantation). Conversely, due to slower growth rates, we don't exactly know when GB138 primary cells start to invade the SVZ. This lack of data might be at the origin of a delay in AMD3100 treatment, potentially explaining the presence of GB138 primary cells in the SVZ environment. The second explanation is to be found in the expression of CXCR7. If we are certain that CXCR7 is not expressed by U87MG cells, the expression of CXCR7 on GB138 primary cells remains an open question. In this case,

the AMD3100 blockade of CXCR4 allowed to significantly decrease the number of GB138 primary cells reaching the SVZ, but wasn't sufficient enough to totally hamper their migration process.

Finally, we would like to draw attention on the population of cancer cells that we isolated from the SVZ. Those distant invaders retain the ability to initiate tumor growth, display an enhanced mesenchymal phenotype, are resistant to radiation and could therefore be incriminated for GBM deadly relapses [208]. *In vitro* migration bio-assays revealed that GBM cells isolated from the SVZ display stronger migration abilities in response to both CXCL12 and SVZ-CM compared to GBM cells isolated from the tumor mass. Moreover, the impact of AMD3100 in reducing cell migration was more significant on the population of cancer cells isolated from the SVZ. Deeper investigations on this particular cell subtype are needed to potentially provide new insights in CSC biology and to better encompass the molecular mechanisms underlying GBM relapses. Of note, it would be interesting to study/compare the intracellular molecular events triggered in GBM cells isolated from the SVZ and the tumor mass upon CXCL12 stimulation. In this line, we already conducted a mass-spectrometry analysis of the U87MG cells phosphoproteome after an hour of stimulation with exogenous CXCL12. These data notably highlighted the potential implication of key regulators from the MAPK and NF-κB pathways in GBM migration abilities and are actually the topic of another PhD program ongoing in the lab.

3. The SVZ Niche as an Evil Driving Force in GBM

a. The Suitable Soil

To date, our understanding of the tumor microenvironment (the appropriate soil) and its role in CSC (the seed) regulation is still limited. It is however of great therapeutic interest to unravel the mechanisms and mediators provided by the niche micro-environment which specifically define the CSC fate. Indeed, the ability of the niche to act as a suitable soil in which CSC can flourish is a critical process in tumor relapses/metastases. Further investigations are therefore needed to enhance our understanding of the basic biology behind cancer and to potentially identify new therapeutic targets disrupting the molecular crosstalks between the seed and the soil.

In this context, we strongly believe the SVZ environment to play a central role in the course of the disease (see Chapter III, section 3). Ever since Picirillo's work, the *in vivo* model of brain cancer invasion we developed has grown in stature. As already mentioned, Picirillo and teammates reported the first evidence of a GSC population residing in the human SVZ [210]. These findings gave credit to our *in vivo* model since we specifically demonstrated that a fraction of GBM cells enriched in GBM-initiating cells (or GSC) invades the adult SVZ in a CXCL12/CXCR4-dependent manner. Relying on shared hallmarks betwen GSC and SVZ-residing NSC (see Chapter II, section 2), we hypothesized this neurogenic niche to act as a reservoir of GSC potentially involved in GBM deadly relapses. In the facts, GBM relapses occur most of the time within 2 cm from the initial margins of the resection cavity following concomitant radio/chemotherapy [422]. With regard to the initial lesion site, Ellingson and colleagues recently demonstrated, on a cohort of 507 patients, that most GBM originally grow into periventricular white matter regions adjacent to the SVZ [29]. Together these data suggest the implication of the SVZ niche in both the onset and the course of the disease. So how exactly does the SVZ provide GSC with a suitable environment?

In the SVZ stem cell niche, NSC, transit-amplifying cells and neuroblasts (see Chapter III, section 2) specifically interact with blood vessels [423, 424]. In contrast to most blood vessels in the brain, capillaries in the SVZ are not completely enwrapped by pericytes and astrocytic end-feet. This consequently results in a unique, incomplete blood-brain barrier (BBB) which is permeable to blood-derived small molecules and systemic influences [425]. NSC extend a long basal process that makes contact with these capillaries [426], while transit-amplifying cells directly contact vascular endothelial cells with their cell body at the incomplete BBB [424]. The SVZ vascular network is therefore a key component of the adult SVZ stem cell niche, with NSC and transit-amplifying cells uniquely poised to receive spatial cues and regulatory signals from the circulating blood. Just like NSC, GSC also rely on a specific vascular niche to control the balance between their self-renewal and differentiation abilities [154]. This balance is finely regulated by changes in blood flow or EC-released factors including brain-derived neurotrophic factor or nitric oxide [427, 428]. Activated Notch signaling by nitric oxide promotes tumor progression along with increased GSC self-renewal properties and growth of the tumor vasculature *in vivo* [429]. In a similar fashion, Zhu and co-workers demonstrated the endothelial release of Notch-ligand Dll4 (Delta-like ligand 4) in the vascular niche to nurture GSC self-renewal and proliferation [430]. Consistent with this observation, both SVZ endothelial and ependymal cells release a significant amount of pigment

epithelium-derived factor (PEDF) within the niche [428]. This growth factor is known to promote self-renewing cell division and specifically maintains NSC multipotency by enhancing Notch-dependent transcription [431]. Last but not least, angiopoietin-1 (Ang-1), a potent angiogenic growth factor found in the SVZ, is known to actively regulate tumor-induced angiogenesis in several GBM models [432]. Interestingly enough, Ang-1 regulation of expression has recently been demonstrated to be CXCL12/CXCR4 dependent in human malignant brain tumors [347]. Collectively, these data suggest how blood vessel-derived factors found in the SVZ stem cell niche may create a suitable cellular and molecular environment for hosting GSC and how these factors could control various aspects of gliomagenesis, specifically regulating GSC features.

b. A Breeding Ground for Therapeutic Resistance

In the second part of this work, we demonstrated that GBM cells hiding in the SVZ are partially protected from irradiation. Indeed, using our *in vivo* model of brain cancer invasion, we found that 12% of GBM cells initially nested in the SVZ still remain in that environment after radiotherapy. As a reminder, GBM cells located in the SVZ are known to be enriched in tumor-initiating capacities and were therefore characterized as GSC [208]. Since these cells are known to be intrinsically resistant to radiation (see Chapter II, section 3) [124], it basically makes sense to find a remaining fraction of tumor cells in the SVZ environment after irradiation. We anyway questioned the role of the SVZ stem cell niche in GBM extrinsic resistance to radiotherapy. In other words, besides the intrinsic abilities of GSC to be resistant to radiation, we made the hypothesis that the SVZ environment provides extrinsic radio-resistant inputs to GBM cells. We particularly believe the composition of the niche to tightly regulate GSC fate specification and protection. In this line, Piccirillo and colleagues revealed that GSC isolated from the human SVZ are specifically resistant to supra-maximal chemotherapy doses along with differential patterns of drug response between TM and SVZ GBM cells [210]. Together, these findings allow to speculate on the potential role of the NSC niche as a reservoir of radio/chemo-resistant GBM cells involved in tumor relapses.

We managed to transpose the *in vivo* observations we made in an *in vitro* protocol of radiotherapy using the soluble environment of the SVZ (SVZ-CM). This experimental protocol

allowed to highlight a certain level of GBM resistance to radiation (10Gy) following a SVZ-CM stimulation. Additionally, we specifically pinpointed SVZ-released CXCL12 and CXCR4 expressed by GBM cells as key mediators involved in GBM resistance to irradiation *in vitro*. In the light of our findings, CXCR4 has recently been described as a new biomarker for radio-resistant cancer stem cells [370]. Furthermore, pharmacological inhibition of the CXCL12/CXCR4 signaling concomitant with radiotherapy elegantly abrogated GBM regrowth in mice by preventing the development of functional tumor blood vessels post-irradiation [433]. In a similar way, Domanska and colleagues described the inhibition of CXCR4-dependent protective signals from stromal cells to render prostate cancer cells more sensitive to ionizing radiations [434]. Characterization of both CXCL12 and CXCR4 antagonists in pre-clinical cancer models as well as their potential therapeutic benefits in combination with radiotherapy may therefore contribute to better understand the role of CXCL12 and CXCR4 in GBM resistance to treatment and facilitate the translation of these inhibitors to the clinic.

We demonstrated a key role of the CXCL12/CXCR4 signaling axis in GBM resistance to irradiation but the mechanisms specifically underlying these findings were yet to be determined. To tackle down the issue, we focused on the mesenchymal status of GBM cells. Indeed, the acquisition of mesenchymal traits (or epithelial to mesenchymal transition - EMT) is usually in line with poor outcomes and corresponds to the most rebellious subtype of GBM to therapy (see Chapter I, section 5.5) [10, 68]. For the sake of clarity, we'd rather use the term "mesenchymal activation" instead of EMT as GBM may originally display basic mesenchymal properties. This being said, GBM are also known to frequently shift toward a mesenchymal phenotype upon recurrence [10]. With these considerations in mind, we demonstrated GBM cells to express a basal level of Vimentin and N-cadherin, further suggesting the mesenchymal origin of our GBM populations. Interestingly, these two mesenchymal proteins were specifically up-regulated upon CXCL12 stimulation. Supporting our findings, the specific inhibition of the CXCL12/CXCR4 signaling axis in GBM has recently been reported to affect the *in vitro* expression of mesenchymal biomarkers including Vimentin, Snail and N-cadherin [435]. Of importance, we also showed that GBM cells located in the SVZ display a higher expression level of Vimentin and N-cadherin compared to GBM cells located in the tumor mass. Many studies, reviewed in [375], have established a sharp link between the acquisition of mesenchymal traits and resistance to chemotherapy in several human tumors. In contrast, very less is known about the involvement of mesenchymal activation in radio-resistance. In this line, a very elegant study reported GBM mesenchymal activation to promote radio-resistance in a

NF-κB-dependent manner [374], further suggesting this transcription factor to regulate the acquisition of mesenchymal properties. Consistent with these findings, we have demonstrated the nuclear translocation and activation of NF-κB (p65/p50) in GBM cells upon CXCL12 stimulation (Goffart et *al*., unpublished results). Whether NF-κB regulates the mesenchymal activation of SVZ-nested GBM cells is yet to be determined. Our findings anyway provide the very first evidence that the SVZ environment actively participates to the reinforcement of the tumor mesenchymal roots (probably through the secretion of CXCL12). Our findings also highlight a potential mechanism by which this specific environment contributes to GBM resistance to radiotherapy.

These data corroborate with the growing body of clinical evidence correlating delivered doses of radiotherapy to the SVZ with increased PFS and OS in newly diagnosed GBM patients (see Chapter III, section 6) [436-438]. In this line, a very recent retrospective study evaluated the influence of tumor location on recurrence behavior, PFS and OS with respect to the SVZ after radiotherapy. Patients with SVZ-infiltrating GBM specifically showed impaired PFS and OS and presented higher risks of distant/multifocal progression [214]. These findings could potentially be explained by the persistence of radio-resistant GSC within the SVZ environment and readdress the already controversial debate on SVZ dosimetry (see Chapter III, section 6).

If the acquisition of mesenchymal features is typically associated with increased therapeutic resistance in cancer, it also leads to a loss of cell-cell interactions, reduced cellular adhesion, active production of ECM proteases, increased cytoskeletal dynamics and changes in transcription factor expression. All of these changes aim toward a unique goal: to strengthen cell migration/invasion. With that in mind, GBM mesenchymal activation doesn't really make sense if we consider the SVZ environment as a potential "metastatic" niche for GBM cells. In other words, why would GBM cells acquire stronger migration abilities in an environment where they are supposed to settle down and to be done with their invasive process? Well, maybe we are wrong. Maybe "taking possession" of the SVZ is modelled on the Cheshire cat (see Chapter III, section 6) and is just a spark in GBM overall invasive progression. Relying on the facts that the BBB is physiologically incomplete in the SVZ and that GBM cells acquire stronger migration abilities in that environment, it is tempting to hypothesize that the SVZ invasion only represents the non-hidden part of the iceberg, quickly leaving room for something bigger: the systemic invasion. GBM has indeed been thought of as a non-metastatic disease for too long (Lombard A., Goffart N., and Rogister B. under review). Very recent findings tackled down the dogma and demonstrated the hematogenous dissemination of GBM [439, 440].

Curiously, GBM circulating cells were further shown to be exclusively enriched in mesenchymal markers and maintained a non-differentiated phenotype as compared to the primary tumor [439]. In these elegant studies, the authors suggested GBM cells to enter the circulation due to the rupture of the BBB encountered during the course of the disease. We of course agree with this statement but would also like to draw attention on the SVZ capillaries as potential open windows involved in GBM systemic dissemination and metastatic spread. Of importance, EMT is regarded as a key step in the appearance of several tumor metastases. In this line, we demonstrated that GBM cells within the SVZ represent a unique cell population exhibiting tumor-initiating properties and the signature of a mesenchymal activation. We also validated the concept of circulating tumor cells using our *in vivo* model of brain cancer invasion as 100% of the mice bearing xenografts presented GBM cells in their blood (Kroonen and Goffart, unpublished data).

Whether CXCL12 and CXCR4 play a role in GBM intra/extravasation and distant metastases is yet to be investigated. However, scientific evidence already support the CXCL12/CXCR4 axis to promote metastasis and extravasation in breast, lung and prostate cancer [441-443]. In addition, the expression of CXCR4 has specifically been highlighted on circulating-tumor cells released from tumors into the blood stream, further underlying tumor spread to CXCL12-positive distant niches [444, 445]. In colon carcinoma patients, the amount of CD133/CXCR4-positive cells found in blood was shown to correlate with an increased metastatic potential and poor prognosis [446]. The take home message from these independent studies indicates that CXCR4-positive cancer cells are likely to be found in the blood stream of patients and could therefore endorse a key role in metastatic dissemination. A lot of work remains to be done in order to better characterize GBM-circulating cells as well as their potential implication in brain tumor distant metastases. Let's just take one more line to imagine the tremendous impact these cells would have in the field of oncology if they could ever be able to repopulate the brain after multimodal therapy…

… To be continued

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Appendix

Appendix

- **Paper 1**

Adult Mouse Subventricular Zones Stimulate Glioblastoma Stem Cells Specific Invasion Through CXCL12/CXCR4 Signaling

- **Paper 2**

Mesenchymal Activation Mediated by CXCL12 in the Subventricular Zone Promotes Glioblastoma Resistance to Radiotherapy (Submitted)

- **Paper 3**

Glioblastoma-Initiating Cells: Relationship with Neural Stem Cells and the Micro-Environment

- **Paper 4**

Glioblastoma Stem Cells: New Insights in Therapeutic Strategies

- **Paper 5**

Glioblastoma Circulating Cells: Reality, Trap or Illusion? (In Press)

Paper 1

Adult Mouse Subventricular Zones Stimulate Glioblastoma Stem Cells Specific Invasion Through CXCL12/CXCR4 Signaling

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Adult mouse subventricular zones stimulate glioblastoma stem cells specific invasion through CXCL12/CXCR4 signaling

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Background. Patients with glioblastoma multiforme (GBM) have an overall median survival of 15 months. This catastrophic survival rate is the consequence of systematic relapses that could arise from remaining glioblastoma stem cells (GSCs) left behind after surgery. We previously demonstrated that GSCs are able to escape the tumor mass and specifically colonize the adult subventricular zones (SVZs) after transplantation. This specific localization, away from the initial injection site, therefore represents a high-quality model of a clinical obstacle to therapy and relapses because GSCs notably retain the ability to form secondary tumors.

Method. In this work, we questioned the role of the CXCL12/CXCR4 signaling in the GSC-specific invasion of the SVZs.

Results. We demonstrated that both receptor and ligand are respectively expressed by different GBM cell populations and by the SVZ itself. In vitro migration bio-assays highlighted that human U87MG GSCs isolated from the SVZs (U87MG-SVZ) display stronger migratory abilities in response to recombinant CXCL12 and/or SVZ-conditioned medium (SVZ-CM) compared with cancer cells isolated from the tumor mass (U87MG-TM). Moreover, in vitro inhibition of the CXCR4 signaling significantly decreased the U87MG-SVZ cell migration in response to the SVZ-CM. Very interestingly, treating U87MG-xenografted mice with daily doses of AMD3100, a specific CXCR4 antagonist, prevented the specific invasion of the SVZ. Another in vivo experiment, using CXCR4-invalidated GBM cells, displayed similar results.

Conclusion. Taken together, these data demonstrate the significant role of the CXCL12/CXCR4 signaling in this original model of brain cancer invasion.

Keywords: cancer stem cells, CXCL12, invasion, stem cell microenvironment.

Primary brain tumors are among the most refractory of malignancies. Their most aggressive form, glioblastoma multiform (GBM, WHO grade IV), is also the most common and lethal subtype.[1] Although multimodal therapies have been developed, the overall median survival of GBM patients hardly reaches 15 months from the time of diagnosis.[2] This poor survival rate is the consequence of tumor recurrence that systematically occurs despite classical therapeutic strategies. Trying to understand the origin of GBM relapses seems mandatory, in this context, for a better understanding of the tumor's biology and improving the patients' quality of life.

The infiltrative patterns of GBM make tumor cells hard to target. Furthermore, recent studies using orthotopic xenografts have demonstrated that GBM cells are able to escape the tumor mass and specifically invade the subventricular zones (SVZs) of the adult brain.[3,4] In that environment, GBM cells were first shown to be highly tumorigenic and were later characterized as glioblastoma stem cells (GSCs). The SVZ is known to be the major source of neural stem cells and progenitors in adults and functions as a supportive niche promoting self-renewal and inhibits differentiation.[5,6] This "seed-and-soil" relationship has also been adapted to cancer stem cell research because GSCs also rely on a specific

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environment or niche to maintain their stem cell properties and their ability to drive tumor growth.[7,8]

In this work, we questioned the role of the CXCL12/CXCR4 signaling in the GSC-oriented invasion of the SVZ. CXCR4 is known to be expressed by highly malignant gliomas,[9,10] be involved in tumor cell proliferation,[11] and be associated with a poor survival.[12] Recent studies have shown that CXCL12 and CXCR4 enhance tumorigenesis through increased proliferation of tumor cells[13] and that CXCL12 boosts the release of vascular endothelial growth factor (VEGF) from GSC, leading to tumor growth-induced angiogenesis.[14] When taken altogether, those facts led to investigating the eventual role of the CXCR4 signaling in the specific invasion of the SVZ by human GSC. Counteracting with the invasion abilities of GSC would make those cells more easily targeted by classical therapeutic strategies and would definitely improve the survival rate for GBM patients.

Materials and Methods

Cell Culture

Primary GBM cultures (GBM1, GBM2, and GB138) were established from consenting participants and validated as previously described.[3,15] Human GBM cell lines (U87MG ATCC HTB-14, U373 [a generous gift from Florence Lefranc], and LN18 ATCC CRL-2610) were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Invitrogen). Details can be found in Supplemental Experimental Procedures.

Ethics Statement

Participants gave their informed consent for use of GBM specimens. The use of human tissue has been allowed by the "Comité d'éthique Hospitalo-Universitaire du CHU de Liège". This use concerns only residual material after surgical tumor ressection.

Animals

Adult P40 female immunodeficient nude mice, Crl:NU-Foxn1^{nu}, obtained from Charles River Laboratories, were used for xenografts and ventricular surface studies. All animals were cared for in accordance with the Declaration of Helsinki and following the guidelines of the Belgium Ministry of Agriculture in agreement with European Commission Laboratory Animal Care and Use Regulation (86/609/CEE, CE of J n_L358, 18 December 1986). The athymic nude mice were housed in sterilized, filter-topped cages and were processed as approved by the Animal Ethical Committee of the University of Liège.

Whole Mount Dissection

The lateral walls of the ventricles were dissected from the caudal aspect of the telencephalon, as previously described.[16] Detailed procedures can be found in Supplemental Experimental Procedures.

Intracranial Transplantation

Intracranial xenografts were generated, as described previously.[3] Detailed procedures can be found in Supplemental Experimental Procedures.

In Vitro Migration Bio-assays

Chemotaxis assays were performed using a 96-well chemotaxis chamber with 10 μm pore size (NeuroProbe). GBM cell lines were labeled using a Cell Tracker Green (CTG) dye (Invitrogen) at a final concentration of 5 μM in prewarmed DMEM for 30 minutes. The medium was replaced with DMEM and incubated for 30 minutes at 37°C. Cells were washed 3 times with phosphate-buffered saline (PBS) and seeded in the upper chamber (25 000 cells in 25 μl of serum-free medium without growth factors). The lower chamber was filled with 28 μl of serum-free medium, SVZ-conditioned medium, SVZ-conditioned medium containing AMD3100 (25 μg/ml, Sigma), or serum-free medium with different concentrations of human recombinant CXCL12 (Peprotech). After incubation at 37°C for 16 hours, cells in the lower chambers were fixed with 4% paraformaldehyde (PFA) for 15 minutes. Chambers were rinsed, and the total number of migrating cells was quantified by counting the number of CTG-positive cells per well ($n = 3$) for each condition.

Time-lapse Analysis

Live chemotaxis was measured by means of μ-Slides (Ibidi GmbH) according to the manufacturer's instructions.[17] Detailed procedures can be found in Supplemental Experimental Procedures.

Western Blot Analysis

Protein extracts were resolved with Novex 10% Bis-Tris gels (NuPAGE, Invitrogen) and transferred onto a PVDF membrane (Roche) according to standard protocols. Blots were then probed with primary and secondary antibodies. Blots were imaged with the ImageQuant 350 scanning system (cooled-CCD camera, GE Healthcare). Detailed procedures and buffer composition can be found in Supplemental Experimental Procedures.

Gene Expression Profiling Using Real-time PCR Arrays

SVZs were dissected as previously described ($n = 12$), and total RNA was isolated using Trizol reagent (Invitrogen) and then repurified using a column (RNeasy Mini Kit; Qiagen) according to the manufacturer's protocol. PCR array analysis was performed using RT^2 profiler PCR array (mouse chemokines and cytokines, PAMM-150, SABiosciences). Detailed procedures can be found in Supplemental Experimental Procedures.

Real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Detailed procedures can be found in Supplemental Experimental Procedures.

Processing of Tissue Sections and Cell Cultures Before Immunostaining

Mice were anesthetized with an injection of Nembutal (pentobarbital 60 mg/mL, Ceva Sante Animal) before an intracardiac perfusion with a NaCl 0.9% solution (Prolabo, VWR International) followed by 4% PFA at 4°C (4,3 g/L NaOH, 40 g/L paraformaldehyde, 18.8 g/L

NaH_2PO4). Brains were collected, postfixed in 4% PFA, and cryoprotected overnight in a solution of PBS/sucrose (20%). Brains were frozen at −20°C in a 2-methylbutane solution (Sigma) and cut into 16 μm thick coronal sections using a cryostat. For immunocytofluorescence, cells were placed on coverslips previously coated with polyornithine for 3 hours (0.1 mg/mL, Sigma). Cells were washed in PBS, fixed in 4% PFA for 15 minutes, and washed in PBS.

Immunostaining

Brain coronal sections or GBM cells were permeabilized, and unspecific binding sites were blocked using 10% donkey serum and 0.1% Triton X-100 PBS solution. Tissue sections or cells were incubated with primary antibodies diluted in PBS containing 0.1% donkey serum and 0.1% Triton X-100, followed by a second incubation with RRX- or FITC-conjugated secondary antibodies (1:500, Jackson Immunoresearch Laboratories). Detailed procedures can be found in Supplemental Experimental Procedures.

Enzyme-linked Immunosorbent Assay Analysis

CXCL12 concentrations in SVZ/cerebellum/olfactory bulb (OB)-conditioned medium were analyzed by sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems).

Plasmids, Lentiviral Vectors Generation, and Transduction

Detailed information and procedures can be found in Supplemental Experimental Procedures.

Bioluminescence Imaging

Immunodeficient nude mice bearing intracranial xenografts were injected intraperitoneally with D-luciferin (150 mg/kg, Sigma). After anesthesia using 2.5% isoflurane, mice were imaged with a charge-coupled device camera-based bioluminescence imaging system (IVIS 50, Xenogen; exposure time 1–30 s, binning 8, field of view 12, f/stop 1, open filter). Signals were displayed as photons/s/cm^2/sr. Regions of interest were defined manually, and images were processed using Living Image and IgorPro Software (Version 2.50). Raw data were expressed as total photon flux (photons/s).

Image Acquisition and Data Analysis

Immunostained sections were imaged using a laser-scanning confocal microscope equipped with a krypton/argon gas layer (Olympus Fluoview 1000). Zeiss Axiovert 10VR microscope (Carl Zeiss), coupled with Mercator software (Explora Nova), was used for cell counting and 3D reconstructions. Figures were composed and examined using ImageJ.

Statistical Analysis

Quantitative data are expressed as mean ± SEM. Two-way ANOVA, followed by a Tukey posttest was used, and a P value <0.05 was considered statistically significant. Student t tests were performed for 2 groups were compared using Statistica 10.0 software.

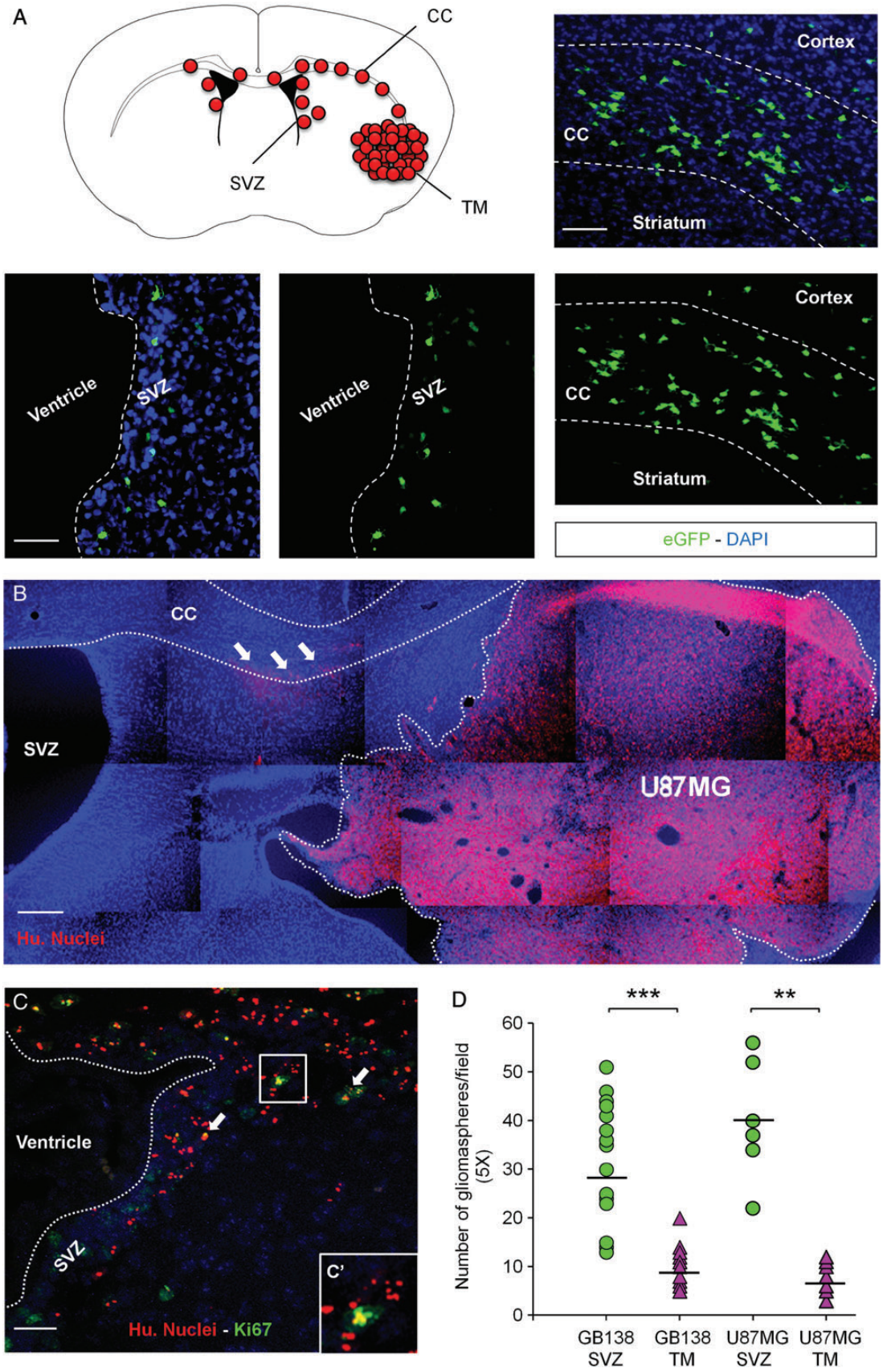
Results

In Vivo Model of GSC Invasion

We previously demonstrated that human GSCs are able to invade the SVZ environment once injected in the striatum of immunodeficient mice.[3] Using this model of cancer cell invasion, we grafted human primary GBM cells (GB138) expressing eGFP in the striatum of mice tolerating xenografts. Twelve weeks after the injection, as the mice developed tumors (TM), we identified primary GB138 cells in the SVZ environment. As expected, those cells migrated along the corpus callosum (CC), one of the main white matter structures connecting both left and right hemispheres together (Fig. 1A). We then validated this specific model of SVZ invasion with human U87MG cells, which were injected into the striatum of immunodeficient mice as well. Three weeks after the injections, we identified U87MG cells in the CC of mice using specific antihuman nuclei antibodies (white arrows, Fig. 1B). By the end of the fourth week after the graft, human U87MG cells had colonized the SVZ environment, with some of the cells retaining their ability to proliferate as shown by a Ki67-positive staining (white arrows, Fig. 1C and C′). Relying on this specific model of brain cancer invasion, we isolated U87MG and GB138 cells from the TM (U87MG-TM and GB138-TM) and from the SVZs (U87MG-SVZ and GB138-SVZ). We tested the ability of those 4 cell subtypes to form gliomaspheres (Fig. 1D). Being able to form gliomaspheres is indeed part of the cancer stem cell definition.[18] Interestingly, both U87MG-SVZ and GB138-SVZ cells were better to grow as floating spheres compared with U87MG-TM cells ($P = .002$) and GB138-TM cells ($P = .0001$).

Expression of Chemokines in the SVZ Environment

In order to identify potential targets involved in the GSC-specific invasion of the SVZ, we looked for soluble factors secreted by the SVZ environment that could eventually play a role in this migration phenotype. To do so, total mRNA extractions from 12 independent SVZ whole mounts were performed, and a wide scale RT-qPCR array was conducted. This analysis highlighted high mRNA levels (*Ccl12, Ccl19, Cxcl12* and *Cx3cl1* - [yellow rim of the graph]), basal mRNA levels (*Ccl5, Ccl17, Cxcl10* and *Cxcl16* [purple rim of the graph]), and low mRNA levels (*Xcl1, Ccl1, Cxcl3* and *Cxcl9* [white rim of the graph]) of cytokines and chemokines present in the SVZ environment (Fig. 2A). This database provides a "big picture" summary of the different factors that could be involved in the GSC-specific invasion of the SVZ. From those data, we identified CXCL12 as the main target of our study. Indeed, CXCL12 has already been shown to be involved in glioma cell proliferation.[19,20] We first confirmed CXCL12 mRNA expression on SVZ whole mounts, using mesenchymal stem cells as a positive control (Fig. 2B). We studied the expression of CXCL12 on brain coronal sections of mice previously injected with human U87MG cells and highlighted a close relationship between the expression of CXCL12 and the presence of U87MG GSC in the SVZ (Fig. 2C). We confirmed that observation on coronal sections of brains injected with human GB138 primary cells labeled with eGFP (Fig. 2D and E). We managed to put to light a gradient of CXCL12 within the SVZ environment (Fig. 2F). To do so, we transformed each CXCL12 acquisition from the SVZ environment into binary images and quantified the CXCL12 expression in



predefined areas (A, SVZ; B, transition SVZ-striatum; C, striatum). We systematically found a constant decrease of the CXCL12 expression starting from area A to C. This suggests that CXCL12 is mostly secreted in the SVZ and diffuses towards the striatum along a decreasing concentration gradient. To further explore the expression of CXCL12, we decided to compare, by ELISA, the secretion of CXCL12 within the SVZ with other regions of the brain such as the cerebellum and the OBs. We found a significant increase of the CXCL12 expression within the medium conditioned by SVZ whole mounts for 60 hours compared with 24 hours ($P = .0006$). Moreover, a significantly higher amount of CXCL12 was found in the medium conditioned by SVZ whole mounts for 60 hours compared with media conditioned either by OB or cerebellum whole mounts for 60 hours ($P = .005$) (Fig. 2G). This shows how specific the CXCL12 expression is to the SVZ region. We finally demonstrated that the blood vessels overlaying the lateral wall of the ventricle (lectin staining) and the astrocytes (glial fibrillary acid protein [GFAP] staining) were sources of CXCL12 in the adult brain (Fig. 2H).

Expression of CXCR4 by Human GBM Cells

We then tackled the expression of CXCL12 receptors on human GBM cells. Using reverse transcription (RT-)PCR and Western blot approaches, we highlighted the expression of CXCR4 on 3 GBM cell lines (U87MG, U373, and LN18) and 2 GBM cell populations in primary cultures (GBM1 and GBM2) (Fig. 3A and B). We also checked the CXCR4 expression profile on U87MG cells, U87MG cells isolated from the TM (U87MG-TM), U87MG cells from the SVZ (U87MG-SVZ), and U87MG cells cultured as floating spheres (U87MG NS). We noticed a comparable expression of CXCR4 in each of these cell populations (Fig. 3C). We then characterized the U87MG spheres for the expression of CXCR4, together with immature markers and differentiation markers. Immunostained spheres revealed that CXCR4 is mainly associated with proteins such as Nestin, Sox2, βIII-Tubulin, and GFAP on GBM cells (Fig. 3D). We finally characterized gliomaspheres from U87MG-TM and U87MG-SVZ cells and labeled them with different GSC markers such as Prominin-1 (CD133) or Integrin α6 (ITGA6).[21] We demonstrated here that both gliomasphere subpopulations expressed important levels of CXCR4, Nestin, CD133, and ITGA6 (Fig. 3E).

We know that CXCL12 can bind a second chemokine receptor, CXCR7.[22] This recently discovered receptor is the center of attention in many publications on GBM, but its role in tumorigenesis remains unclear.[23,24] As far as we are concerned, we have not been able to detect a convincing CXCR7 expression by immunohistofluorescence or Western blot analyses (data not shown), making it unlikely to be involved in our model.

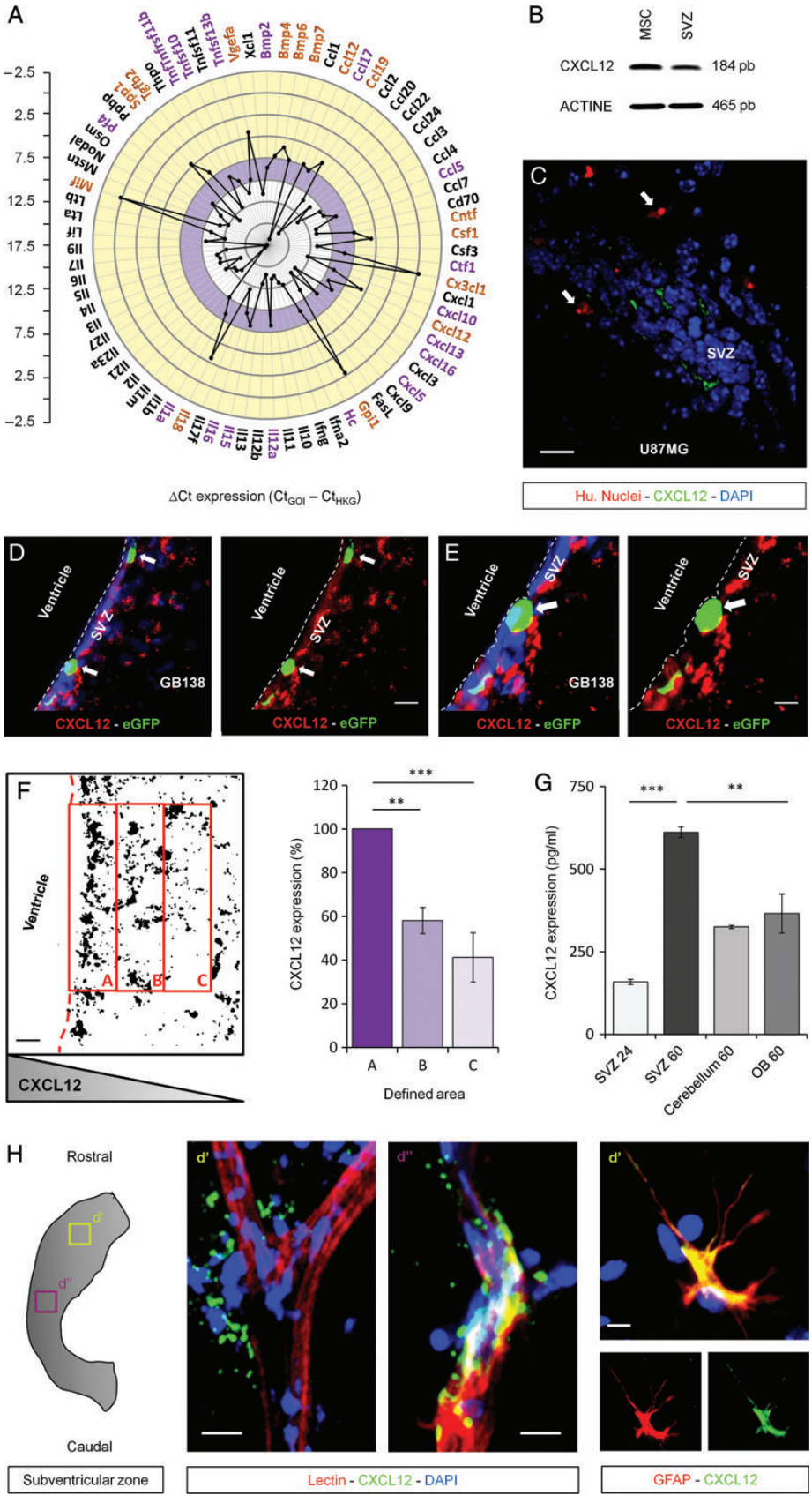
AMD3100 Disrupts Chemotactic Effects of SVZ-conditioned Medium on GBM Cells

To investigate whether human GBM cells home to the SVZ through secreted factors in a direct manner, we brought a modified Boyden chamber assay into focus. Human GBM cells were suspended in serum-free medium and seeded on top of a porous membrane, separating them from a bottom chamber filled with test or control media, which allowed the cells to move into the bottom part of the chamber in response to the chemotactic agent of interest. We first tested the ability of U87MG cells to migrate in response to various concentrations of CXCL12 and serial dilutions of SVZ-CM. U87MG cells displayed a dose-dependent migration behavior in response to both stimulations (Fig. 4A and C). To confirm the U87MG tropism towards a gradient of CXCL12 and SVZ-CM, we seeded U87MG cells in chemotaxis μ-Slides. We then recorded and analyzed the U87MG cell tracks over a period of 20 hours in response to both stimulations. The overall distribution of migration angles was analyzed and revealed significant clusters of migration direction in response to the CXCL12 gradient ($P < .001$) (Fig. 4B) and the SVZ-CM gradient ($P = .0006$) (Fig. 4D).

We then evaluated the impact of AMD3100 on the U87MG cell migration using our modified Boyden chamber assays. CXCL12/CXCR4 signaling can indeed be blocked using AMD3100,[25] a bicyclam noncompetitive antagonist of CXCR4.[26] Adding AMD3100 (25 nM) in the SVZ-CM was followed by a significant reduction (−28.2%) of the number of U87MG cells ($P = .005$) migrating through the filter. AMD3100 also inhibited the migration abilities of GBM primary cells in response to the SVZ-CM (−38.6%, GBM2; $P = .0001$) (Fig. 4E). The impact of AMD3100 was once again confirmed on U87MG cells using cell-track recordings and time-lapse analyses. AMD3100 clearly disrupted the U87MG distribution of migration angles in response to SVZ-CM ($P = .697$) (Fig. 4F) compared with SVZ-CM alone (Fig. 4D). However, AMD3100 did not impact the mean accumulated distance or velocity of U87MG cells, suggesting that other chemokines in the SVZ-CM could also play a role in the in vitro U87MG migration behavior in response to SVZ-CM (Fig. 4G and H).

We finally compared how U87MG cells, U87MG-TM cells (isolated from the tumor mass), and U87MG-SVZ cells (isolated from the SVZ) behaved in response to recombinant CXCL12 or SVZ-CM supplemented or not with AMD3100. Surprisingly, U87MG-SVZ cells displayed greater migration ability in response to recombinant CXCL12 than U87MG cells or U87MG-TM cells ($P < .0001$). Similarly, U87MG-SVZ cells were also more attracted by the SVZ-CM in comparison with the other cell populations ($P < .0001$). Interestingly, the in vitro inhibition of migration by AMD3100 was also more obvious on the U87MG-SVZ cell population compared with their counterparts isolated from the tumor mass. Using AMD3100 (25 nM) allowed to decrease the

Fig. 1. Invasion of the subventricular zone (SVZ) by U87MG cells and GB138 primary cells after intrastriatal implantation. (A) Human GB138 cells specifically colonized the SVZ environment after migration through the corpus callosum (CC). (B) Human U87MG cells invaded the largest part of the right striatum 3 weeks after the graft. At this point, U87MG cells had already escaped the tumor mass (TM) and began to migrate along the CC (white arrows). (C) U87MG cells finally invaded the SVZ environment 4 weeks after the injection, with some of them still being in a proliferation state (white arrows, magnified square). (D) U87MG and GB138 cells isolated from the SVZ displayed stronger abilities to form gliomaspheres than U87MG or GB138 cells isolated from the TM. Human U87MG cells were detected using an antihuman nuclei antibody (Hu. Nuclei - red). GB138 cells were engineered to express the green fluorescent protein (eGFP - green). Cell nuclei were counterstained with DAPI (blue). Scale bars = 500 μm for B, 40 μm for A and C. ** $P < .01$, *** $P < .001$.



U87MG-TM and U87MG-SVZ migration levels up to 17.7% (not statistically significant) and 44.2% respectively ($P < .0001$) (Fig. 4I), suggesting a stronger impact of the drug on GBM cells isolated from the SVZ and previously characterized as a population enriched in GSC abilities.[3]

Depletion of CXCR4 Inhibits the GSC Invasion of the SVZ

We generated stable CXCR4-invalidated U87MG cells using 2 different shRNA-mediated knockdowns. Those cells were also engineered to express a fusion protein of firefly luciferase and eGFP (U87MG-EIL-shCXCR4* and U87MG-EIL-shCXCR4**). Control cells for this experiment were built through expression of a scrambled shRNA (U87MG-EIL-sc).

We first confirmed the depletion of CXCR4 by Western blot analysis and validated those data by immunocytofluorescence (Fig. 5A and B). The expression of CXCR4 was downregulated by 41% and 64%, respectively, in the U87MG-EIL-shCXCR4* and U87MG-EIL-shCXCR4** cell populations (Fig. 5A). We then tested the functionality of those 2 CXCR4-depleted cell populations using modified Boyden chambers to evaluate chemotaxis in response to recombinant CXCL12. The migration of both CXCR4-depleted U87MG cells was significantly reduced, regardless of the remaining level of CXCR4 (Fig. 5C). We finally grafted the 2 populations of CXCR4-depleted U87MG cells and U87MG control cells into the striatum of immunodeficient mice. As expected, human U87MG cells, labeled with eGFP, were found in the CC and the SVZ region of control mice after 4 weeks (Fig. 5D). Surprisingly, this invasion phenotype was totally hampered in the CXCR4 knockdown conditions, in which no eGFP signal could be detected in the SVZ environment (Fig. 5E). There were no differences observed in mean body mass between groups (data not shown).

AMD3100 Inhibits the Specific GSC Invasion of the SVZ

We injected luciferase-expressing U87MG cells into the right striatum of immunodeficient nude mice. Animals bearing xenografts were separated into 2 homogeneous groups at day 25 post injection according to in vivo bioluminescence data. Indeed, U87MG cells expressing the luciferase enzyme allowed to monitor noninvasive imaging of tumor-associated bioluminescence and quantification of tumor growth over time.[27] Half of the cohort was treated twice a day with intraperitoneal injections of AMD3100 at a concentration of 1.25 mg/kg, whereas the other half of the cohort was treated with PBS (control). The treatment started at day 25 in order to minimize the already-known impact of AMD3100 on tumor growth[11] and, more specifically, to treat the mice during the particular window of time when human GSC started to invade the SVZ.[3] Growth curves established from serial measurements of bioluminescence revealed a significant antitumor effect of AMD3100 in U87MG-xenografted mice ($P < .01$) (Fig. 6A). This observation was consistent with the fact that tumor volumes were on average 28% smaller in the AMD3100-treated group after histological examinations (data not shown). This reduction of tumor volume was nevertheless not statistically significant due to the high variability between animals bearing xenografts ($P = .65$).

Regarding the invasive abilities of U87MG cells, the number of GBM cells in the CC of AMD3100-treated animals was significantly reduced compared with control animals ($P < .001$) (Fig. 6B and C). More importantly, we were not able to detect the presence of any U87MG cells in the SVZ environment of AMD3100-treated animals ($P < .01$) (Fig. 6B and C). We then showed that AMD3100 does not induce U87MG cell death in xenografted animals after treatment (Supplementary data, Fig. S2). In this way, we demonstrated that the inhibition of SVZ invasion by U87MG GSC is CXCR4 dependent and not a consequence of a loss of cell viability. On the other hand, U87MG cells had left the tumor mass and specifically invaded the SVZ environment through the CC in the control animals (Fig. 6C).

Discussion

The hypothesis of cancer stem cells has often been proposed to explain therapeutic failure and recurrence in a variety of cancers including GBM.[28] In this case, it has been suggested that GSCs are both radioresistant[29,30] and chemoresistant.[31] Previously, we demonstrated that one of the known neurogenic zones of the adult brain, the SVZ, was able to specifically attract and host GSCs.[3] We correlated this observation with the fact that cancer stem cells often tend to hide in very specific niches, which could help maintain their stem-like abilities and capacity to drive tumor growth[7,8] as well as influence their intrinsic resistance to treatments.[32] Moreover, this observation creates an interesting parallel between GSCs and neural stem cells, which also remain located in specific neurogenic zones in order to maintain their stem cell properties.[33] A recent clinical study strengthened the link between neurogenic niches and malignant gliomas even more. This study demonstrated that patients whose bilateral SVZs received greater than the median SVZ radiation dose showed significant improvement in progression-free survival.[34] Taken together, these data suggest that the SVZ environment could generously host and protect GSCs from radiation and chemotherapy and therefore play a key role in GBM relapses.[35,36] We

Fig. 2. Expression of CXCL12 by the subventricular zone (SVZ) environment. (A) RT-qPCR screening notably displayed a high expression level of CXCL12 in the SVZ environment (yellow rim of the graph). (B) This observation was validated by RT-PCR using mesenchymal stem cells (MSC) as a positive control. (C–E) The expression of CXCL12 was next demonstrated on brain coronal sections. This expression was consistent with the presence of U87MG cells (Hu. Nuclei) or GB138 primary cells (eGFP) in the SVZ (white arrows). (F) CXCL12 acquisitions were processed as binary images. The mean intensity, with foreground 255 and background 0, in predefined areas of the SVZ environment (A, B, and C) was calculated. A constant decrease of the CXCL12 expression was observed starting from area A to end at area C, suggesting that CXCL12 is secreted along a decreasing concentration gradient. (G) CXCL12 levels were evaluated by ELISA in conditioned media from SVZ, cerebellum, and olfactory bulb (OB) whole mounts for 24 or 60 hours. (H) CXCL12 was expressed by astrocytes and endothelial cells within the adult SVZ. Immunostaining on organotypic whole mounts showed a closely related expression of CXCL12 (green) with the vasculature and astrocytes (red). SVZ blood vessels and astrocytes were respectively stained using a FITC-coupled lectin or a specific anti-GFAP antibody (red). Cell nuclei were counterstained with DAPI (blue). Scale bars = 20 μm for C and D and 10 μm for E and H. Caption indicates where pictures and materials were taken.

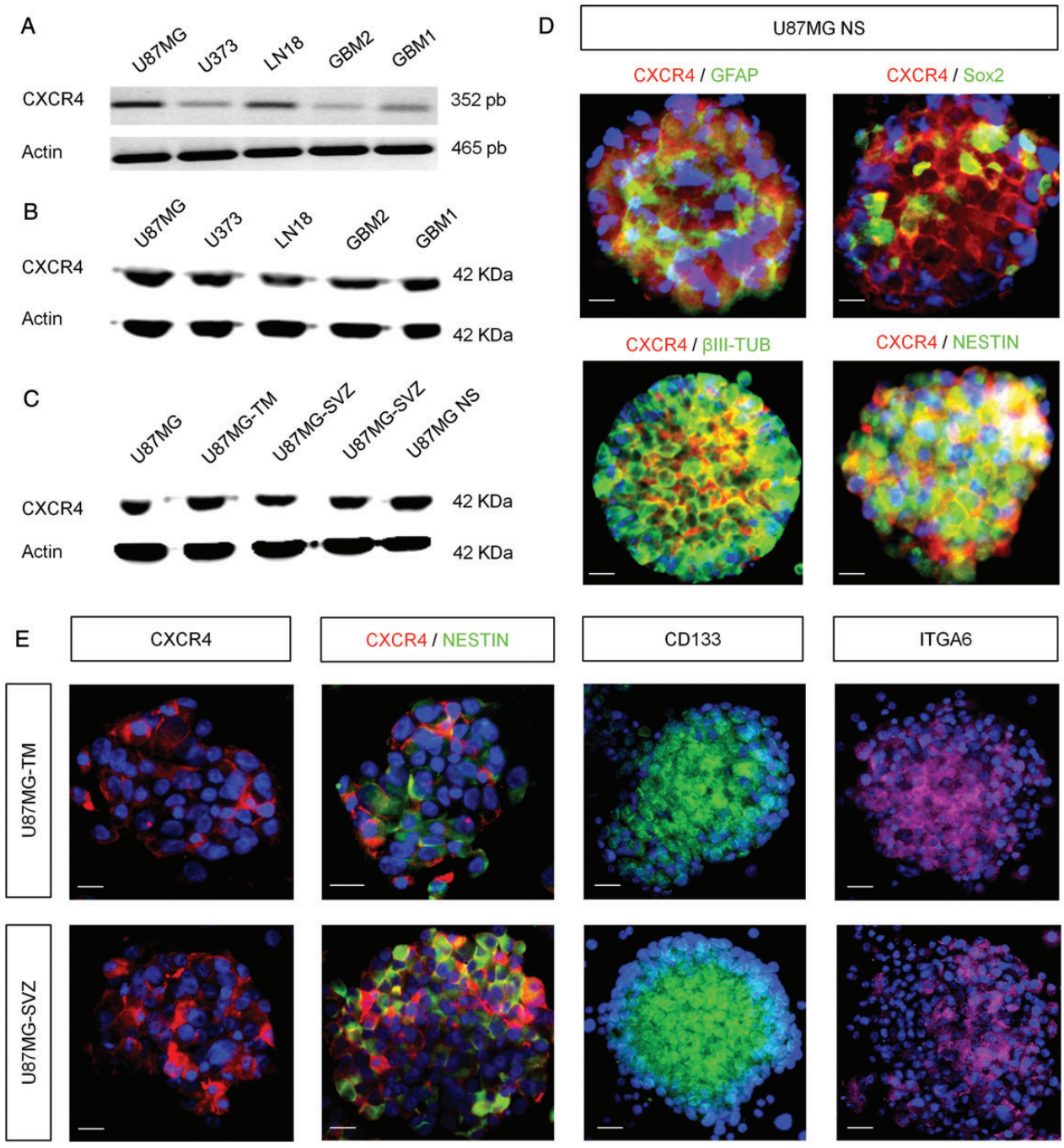


Fig. 3. Expression of CXCR4 by various GBM cell types. (A) *CXCR4* mRNA expression was found in 3 human GBM cell lines (U87MG, U373, and LN18) and 2 human primary cultures (GBM 1 and 2). (B and C) Western blot analyses displayed a strong CXCR4 expression pattern in GBM cell lines and primary cultures as well as in U87MG cells isolated from the tumor mass (U87MG-TM) or the SVZ (U87MG-SVZ) and U87MG floating gliomaspheres (U87MG NS). (D) Human U87MG NS showed a combined expression of CXCR4 (red) with GFAP, Sox2, Nestin, and βIII-tubulin proteins (green). E) U87MG-TM and U87MG-SVZ gliomaspheres specifically expressed CXCR4 (red) as well as GSC markers such as CD133 (green), Nestin (green), and Integrin α6 (ITGA6) (pink). Nuclei were counterstained with DAPI (blue). Scale bars = 15 μm for D and E.

should also keep in mind that the cell of origin for GBM, whether it derives from neural stem cells located in the SVZ or another cell type, remains undefined to date.[37–39] In this context, we decided to decipher the molecular mechanisms that underlie the oriented migration of GSCs to the SVZ. Indeed, prospective identification and targeting of GSCs seem mandatory to fully understand their biology, to counteract GBM relapses, and to develop new powerful therapeutic strategies.

As a starting point, we hypothesized that chemokine receptor CXCR4 would play a role. It is known that CXCR4 is expressed by

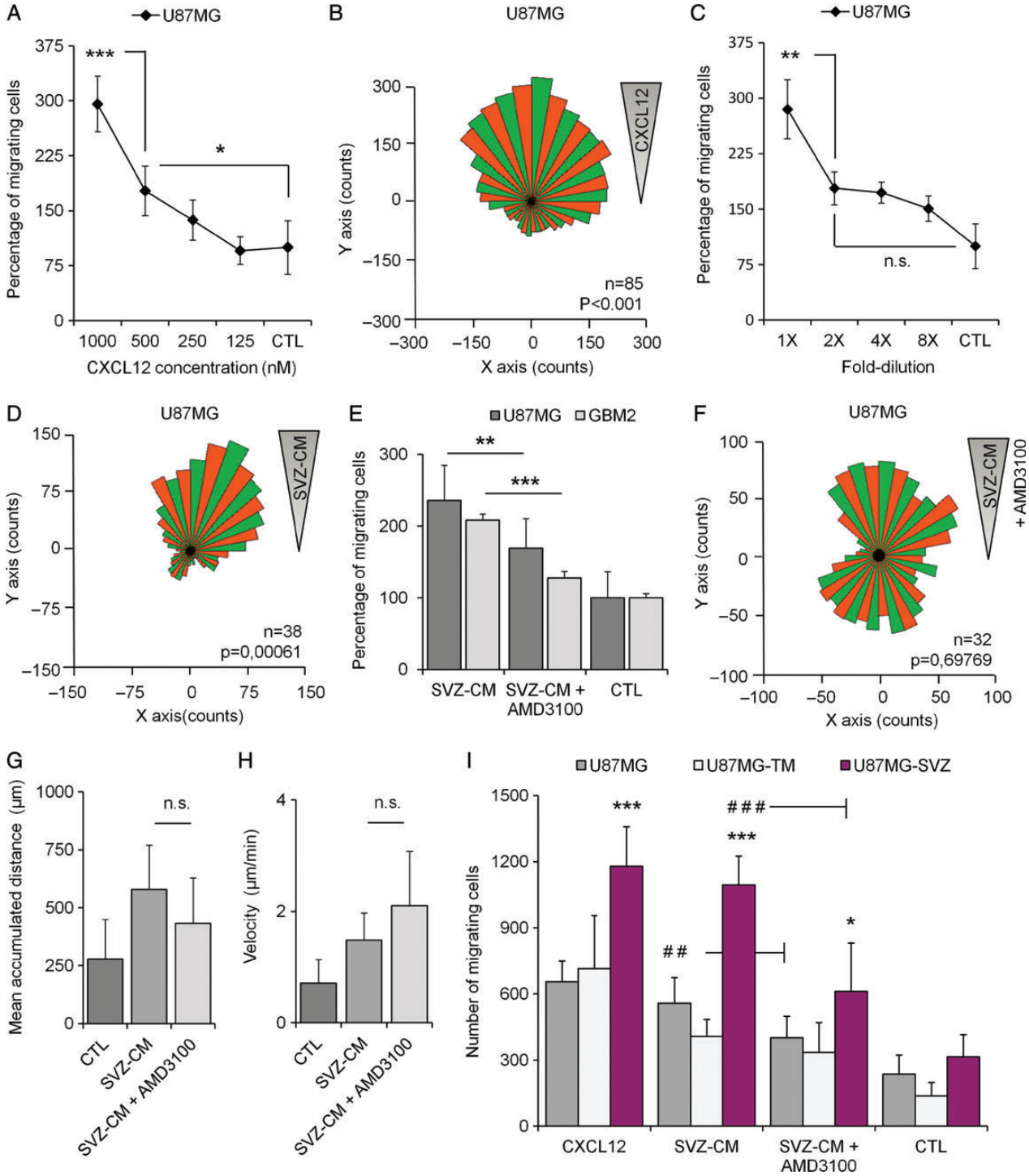


Fig. 4. In vitro migration of GBM cells in response to recombinant CXCL12 and subventricular zone-conditioned medium (SVZ-CM). (A and B) Recombinant CXCL12 triggered migration and chemotaxis of human U87MG cells. (C and D) SVZ-CM triggered migration and chemotaxis of human U87MG cells. (E) The migration of U87MG cells and human GBM cells in primary culture (GBM2) in response to SVZ-CM was significantly reduced by using AMD3100, a specific CXCR4 antagonist. (F) AMD3100 disrupted chemotaxis of U87MG cells in response to SVZ-CM. (G and H) AMD3100 did not impact parameters such as the mean accumulated distance (µm) and velocity (µm/min) of U87MG cells. (I) U87MG-SVZ cells showed greater migration abilities in response to recombinant CXCL12 and SVZ-CM compared with U87MG cells or U87MG-TM cells. Moreover, AMD3100 clearly inhibited the migration of U87MG-SVZ cells in response to SVZ-CM. Graphs are mean values ± SEM and are representative of 3 independent experiments. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; ns, not significant.

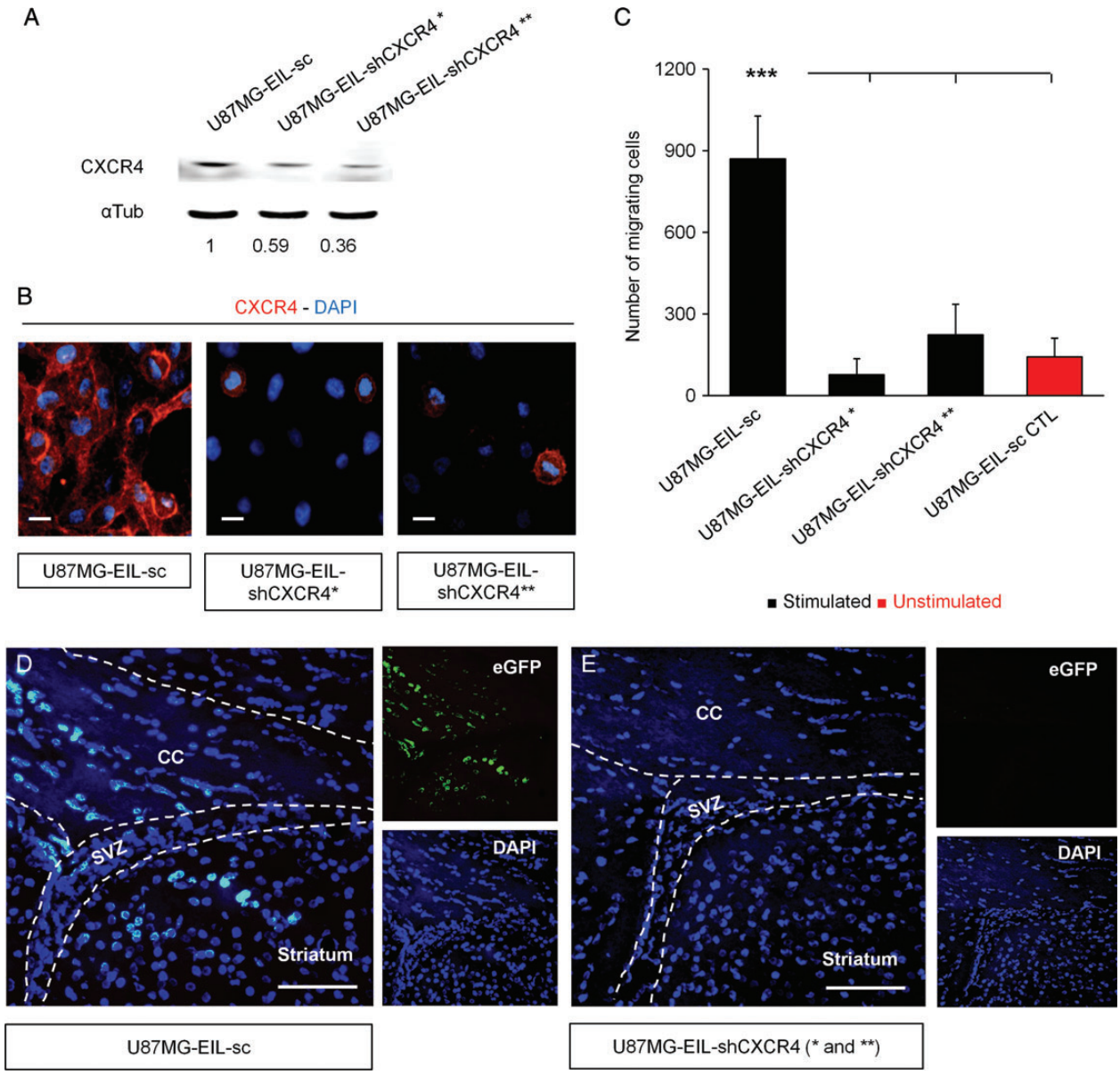


Fig. 5. Inhibition of the SVZ invasion by CXCR4-depleted U87MG cells. (A and B) Western blot and cytological analyses of the CXCR4 expression in U87MGeGFP-Ires-Luc cells infected with lentiviruses encoding either a scrambled shRNA (U87MG-EIL-sc) or 2 short hairpins-RNA directed against CXCR4 (U87MG-EIL-shCXCR4* and U87MG-EIL-shCXCR4**). CXCR4 expression was declined up to 41% in the U87MG-EIL-shCXCR4* cells and up to 64% in the U87MG-EIL-shCXCR4** cells. (C) CXCR4 knockdown also decreased the U87MG cells in vitro migration in response to recombinant CXCL12 (1000nM) compared with the absence of CXCL12 (U87MG-EIL-sc CTL). (D and E) Animals were injected with either control U87MG cells (U87MG-EIL-sc, $n = 5$ mice) or with CXCR4-depleted U87MG cells (U87MG-EIL-shCXCR4* and U87MG-EIL-shCXCR4**, $n = 5$ mice per group). Human U87MG cells invaded the corpus callosum (CC) and the SVZ of mice grafted with U87MG-EIL-sc. No eGFP signal could be detected in the CC or the SVZ of both shRNA conditions. Scale bars = 10 μm and 100 μm, respectively, for B and D-E. ***$P < .001$.

different types of cancer cells.[9] This receptor is notably involved in cancer cell proliferation and invasiveness.[11,40] The expression level of CXCR4 is usually associated with a poor prognosis in GBM patients[12] and has been shown to be a key mediator in GBM invasion.[10] Recent findings showed that inhibition of the CXCL12/CXCR4 autocrine/paracrine loop reduces viability of human glioblastoma stem-like cells affecting self-renewal activity.[41] In this work, SVZ whole mounts showed a staining pattern consistent with the expression of CXCL12 by adult SVZ blood vessels and released in the nearby environment. Interestingly, it has also been suggested that GSCs are maintained in vascular niches.[42] We observed that the expression of CXCL12 in the SVZ is closely related to capillaries which, in turn, have been shown to play a crucial role in maintaining neural stem cell stemness in the adult brain.[43,44] It could be tempting to hypothesize that those capillaries are also active on GSCs, notably by secreting

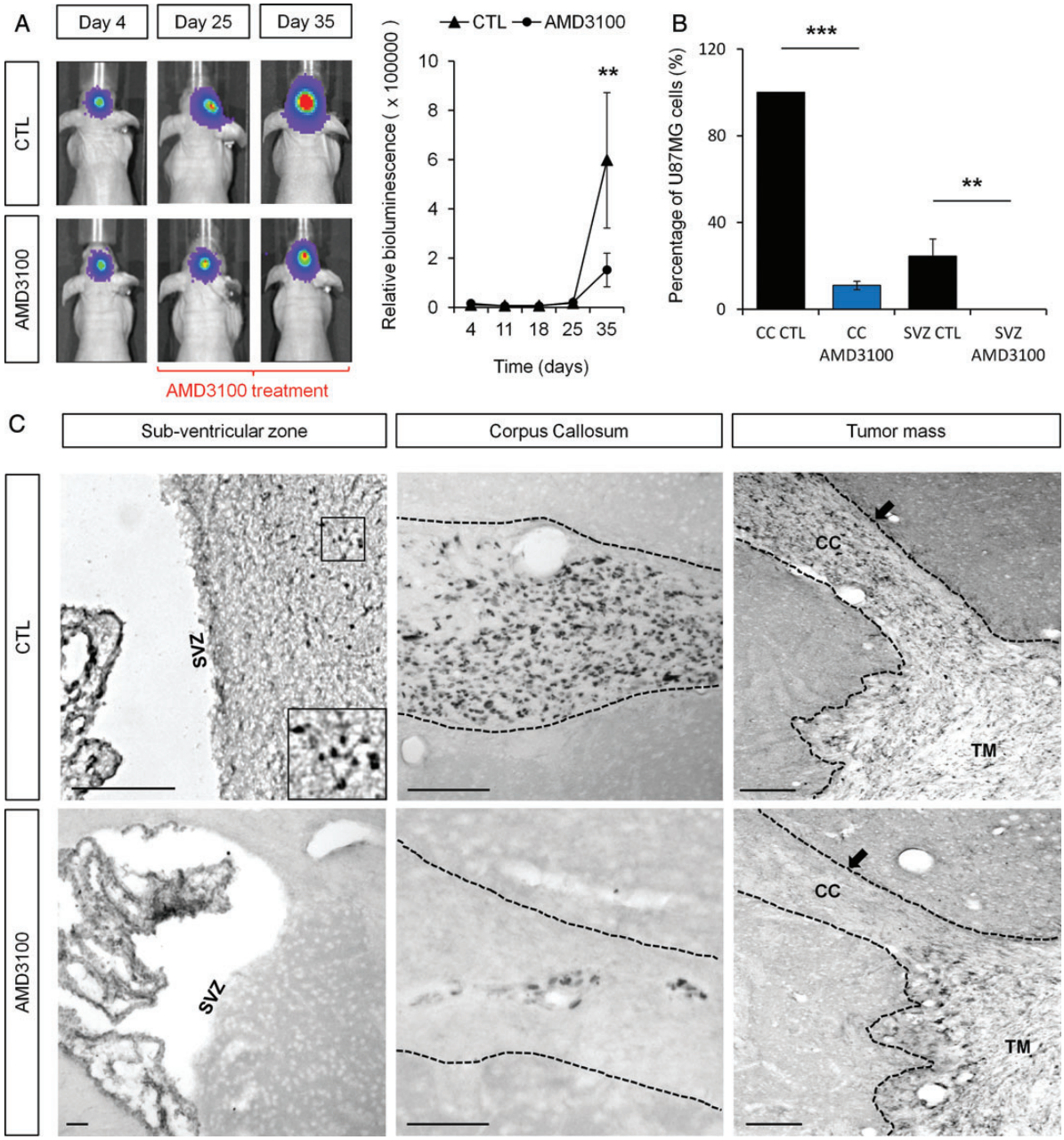


Fig. 6. AMD3100 inhibited the U87MG GSC invasion of the subventricular zone (SVZ). (A) AMD3100-treated animals showed significantly smaller amounts of relative bioluminescence compared with the control group ($P < .01$) at the end of the treatment period. (B and C) Only a few U87MG cells were found in the corpus callosum (CC) of AMD3100 treated-mice, whereas U87MG cells fully invaded the CC of PBS-treated mice (control). U87MG cells could not be found in the SVZ area in AMD3100-treated animals, whereas U87MG cells from the control group invaded the SVZ as expected. U87MG cells are labeled with a specific antihuman nuclei antibody. Scale bar = 100 μm for C. Graphs are mean values ± SEM. $^{**}P < .01$, $^{***}P < .001$.

CXCL12. In the same line, it has recently been demonstrated that brain endothelial cells secrete factors that are thought to support the expansion of GSCs by recruiting the mTOR pathway.[45] It would therefore be interesting to examine the role of the mTOR pathway in GSC invasion as a future perspective.

Human U87MG cells and GBM primary culture cells display in vitro migration abilities in response to the SVZ soluble environment (SVZ-CM). This phenotype was shown to be partially inhibited using AMD3100, a CXCR4-specific inhibitor. That observation clearly suggested the eventual role played by other chemokines in the SVZ-CM. Indeed, different mRNA levels of chemokines were detected in the SVZ environment. Several of those chemokines were found in the SVZ-CM as well. A proteome profiler analysis showed that CXCL1 was the most highly

expressed chemokine in the SVZ-CM (Supplementary data, Fig. S1). This new target could be really helpful for better characterizing the controversial cell type responsible for the onset of gliomagenesis. CXCL1 has indeed been shown to promote proliferation of early oligodendrocyte progenitor cells,[46] which in turn have recently been suggested to be at the origin of malignant brain tumors.[47] Consequently, it would be worthwhile to investigate the status of CXCL1 as a target that might either support the CXCL12/CXCR4-dependent migration of GSC to the SVZ or play another role in GBM biology.

We also think it is mandatory to direct major attention to the cancer cell population isolated from the SVZ. Those cells, located away from the initial tumor mass, retain their ability to initiate development of new tumors when secondarily injected into new animals and could therefore be at the origin of GBM relapse.[3] In-depth investigation on this cell subtype could provide tremendous new insights on the biology of cancer stem cells. In vitro migration bio-assays showed that U87MG cells isolated from the SVZ display enhanced migration abilities in response to CXCL12 and SVZ-CM compared with U87MG cells isolated from the tumor mass. Future analysis of the molecular response to both stimulations in these 2 different cell subtypes thus makes sense for a better understanding of the molecular mechanisms that could lead GBM to systematically relapse and to improve current treatments.

"Inhibition of the in vivo CXCL12/CXCR4 signaling, using either lentiviral vectors allowing the expression of specific CXCR4 shRNAs or blocking CXCR4 with a specific antagonist (AMD3100) led to the same observation: GSCs were not able to home to the SVZ anymore." Tumor volumes in the AMD3100-treated animals were on average 28% smaller than tumors in the control group but most importantly as the main message of the present work, AMD3100 partially blocked the CC invasion and prevented U87MG cells to reach the SVZ environment. From a clinical aspect, the safety of AMD3100 (also known as Plerixafor or Mozobil) has been evaluated in clinical trials[25] and has raised great hope for new potential clinical implications. This FDA-approved drug has successfully been tested in patients with non-Hodgkin's lymphoma and multiple myeloma, in order to support optimal stem cell mobilization for autologous stem cell transplantation.[48,49] Plerixafor, in combination with Avastin (an anti-VEGF antibody) is now under intense investigation in a phase I clinical trial with the aim of preventing the growth of recurrent high-grade gliomas (NCT01339039[50]).

Taken together, our observations reported in the present manuscript, as well as our previous work,[3] have demonstrated that the SVZ environment is able to attract and harbor GSCs via the CXCL12/CXCR4 pathway. By this chemokine signaling system, it is therefore tempting to speculate if neurogenic zones in the adult brain may constitute a reservoir for tumor recurrence. Counteracting with the molecular mechanisms, which drive the migration of GSCs to neurogenic zones, might therefore be of great importance for future definition of therapeutic strategies.

Supplementary Material

Supplementary material is available at *Neuro-Oncology Journal* online (http://neuro-oncology.oxfordjournals.org/).

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Conflict of interest statement. The authors declare that they have no conflicts of interest.

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Supplemental Information

Supplemental Data

Chemokine Profiler Array on SVZ-CM

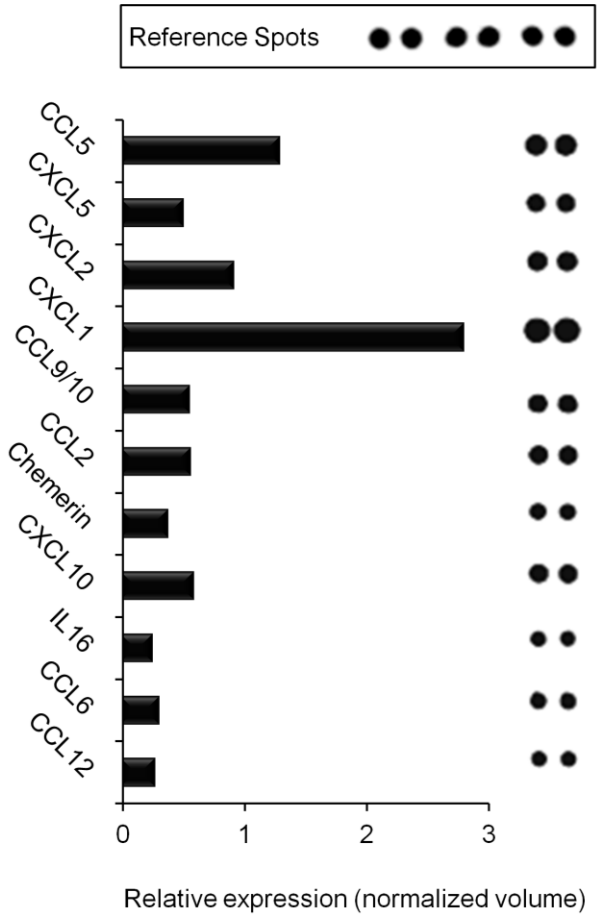

Figure S1. A proteome profiling analysis of culture media conditioned for 60 hours with 12 different SVZ whole-mounts was performed and allowed to draw a non-exhaustive list of chemokines secreted by the SVZ environment such as CCL2, CCL5, CCL6, CCL9/10, CCL12, CXCL1, CXCL2, CXCL5, CXCL10, IL16 and Chemerin.

CXCR4 Blockade Effects on Survival

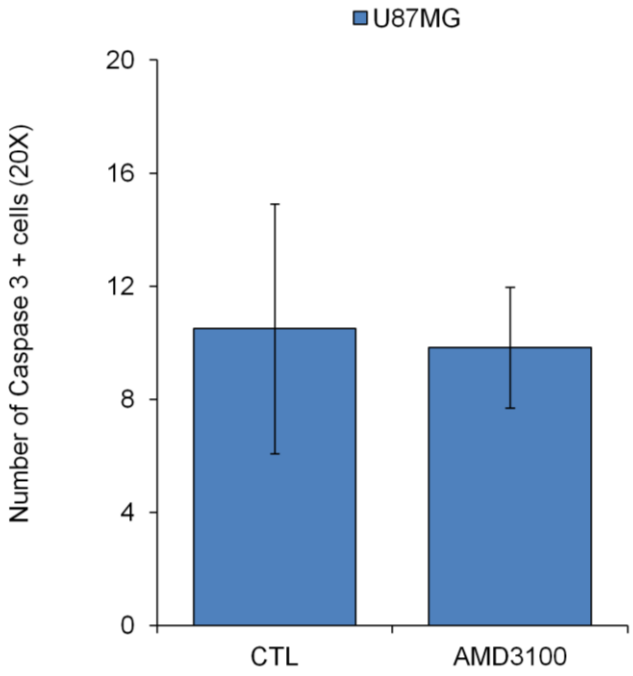

Figure S2. CXCR4 blockade with AMD3100 does not reduce survival of GBM cells. Activated caspase 3 stainings on brain coronal sections from animals previously injected with U87MG cells were performed in the control group (PBS-treated animals) or the AMD3100 treated group in order to check whether AMD3100 was able to induce tumor cell death at 35 days post-tumor cell implantation. A loss of tumor cell viability could indeed account for the inhibition of the U87MG cell invasion of the SVZ. We showed here that AMD3100 treatment does not increase the number of apoptotic cancer cells in U87MG transplanted animals. In this way, we demonstrated that the inhibition of the U87MG cell migration towards the SVZ is not a consequence of a loss of cell viability.

Supplemental Experimental Procedures

Cell culture

To stimulate the formation of floating spheres, cells were cultivated in DMEM/F12 serum-free medium containing B27 without vitamin A (Invitrogen®) and supplemented daily with recombinant human epidermal growth factor and recombinant human fibroblast growth factor 2 (EGF, 20 ng/mL and FGF-2, 10 ng/mL, respectively, Preprotech®, Rocky Hill, NJ, USA). U87MG cells and primary GB138 cells were isolated from the SVZ and striatum after dissecting both regions. The wholemounts were then incubated in papaïn (Worthington®, Lakewood, NJ, USA) for 30 min at 37°C and ovomucoïd (Worthington®) was next added to stop the dissociation. Cells were then mechanically dissociated and plated in six-well dishes in DMEM/F12 serum-free medium containing B27 without vitamin A (Invitrogen®) and supplemented with recombinant human epidermal growth factor and recombinant human fibroblast growth factor 2 (EGF, 20 ng/mL and FGF-2, 10 ng/mL, Preprotech®) at the density of 25.000 cells/mL. Human cells were selected with one passage allowing the selection of more than 99% of human cells. The human origin of those newborn spheres was moreover tested by immunocytostainings using a specific anti-human nuclei antibody (Millipore®). Cultures were maintained at 37°C under humidified atmosphere containing 5% carbon dioxide.

Whole-Mounts Dissection and Processing

The hippocampus and septum were removed. The dissected lateral walls were cultured for 60 hours in DMEM/F12 (Invitrogen®) and supplemented with recombinant human epidermal

growth factor and recombinant human fibroblast growth factor 2 (EGF, 20 ng/mL and FGF-2, 10 ng/mL, respectively, Preprotech®) in order to prepare whole-mounts conditioned medium. The different conditioned media were then centrifuged and directly frozen. For immunostainings, SVZ wholemounts were fixed in 4% PFA/0.1% Triton-X overnight at 4°C. The ventricular walls were finally dissected from underlying parenchyma as slivers of tissue from 200 to 300 µm in thickness, mounted on microscope slides with mounting media (Vectashield® with DAPI, Vector laboratory, Burlingame, CA, USA) and coverslipped.

Intracranial transplantation

Crl:NU-Foxn1^{nu} mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/mL, Pfizer®, Bruxelles, Belgium)/xylazine (Sedativum 2%, Bayer®, Bruxelles, Belgium) solution (V/V). The cranium was exposed and a small hole was drilled 2.5 mm lateral and 0.5 mm anterior to the bregma with a size 34 inverted cone burr (Dremel). Mice were positioned in a stereotactic frame and 50,000 cells in 2 µl PBS were injected into the right striatum through a 27-gauge needle over 1 min at 3 mm below the *dura mater*. The incision was closed with Vetbond (3M). U87MG GBM cells were grown in adherent culture conditions (DMEM containing 10% of fetal bovine serum) prior to injection.

Time lapse analysis

Human GBM cells were incubated on the µ-Slides for 3 hours at 37°C in a humid atmosphere. CXCL12 (1000nM), SVZ-CM or SVZ-CM supplemented with AMD3100 (25µg/ml) were used as chemo-attractants and added to the upper reservoir. Images were collected every 10 min for 20 hours on a Nikon A1R confocal microscope with a 10X objective equipped with a

CO_2- and temperature-controlled chamber. Data were analyzed for cell migration with Manual Tracking, a plug-in of ImageJ® (public domain Java image processing program, author: Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA) and the Chemotaxis and Migration tool from Ibidi GmbH®. Rose diagrams, which show distribution of migration angles calculated from x-y coordinates at the beginning and end of the cell tracks, were plotted and followed by the Rayleigh test to determine significant clustering of migration directions.

Western blot analysis

Protein extracts were obtained by lysing GBM cells in lysis buffer [10mM Hepes, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT and 10% NP-40, pH 7.9] supplemented with Complete Protease Inhibitors (Roche®, Brussels, Belgium). The proteins (50 µg) were resolved with Novex 10% Bis-Tris gels (NuPAGE®, Invitrogen) and transferred onto a PVDF membrane (Roche®) according to standard protocols. Blots were then probed with polyclonal anti-CXCR4 antibody (Abcam®, Cambridge, UK). Total protein loading per lane was evaluated with anti-β-Actin antibody (Abcam®). This was followed by incubation with HRP conjugated secondary antibodies (Santa Cruz Biotechnology®, Santa Cruz, CA, USA) and enhanced chemiluminescent (ECL) substrate (SuperSignal West Pico, Thermo® Scientific, Rockford, IL, USA). Blots were imaged with the ImageQuant 350 scanning system (cooled-CCD camera, GE Healthcare®, Diegem, Belgium).

Gene expression profiling using real-time PCR arrays

Remaining genomic DNA was removed from samples using DNAse (Quiagen®). 1 µg of total RNA was subjected to first-strand cDNA synthesis using RT^2 First Strand Kit (SABiosciences®, Frederick, MD, USA). PCR array analysis was performed using RT^2 profiler PCR array (SABiosciences®, mouse chemokines and cytokines, PAMM-150) on the ABI® 7000 (Roche®) using ready-to-use RT^2 SYBR Green qPCR master mix (SABiosciences®). Thermocycler parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Screening of relative mRNA expression was performed using the ΔCt method.

RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen®) according to the manufacturer's protocol. Reverse transcription reactions were performed using 5 µg of DNA-free RNA incubated with random primers (Promega®, Madison, WI, USA) for 10 min at 70°C, followed by 60 min at 37°C with a mixture of dNTP 10mM (Promega®), M-MMLV-Reverse Transcriptase (Promega®), DTT 10mM (Invitrogen®) and 5X reaction buffer (Tris-HCl 50mM, KCl 75mM, $MgCl_2$ 3mM, pH 8.3). PCR was performed using *CXCR4* (Rev: TTAGCTGGAGTGAAAACTTGA; For: GGCCCTCAAGACCACAGTCA) and *Cxcl12* (Rev: TAATTACGGGTCAATGCACA; For: GCTCTGCATCAGTGACGGTA) specific primers as well as *ACTIN* specific primers (Rev: CCTTCTGCATCCTGTCAGCAATG; For: ACACTGTGCCCATCTACGAGGG) as an internal standard. PCR were performed using Taq Polymerase (Promega®), 5X enzyme buffer (Promega®) and dNTP 10 mM (Promega®). PCR conditions were as follows: 5 min at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. PCR products were resolved on 1% agarose gels and stained with Midori-Green® (LabGene Scientific®, Châtel St. Denis, Switzerland).

Immunostainings

Brain coronal sections or cells on coverslips were permeabilized and unspecific binding sites were blocked for an hour at room temperature using a 10% donkey serum and 0.1% Triton X-100 PBS solution. Tissue sections or coverslipped cells were then incubated overnight at 4°C with primary antibodies diluted in PBS containing 0.1% donkey serum and 0.1% Triton X-100. Primary antibodies were directed against CXCR4 (rabbit polyclonal IgG, 1:250, Abcam®, goat polyclonal IgG, 1:200, Santa-Cruz®), CXCL12 (goat polyclonal IgG, 1:200, Santa-Cruz®), eGFP (rabbit polyclonal IgG, 1/1000, Abcam®), NESTIN (chicken polyclonal IgG, 1:250, Novus BioloGSCals®, Littleton, CO, USA), GFAP (mouse monoclonal IgG, 1:500, Sigma®), Human Nuclei (mouse monoclonal IgG, 1:250, Millipore®, Brussels, Belgium), SOX2 (goat polyclonal IgG, 1:250, Santa-Cruz®), β-III-tubulin (rabbit polyclonal IgG, 1:1000, Covance®, Belgium) and Ki67 (rabbit polyclonal IgG, 1:200, Abcam®), CD133 (rabbit polyclonal IgG, 1:200, Abcam®) and ITGA6 (rabbit polyclonal IgG, 1:200, Novex®). Brain slides were incubated for an hour at RT with RRX- or FITC-conjugated secondary antibodies (1/500, Jackson Immunoresearch Laboratories®, West Grove, PA, USA) and finally coverslipped in a mounting solution containing DAPI (Vectashield®).

Plasmids, lentiviral vectors generation and transduction

Gene/shRNA transfer lentiviral plasmids were *CXCR4* shRNA plasmids (Sigma®, TRCN0000004056 and TRCN0000256866), *eGFP* shRNA plasmid (Sigma®, SHC005) and pLenti6-eGFP-IRES-Luc. This last plasmid allows the dual eGFP and firefly luciferase (Photinus pyralis) expression and was generated by cloning eGFP sequence and the IRES of the encephalomyocarditis virus (ECMV) sequence (from pIRES2-eGFP Vector, Clontech®,

Mountain View, CA, USA) and Luciferase gene (from pGL3-Basic, Promega®) into the pLenti6/V5-D-Topo (Invitrogen®). Lentiviral vectors were generated by co-transfecting Lenti-X 293T cells (Clontech®) with a pSPAX2 (Addgene®, Cambridge, MA, USA) and a VSV-G encoding vector. Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus was generated along with the gene/shRNA transfer lentiviral plasmid (*CXCR4* or *eGFP* shRNAs plasmids or pLenti6-eGFP-IRES-Luc). Viral supernatants were collected 48h, 72h and 96h post transfection, filtrated and concentrated 100 x by ultracentrifugation. The lentiviral vectors were then titrated with qPCR Lentivirus Titration (Titer) Kit (ABM®, Richmond, BC, Canada). Human U87MG cells were first transduced with lentiviral vectors (15 TU/cell) allowing the dual expression of eGFP and luciferase. Transduced cells were selected with 1mg/mL Blasticidin (Sigma®). These cells were then transduced with shRNA lentiviral vectors (shRNA *CXCR4* or eGFP, 15 TU/cell) and selected with 1mg/mL Puromycin (Sigma®).

Chemokine profiler array

Screening for different chemokines in the SVZ-CM was performed with the proteome profiler™ array (mouse chemokine array kit, R&D Systems®, Minneapolis, MN, USA). The SVZ-CM was diluted and mixed with a cocktail of biotinylated detection antibodies for an hour. Any protein/detection antibody complex present is bound by its cognate immobilized capture antibody on the nitrocellulose membrane. Streptavidin-Horseradish Peroxidase and chemiluminescent detection reagents are then added and a signal is produced in proportion to the amount of chemokine bound. Dot blots (standardized for loading control) were imaged with the ImageQuant 350 scanning system (cooled-CCD camera, GE Healthcare®).

Paper 2

Mesenchymal Activation Mediated by CXCL12 in the Subventricular Zone Promotes Glioblastoma Resistance to Radiotherapy (Submitted)

Mesenchymal Activation Mediated by CXCL12 in the Sub-ventricular Zone Promotes Glioblastoma Resistance to Radiotherapy.

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Abstract

Patients with glioblastoma multiforme (GBM) have an overall median survival of 15 months despite multimodal therapy. This catastrophic survival rate is the consequence of systematic relapses which may arise from remaining glioblastoma stem cells (GSC) left behind after surgery. We and others have previously demonstrated that GSC are able to escape the tumor mass and specifically colonize the adult sub-ventricular zone (SVZ) after transplantation. This specific location, away from the initial tumor site, may therefore represent a high-quality model of clinical obstacle to therapy and relapses since GSC retain the ability to form secondary tumors. Relying on recent findings demonstrating the existence of GSC in the human SVZ and their potential implication in therapeutic resistance, we wondered whether the SVZ could endorse the role of an efficient GSC reservoir, potentially involved in malignant brain tumor relapses. In this context, we demonstrated SVZ-nested GSC to be specifically resistant to radiation *in vivo*. Interestingly, these cells displayed an enhanced mesenchymal phenotype compared to GBM cells present in the tumor mass. These mesenchymal traits were further shown up-regulated upon CXCL12 stimulation *in vitro*. In this line, we and others previously reported the SVZ to be source of CXCL12 in the adult brain. SVZ-released CXCL12 was finally demonstrated to mediate GBM resistance to irradiation *in vitro.* Taken together, these data suggest the critical role undertaken by the SVZ-released CXCL12 in mediating GBM resistance to radiotherapy through GBM mesenchymal activation and underpin the adult SVZ stem cell niche as a potential environment involved in GBM radio-resistance.

Key words: Glioblastoma, Radio-resistance, Sub-ventricular Zone, Mesenchymal Activation, CXCL12.

Introduction

Primary brain tumors are considered as one of the nastiest scourges faced in oncology. Their most aggressive form, glioblastoma multiforme (GBM, WHO grade IV), is also regarded as the most common and lethal subtype [1]. The overall median survival of GBM patients indeed hardly reaches 15 months from the time of diagnosis despite the development of multimodal therapies [2]. Poor survival rates are here the consequence of GBM systematic relapses. Therefore, trying to better understand the origin of those relapses may be of clinical benefit to improve both outcomes and the patients' quality of life.

Infiltrative patterns make GBM tumors hard to target. In this line, recent experimental evidence reported a fraction of GBM cells to escape the tumor mass and specifically invade the sub-ventricular zone (SVZ) of the adult brain [3, 4]. This specific *in vivo* model of brain cancer invasion has recently been shown to rely on SVZ-released CXCL12 [5]. Of importance, SVZ-nested GBM cells were demonstrated highly tumorigenic and further characterized as GBM stem cells (GSC) [3]. Being away from the initial tumor site and retaining significant tumor-initiating abilities, GBM cells located in the adult SVZ could therefore represent a reliable example of clinical obstacle to therapy and recurrence. Furthermore, the recent identification of GSC potentially involved in chemo-resistance within the human SVZ has brought the topic into sharp focus [6] and strongly supports the need for a better characterization of the communication lines between the SVZ environment and GSC.

Relying on these considerations and on the fact that GSC display intrinsic resistance to irradiation (IR) [7], we here questioned the influence of SVZ-related factors in GBM radio-resistance. We particularly investigated the potential role of SVZ-released CXCL12 in GSC

extrinsic resistance to irradiation. Chemokine receptor CXCR4 has indeed been identified as a potential biomarker for radio-resistant cancer stem cells [8]. In a similar fashion, the CXCL12/CXCR4 signaling axis has recently been reported to regulate the *in vitro* mesenchymal activation of GBM cells [9], a process further described to sustain GBM radio-resistance [10]. In this work, we demonstrated that SVZ-nested GSC are specifically resistant to IR *in vivo*. Interestingly, these cells displayed an enhanced mesenchymal phenotype compared to GBM cells isolated from the tumor mass. These mesenchymal traits were further shown to be up-regulated upon CXCL12 stimulation *in vitro*. In this line, we and others previously reported the SVZ to be a source of CXCL12 in the adult brain [5, 11]. SVZ-released CXCL12 was finally demonstrated to mediate GBM resistance to IR *in vitro.* Taken together, these data highlight the critical role undertaken by CXCL12 in mediating GBM resistance to radiotherapy through GBM mesenchymal activation and underpin the adult SVZ stem cell niche as a potential environment involved in GBM recurrence.

Materials and Methods

Cell Culture

Human GBM primary cultures (GBM1, GBM2 and GB138) were established from consenting patients and validated as previously described [12]. Human U87MG cell line (U87MG ATCC® HTB-14™) and GBM primary cultures were cultivated in DMEM supplemented with 10% of fetal bovine serum (FBS, Life Technologies®) prior to *in vitro* experiments. Additional details can be found in the Supplementary Data section.

Ethic Statement

Patients gave informed consent for the use of GBM specimens.

Animals

Adult P40 female immunodeficient nude mice, Crl:NU-Foxn1^{nu}, obtained from Charles River® animal facilities (Charles River Laboratories®), were used for xenograft purposes. Animals were taken care in accordance with the declaration of Helsinki and following the guidelines of the Belgium Ministry of Agriculture in agreement with EC laboratory animal care and use regulation (86/609/CEE, CE of J n_L358, 18 December 1986). Athymic nude mice were housed in sterilized filter-topped cages and were processed as approved by the ethical committee of the University of Liège.

Intracranial Transplantation and Radiotherapy Treatment

Intracranial xenografts were generated as described previously [3]. A detailed procedure can be found in the Supplementary Data section.

Mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/mL, Pfizer®)/xylazine (Sedativum 2%, Bayer®) solution (V/V) prior to be irradiated. IR was delivered using fractionated doses (6Gy/day for 5 consecutive days) using an orthovoltage x-ray (Stabilipan®, Siemmens) with a custom immobilization system and a validated dosimetry.

Whole-Mount Dissection

The lateral walls of the ventricles were dissected from the caudal aspect of the telencephalon as previously described [13]. A detailed procedure can be found in the Supplementary Data section.

***In vitro* Radiotherapy Protocol**

GBM cells were seeded on coverslips previously coated with polyornithine (0.1 mg/mL, Sigma®) for 6 hours to allow adhesion and then cultured overnight in serum-free condition. They were supplemented with a 7.5 fold dilution of conditioned medium an hour prior to be irradiated (10Gy) using a Gammacell® 40 Exactor irradiator. This protocol was also performed with SVZ-CM supplemented with a specific anti-CXCL12 blocking antibody (Millipore®) or serum free medium with different concentrations of human recombinant CXCL12 (25 - 50 - 100nM - Peprotech®).

Cells were fixed in paraformaldehyde (PFA - 4% - 4,3g/L NaOH, 40g/L paraformaldehyde, 18.8 g/L NaH_2PO4) an extra hour later and the γH2AX response was finally assessed.

Western Blot Analysis

Protein extracts were resolved with Novex 4-12% Bis-Tris gels (NuPAGE®, Invitrogen®) and transferred onto a PVDF membrane (Roche®) according to standard protocols. Blots were then probed with primary and secondary antibodies finally imaged with the ImageQuant 350 scanning system (cooled-CCD camera, GE Healthcare®). A detailed procedure, antibodies and buffer composition can be found in the Supplementary Data section.

Proliferation Assays

Human GBM cells (7.5×10^3) were seeded in 6-well culture plates (Corning®). Cells were trypsinized and counted using Thoma counting chambers by the trypan blue exclusion method after 7 days.

Clonogenic Assays

Exponentially growing cells were seeded at low density (7.5×10^3) in 6-well culture plates (Corning®) for 6 hours to allow adhesion. GBM cells were then cultured overnight in serum-free condition and supplemented with a 7.5 fold dilution of SVZ-CM an hour prior to be irradiated (4Gy) using a Gammacell® 40 Exactor irradiator. Cells were then allowed to grow for 6 hours. Media were then removed and replaced with DMEM/10% FBS for 7 to 9 days. Live colonies of

more than 20 cells were finally counted in 3 low-power (10×) random microscopic fields. Results represent the mean of 3 independent experiments.

FACS Analysis

GB138 primary cells were collected and incubated with a specific anti-CXCR4 antibody (rabbit polyclonal IgG, 1:500, Abcam®) for an hour. A second incubation period was performed with a FITC-conjugated secondary antibody (1/500, Jackson Immunoresearch Laboratories®) for 45 minutes. GB138 primary cells were finally sorted relying on the expression of CXCR4 (FACS-Aria III, BD®) and cultured in DMEM/10% FBS conditions.

Processing of Tissue Sections Before Immunostainings

Mice were anaesthetized with an injection of Nembutal® (Pentobarbital 60 mg/mL, Ceva Sante Animal®) before an intracardiac perfusion with a NaCl 0.9% solution (VWR International®) followed by paraformaldehyde (PFA) 4% at 4°C (4,3g/L NaOH, 40g/L paraformaldehyde, 18.8 g/L NaH_2PO4). Brains were collected, postfixed in 4% PFA and cryoprotected overnight in a solution of PBS/sucrose (20%). Brains were frozen at -20°C in a 2-methylbutane solution (Sigma®) and cut into 16 µm thick coronal sections using a cryostat.

Immunostainings

Brain coronal sections or GBM cells were permeabilized and unspecific binding sites were blocked using a 10% donkey serum and 0.1% Triton X-100 PBS solution. Tissue sections or cells were

incubated with primary antibodies diluted in PBS containing 0.1% donkey serum and 0.1% Triton X-100 followed by a second incubation with RRX- or FITC-conjugated secondary antibodies (1/500, Jackson Immunoresearch Laboratories®). Detailed procedures can be found in Supplemental Experimental Procedures.

Image acquisition and data analysis

Immunostained sections were imaged using a laser-scanning confocal microscope equipped with a krypton/argon gas layer (Olympus® Fluoview 1000). Zeiss Axiovert 10VR microscope (Car Zeiss®) coupled with Mercator® software (Explora Nova®) was used for cell counting. Figures were composed and examined using ImageJ®.

Statistical Analysis

Quantitative data are expressed as mean ± SEM. Two-way ANOVA followed by a Tukey post-test was used and a p value < 0.05 was considered statistically significant. Student's t-tests were performed for two groups comparison using Statistica 10.0 software.

Results

a. GBM Resistance to Irradiation in the Adult SVZ.

GB138 primary cells were grafted in the striatum of immunocompromised mice. Ten weeks after the implantation, 8 mice were submitted to daily doses of radiation (6Gy) for 5 days. By the end of the 11th week, animals from both control and irradiated groups were sacrificed. The impact of IR was assessed by histological techniques using an anti-human nuclei antibody to specifically detect human GBM cells in brains. Control animals displayed massive infiltration of the CC and SVZ (Figure 1). Surprisingly, a very limited amount of cells was found in the CC of irradiated mice (0.68% of the initial population) whereas no tumor cells were spotted in the vicinity of the injection in irradiated mice (Figure 1). Proportionally to the number of GB138 primary cells which invaded the SVZ of control mice, we detected 12% of remaining GB138 primary cells in the SVZ of irradiated mice (Figure 1). These results strongly suggest the SVZ microenvironment to openhandedly host GBM cells and to protect a bunch of these cells from IR, therefore playing a key role in GBM resistance to treatment and corroborating with late periventricular patterns of recurrence usually observed in patients [14].

b. GBM Resistance to radiation is Mediated by the SVZ-CM *in vitro.*

In order to confirm the *in vivo* findings, we decided to test whether the soluble environment of the SVZ could play a role in GBM resistance to radiotherapy. To do so, we serum starved U87MG cells and GBM1/2 primary cells overnight. We then added a 7.5 fold dilution of SVZ-CM to these

three cell types prior to IR. After an hour of incubation, GBM cells were irradiated (10Gy) and the γH2AX response, a marker of DNA damage response, was assessed at one hour following the end of the radiotherapy protocol. Regardless the nature of the GBM populations we used, addition of SVZ-CM prior to IR significantly decreased the radio-sensitivity of these populations in comparison to control medium (Figure 2A). Consistent with prior studies in human and mouse epithelial cells [15], radio-desensitization was correlated with a significant decrease of γH2AX positivity (Figure 2B). These data indicate that the SVZ-CM abrogates the response to DNA damage in GBM cells, therefore decreasing radiosensitivity.

53BP1 is a DNA damage checkpoint protein that is recruited to DNA double strand breaks to mediate the damage response. It binds to the central domain of p53 and usually interacts with histone H2AX when phosphorylated on serine 139 (γH2AX). We therefore gaged the 53BP1 response following IR (10Gy) in the presence of SVZ-CM or control medium. 53BP1 colocalized with γH2AX at the break sites but no discrepancy was found in terms of 53BP1 expression regarding control and SVZ-CM conditions (Figure 2B).

We then checked whether the radio-protective impact of the SVZ-CM was to be incriminated to the SVZ itself or to other brain regions as well. We therefore irradiated human U87MG cells and GBM2 primary cells in the presence of a 7.5 fold dilution of SVZ, OB or cerebellum (CRBL) conditioned media. As previously observed, we demonstrated a significant decrease of γH2AX-positive cells in response to IR and SVZ-CM in both U87MG cells and GBM2 primary cells. On the other hand, irradiation of U87MG cells and GBM2 primary cells in both OB- and CRBL-conditioned media did not impact the γH2AX response (Figure 2C). Similar numbers of γH2AX-positive cells either supplemented with OB- or CRBL-conditioned media were found in comparison with a dose of 10Gy in control medium. These data underlie that GBM resistance to

IR is specifically mediated by the soluble environment of the SVZ and is therefore not incriminated to random brain regions.

We finally compared the γH2AX response in U87MG cells isolated from the tumor mass (U87MG-TM) and U87MG cells isolated from the SVZ (U87MG-SVZ) in the presence of SVZ-CM or control medium. These GBM sub-populations were pre-incubated for an hour in SVZ-CM or control medium and then received a dose of 10Gy. U87MG-TM cells displayed a γH2AX pattern of expression similar to U87MG cells. Indeed, adding SVZ-CM prior to radiotherapy allowed to significantly decrease the number of γH2AX-positive cells in this sub-population ($p=0.0002$). U87MG-SVZ cells also displayed a significantly lesser extend of γH2AX-positive cells when supplemented with SVZ-CM prior to IR ($p=0.012$) (Figure 3A). Of note, SVZ-isolated U87MG cells have been previously characterized as a sub-population of cancer cells enriched in GSC properties [3, 5]. In this line, GSC are known to be intrinsically radio-resistant as they preferentially activate DNA damage checkpoints and display stronger DNA repair capacities than differentiated GBM cells [7]. Interestingly, we here confirmed that U87MG-SVZ cells are innately more resistant to radiation than U87MG-TM cells as the basal level of γH2AX-positive cells in both GBM sub-populations was significantly lower in GBM cells isolated from the SVZ environment ($p=0.009$) (Figure 3A). A similar conclusion was drawn following clonogenic assays on GB138 primary cells isolated from the TM and from the SVZ. A significant higher number of colonies was indeed raised from GB138-SVZ cells compared to GB138-TM cells after a treatment of 4Gy ($p<0.001$ - Figure 3B). These data strongly support that GBM cells located in the SVZ region represent a sub-population of cancer cells with intrinsic radio-resistance abilities similar to what has been described in the literature so far and could therefore be a source of tumor recurrence after radiotherapy.

c. The SVZ-CM Stimulates GBM Cell Proliferation and is Associated with Increased Survival after Radiotherapy.

We next investigated the SVZ-CM impact on U87MG cells and GBM1 primary cells growing abilities. We seeded 7.500 GBM cells in six-well plates and serum-starved them for 12 hours. A 7.5 fold dilution of SVZ-CM was added to the different cellular monolayers prior to IR (4Gy). Six hours after IR, U87MG cells and GBM1 primary cells were placed in DMEM culture medium supplemented with fetal bovine serum (10%) and respectively cultured at an exponential growth phase for 7 and 9 days. Putting IR aside, we first noticed that media conditioned by SVZ whole-mounts had a significant positive impact on the proliferation of both U87MG cells and GBM1 primary cells. U87MG cells supplemented with SVZ-CM proliferated twice as much (2.03 times more) compared to U87MG cells in control medium ($p<0.001$). GBM1 primary cells proliferated on average 1.6 times more with a SVZ-CM supplementation compared to control medium ($p<0.001$) (Figure 4). We then estimated the impact of a dose of 4Gy on the survival of both GBM populations. Irradiation of U87MG and GBM1 primary monolayers respectively led to a 43.5% and a 45.5% reduction of cell survival ($p<0.001$) (Figure 4).

Surprisingly, the addition of SVZ-CM to U87MG cells prior to IR significantly propelled their proliferation since U87MG cells proliferated 2.5 times more compared to the 4Gy condition in control medium ($p<0.001$). Similarly, irradiated GBM1 primary cells proliferated 1.5 times more when supplemented with SVZ-CM ($p=0.07$) (Figure 4). These data provide evidence that the SVZ-CM actively promotes GBM cell proliferation and is associated with increased cell survival after radiotherapy.

d. Inhibition of SVZ-released CXCL12 Sensitizes GBM Cells to Irradiation *in vitro.*

CXCL12 has recently been suggested to regulate the *in vitro* mesenchymal activation of GBM, especially through Snail and N-cadherin regulation [9]. On the other hand, GBM mesenchymal activation has elegantly been reported to enhance radio-resistance in a NF-κB-dependent manner [10]. Relying on these considerations, we decided to investigate whether SVZ-released CXCL12 undertakes a role in GBM radio-resistance. To do so, we implemented a 7.5 fold dilution of SVZ-CM with a specific CXCL12 blocking antibody or a non-relevant immunoglobulin (IgG) for 45 minutes. We then added these media to U87MG cells and GBM2 primary cells prior to IR (10Gy). We quantified the impact of blocking CXCL12 in the SVZ-CM by assessing the γH2AX response in both GBM populations. As expected, similar smaller amounts of γH2AX-positive cells were found in both SVZ-CM and SVZ-CM/IgG conditions. Interrestingly, the blockade of CXCL12 significantly allowed to sensitize both U87MG cells and GBM2 primary cells to irradiation($p<0.001$) (Figure 5A). However, attention has to be drawn on the fact that the CXCL12 inhibition did not completely restore the number of γH2AX-positive cells encountered without any SVZ-CM supplementation (94.8 ± 2.6% vs 82.9 ± 9.8% ($p=0.005$) and 92.6 ± 8.1% vs 84.6 ± 9.9% ($p=0.02$), respectively for U87MG cells and GBM2 primary cells) (Figure 5A), suggesting the role of other SVZ components in GBM *in vitro* resistance to radiation.

Conversely to the CXCL12 blockade, we conducted rescue experiments to prove the potential of CXCL12 as a radio-protective chemokine for GBM cells. We serum-starved U87MG cells and GBM2 primary cells for 12 hours. We then stimulated these cells for one hour with growing concentrations of recombinant CXCL12 (rCXCL12) (25nM, 50nM and 100nM) prior to a radiation dose of 10Gy and finally assessed the γH2AX response within these conditions. We

noticed a significant decrease of γH2AX-positive U87MG cells starting at 25nM ($p=0.04$ – Figure 5B). A similar observation was made in GBM2 primary cells at 50nM ($p=0.02$) of rCXCL12 (Figure 5B). This slight but constant radio-desensitization was observed up to 100nM in both GBM cell populations ($p<0.001$). Importantly, rCXCL12 never allowed to reach the levels of radio-protection observed with a 7.5 fold dilution of SVZ-CM, suggesting once again the role of other SVZ soluble components in GBM radio-resistance.

To further confirm the potential implication of the CXCL12/CXCR4 axis in GBM resistance to IR, we seeded 7.500 GB138 primary cells sorted for the expression of CXCR4 in six-well plates. Of note, 59.5% of GB138 primary cells were positive for the expression of CXCR4 (Figure 5C). CXCR4-positive and CXCR4-negative GB138 primary cells then received a dose of 4Gy and were finally cultured at an exponential growth phase for 7 days. In this way, we demonstrated that CXCR4-positive GB138 primary cells proliferated 1.6 times more than CXCR4-negative GB138 primary cells ($p=0.03$), suggesting the role of chemokine receptor CXCR4 as a potential biomarker for radio-resistant cancer cells (Figure 5C).

e. CXCL12 Promotes the Mesenchymal Activation of GBM cells *in vitro.*

We have so far demonstrated that the CXCL12/CXCR4 signaling endorses a key role in GBM resistance to radiation but we still don't known the mechanisms which specifically work behind the scene. In this context, CXCL12 has very recently been suggested to regulate the *in vitro* mesenchymal activation of GBM [9] which, in turn, has been suggested to play a key role in GBM radio-resistance. Moreover, the acquisition of mesenchymal traits is usually in line with poor outcomes and corresponds to the most rebellious type of GBM to therapy [16]. Relying on these

findings, we questioned the role of CXCL12 in promoting the mesenchymal activation of GBM cells.

Upon an hour of stimulation with rCXCL12 (20nM), both U87MG cells and GB138 primary cells expressed higher levels of N-cadherin, a protein characteristically involved in mesenchymal activation (Figure 6). The expression of Vimentin, a type III intermediate filament protein specifically expressed in mesenchymal cells, was also upregulated within an hour of stimulation in U87MG cells (Figure 6B). No significant change in Vimentin expression was observed in GB138 primary cells within an hour of stimulation (Figure 6A). Indeed, Vimentin expression started to increase after 4 hours of stimulation in this GBM population, suggesting different post-translational regulations in U87MG cells and GB138 primary cells. These findings nevertheless allowed to highlight the acquisition of a reinforced *in vitro* mesenchymal phenotype upon CXCL12 stimulation in both U87MG cells and GB138 primary cells.

f. GBM Cells Located in the SVZ Maintain Strong Mesenchymal Properties.

Now that we demonstrated SVZ-released CXCL12 to be involved in GBM resistance to radiation *in vitro* and CXCL12 to underlie an enhanced GBM mesenchymal phenotype, we looked for a potential mesenchymal signature in GBM cells nested in the SVZ. Acquisition of mesenchymal properties is indeed considered as a hallmark of therapeutic resistance in cancer [17]. To tackle the issue, we first performed immunohistostainings on coronal sections of brains previously grafted with GB138 primary cells. These stainings allowed to shed the light on the expression of mesenchymal proteins including N-cadherin and Vimentin (red) in GB138 primary cells (green) located in the ventricular walls (Figure 7A). These cancer cells were detected using a

specific anti-human nuclei antibody (Hu. Nu.). Western Blot analyses on GB138 primary cells isolated from the TM and the SVZ were then performed and compared for the expression of mesenchymal markers. This approach specifically revealed a higher expression level of N-cadherin and Vimentin in GB138-SVZ cells compared to GB138 primary cells initially isolated from the TM (Figure 7B). This observation further suggests a selection of cancer cells with stronger mesenchymal properties from the TM to the SVZ or a SVZ-dependent enhancement of mesenchymal traits in GBM cells probably through the release of CXCL12. Anyway, in both cases, GBM cells nested in the SVZ region specifically display stronger mesenchymal abilities, often correlated with therapeutic resistance and poor outcomes.

Discussion

The take home message of the present work states that GBM cells hiding in the SVZ environment are particularly resistant to radiotherapy treatment. Indeed, using an *in vivo* model of SVZ invasion [3], we found that 12% of GBM cells initially nested in the SVZ still remain in that environment after radiotherapy. Of importance, we previously demonstrated that GBM cells located in the SVZ are enriched in tumor-initiating capacities and were therefore characterized as GSC [3]. Since GSC have been widely reported to be intrinsically resistant to radiotherapy [7], it thus makes sense to find a fraction of remaining tumor cells in the SVZ environment after IR. We anyway questioned the role of the SVZ niche in GBM extrinsic resistance to IR. In this context, we believe the composition of the niche to finely regulate fate specification and protection of GSC. In the facts, the SVZ physiologically acts as a supportive niche, promoting neural stem cells self-renewal and inhibiting differentiation [18]. This “seed-and-soil” relationship has also been adapted

to cancer stem cell research as GSC also rely on specific niches to maintain their stem cell properties and their ability to drive tumor growth [19-21]. Furthermore, Piccirillo and colleagues revealed that GSC isolated from the human SVZ are specifically resistant to supra-maximal chemotherapy doses along with differential patterns of drug response between GBM cells isolated from the tumor mass or the SVZ from the same patient [6]. Altogether, these findings allow to speculate on the potential role of the SVZ niche as a reservoir of radio/chemo-resistant GBM cells potentially involved in tumor relapses.

We then managed to transpose the *in vivo* observations we made in an *in vitro* protocol of radiotherapy using the soluble environment of the SVZ (SVZ-CM). This experimental protocol allowed to highlight a certain level of GBM resistance to radiation (10Gy) following a SVZ-CM stimulation. Additionally, we specifically pinpointed SVZ-released CXCL12 and CXCR4 expressed by GBM cells as a key mediators involved in GBM resistance to IR *in vitro*. In the light of our findings, CXCR4 has recently been described as a new biomarker for radio-resistant cancer stem cells [8]. Furthermore, pharmacological inhibition of the CXCL12/CXCR4 signaling concomitant with radiotherapy elegantly abrogated GBM regrowth in mice by preventing the development of functional tumor blood vessels post-irradiation [22]. In a similar way, Domanska and colleagues described the inhibition of CXCR4-dependent protective signals from stromal cells to render prostate cancer cells more sensitive to ionizing radiations [23]. Characterization of both CXCL12 and CXCR4 antagonists in pre-clinical cancer models as well as their potential therapeutic benefits in combination with radiotherapy may therefore contribute to better understand the role of CXCL12 and CXCR4 in GBM resistance to treatment and facilitate the translation of these inhibitors to the clinic.

We have so far demonstrated the key role of the CXCL12/CXCR4 signaling in GBM resistance to IR but the mechanisms specifically underlying these findings were yet to be determined. To tackle down the issue, we focused on the mesenchymal status of GBM cells. Indeed, the acquisition of mesenchymal traits is usually in line with poor outcomes and corresponds to the most rebellious type of GBM to therapy [16, 24]. Moreover, GBM are known to frequently shift toward a mesenchymal phenotype upon recurrence [24]. With these considerations in mind, we demonstrated GBM cells to express a basal level of Vimentin and N-cadherin, further suggesting the mesenchymal origin of our GBM populations. Interestingly, these two mesenchymal proteins were specifically up-regulated upon CXCL12 stimulation. Supporting our findings, the specific inhibition of the CXCL12/CXCR4 signaling axis in GBM has recently been reported to affect the *in vitro* expression of mesenchymal biomarkers including Vimentin, Snail and N-cadherin [25]. Of importance, we also showed that GBM cells nested in the SVZ display a higher expression level of Vimentin and N-cadherin compared to GBM cells located in the TM. Many studies, reviewed in [17], have established a sharp link between the acquisition of mesenchymal properties (epithelial to mesenchymal transition – EMT) and resistance to chemotherapy in many human tumors. In contrast, very less is known about the involvement of EMT in radio-resistance. In this line, a very elegant study reported that GBM mesenchymal activation promotes resistance to irradiation in a NF-κB-dependent manner [10], further suggesting this transcription factor to regulate EMT. Our findings provide the very first evidence that the SVZ environment actively participates to the reinforcement of the tumor mesenchymal roots (probably through the secretion of CXCL12) and highlight a potential mechanism by which this niche contributes to GBM resistance to irradiation.

Our findings corroborate with a growing body of clinical data correlating delivered doses of radiotherapy to the SVZ with increased progression-free survival (PFS) and overall survival

(OS) in patients with newly diagnosed GBM [26-28]. In this line, a very recent retrospective study evaluated the influence of tumor location on recurrence behavior, PFS and OS with respect to the SVZ after radiotherapy. GBM with SVZ infiltration specifically showed PFS and OS decreased rates and presented higher risks of distant progression [14]. These findings could potentially be explained by the persistence of GSC within the SVZ environment after radiotherapy.

Funding

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Conflict of interest

The authors declare they have no conflicts of interest.

Figure Legends

Figure 1. A substantial fraction of GB138 primary cells survive from irradiation in the SVZ stem cell niche. A) GB138 primary cells are removed from the striatum after IR. B) A similar observation was made with regard to the CC as only 0.68% of GB138 primary cells remained in the white matter structure after IR. C) Surprisingly, 12% of the initial amount of GB138 primary cells nested in the SVZ environment persisted in this niche after radiotherapy. D) A minimum of five mice was used in each group for quantification. These results underscore the potential role of the SVZ environment in GBM resistance to radiation. GB138 primary cells were detected using a specific anti-human nuclei antibody (red). Cell nuclei were counterstained with DAPI (blue). The caption shows where pictures were taken. Scale bars = 40 µm for A, B and C.

Figure 2. SVZ-CM mediates GBM resistance to irradiation *in vitro*. A) Culture media were conditioned with SVZ whole-mounts for 60 hours and then added to GBM monolayers prior to irradiation(10Gy). This significantly decreased the sensitivity of U87MG cells and GBM2/GBM3 primary cells to IR by reducing the DNA damage response. B) The latter was specifically assessed by measuring the γH2AX (red) and 53BP1 (green) responses in U87MG cells as well as in GBM2 primary cells. Radio-desensitization was correlated with a significant decrease of γH2AX-positive cells. No significant change in 53BP1 expression was observed. C) Irradiation of U87MG cells and GBM3 primary cells supplemented with OB-conditioned medium (OB-CM) or cerebellum-conditioned medium (CRBL-CM) did not impact the DNA damage response as similar levels of γH2AX-positive cells were found in these conditions compared to a dose of 10Gy in control medium. These data demonstrate that GBM resistance to irradiationis specifically sustained by the

SVZ soluble environment. Cell nuclei were counterstained with DAPI (blue). Scale bars = 10 µm for B. *** $p<0.001$

Figure 3. GBM cells nested in the SVZ are intrinsically resistant to irradiation. A) Irradiation of U87MG cells isolated from the TM (U87MG-TM) and U87MG cells isolated from the SVZ (U87MG-SVZ) supplemented with SVZ-CM highlighted a significant decrease of the number of γH2AX-positive cells found in these subpopulations, further supporting the extrinsic role of the SVZ soluble environment in GBM resistance to IR. Nevertheless, U87MG-SVZ cells were intrinsically more radio-resistant compared to their counterparts isolated from the tumor mass ($p=0.009$) in control medium. B) Clonogenic assays on GB138 primary cells isolated from the TM and SVZ revealed that GB138-SVZ cells more efficiently give rise to colonies compared to GB138-TM cells after IR of 4Gy. These findings strengthen even more the assertion that GBM cells located in the SVZ are enriched in GSC properties. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Figure 4. SVZ-CM stimulates GBM cell proliferation and increases survival after irradiation. Adding SVZ-CM to U87MG cells or GBM1 primary monolayers significantly increased these cells proliferative abilities in the absence of IR. A similar conclusion was drawn following a radiation dose of 4Gy, highlighting the beneficial role of the SVZ-CM on GBM cells proliferation following IR. ** $p<0.01$, *** $p<0.001$

Figure 5. Identification of CXCL12 and CXCR4 as key mediators in GBM resistance to radiation. A) The specific inhibition of CXCL12 in the SVZ-CM prior to IR exposure led to a significant rescue of radio-sensitization in human U87MG cells and GBM2 primary cells. The number of γH2AX-positive cells in both GBM populations was indeed found higher after the

blockade of CXCL12 compared to the SVZ-CM condition supplemented with a non-relevant IgG. B) Growing concentrations of recombinant CXCL12 (25/50/100nM) significantly radio-protected U87MG cells and GBM2 primary cells in a dose-dependent manner, further confirming the role of CXCL12 in GBM radio-resistance. C) GB138 primary cells were sorted relying on CXCR4 expression by FACS. CXCR4-positive GB138 primary cells were then reported to better proliferate following IR compared to CXCR4-negative GB138 primary cells (p=0.03) at the end of an exponential growth phase of 7 days. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Figure 6. CXCL12 sustains the mesenchymal activation of GBM cells *in vitro*. A) Immunocytostainings and Western Blot analyses revealed an up-regulation of N-cadherin in GB138 primary cells upon an hour of stimulation with exogenous CXCL12. The expression of Vimentin increased upon 4 hours of stimulation. B) Similar observations were made in U87MG cells as an up-regulation of both N-Cadherin and Vimentin was found upon an hour of CXCL12 stimulation. These data indicate the potential role endorsed by CXCL12 in the mesenchymal activation of GBM cells *in vitro.* Scale bars = 10µm for A and B.

Figure 7. GBM cells nested in the SVZ display enhanced mesenchymal properties. A) Immunohistostainings on brain coronal sections revealed the expression of N-cadherin and Vimentin (red) in GB138 primary cells (green) located in the SVZ. B) By Western Blot analyses, the expression of these two mesenchymal proteins was shown specifically up-regulated in GB138 primary cells isolated from the SVZ compared to G138 primary cells from the TM. This further suggests the critical role of the SVZ in regulating these mesenchymal traits. Scale bars = 25µm.

Figures

Figure 1.

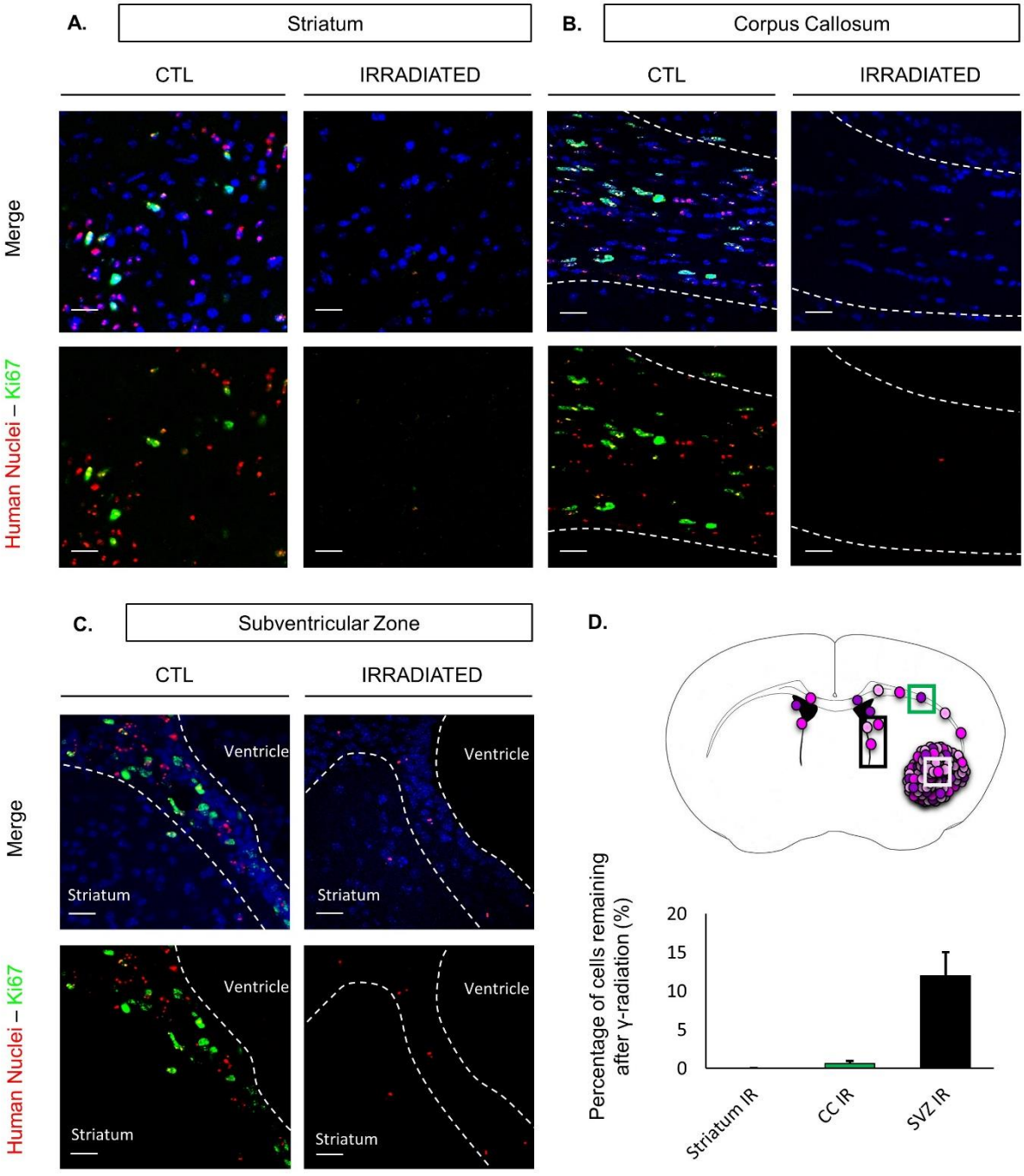


Figure 2.

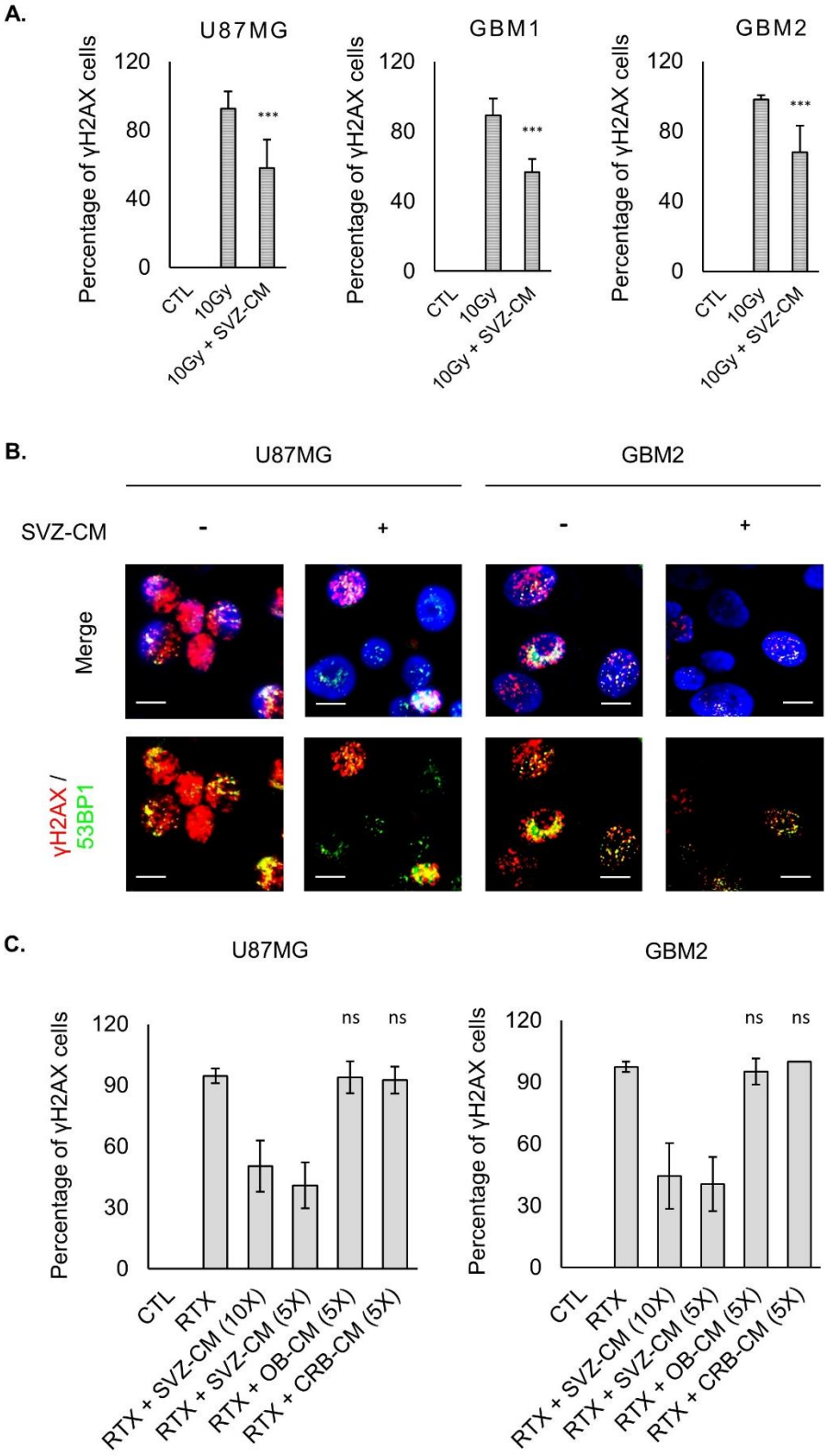

A.
U87MG
GBM1
GBM2
Percentage of γH2AX cells
120
80
40
0
***
CTL
10Gy
10Gy + SVZ-CM
B.
U87MG
GBM2
SVZ-CM
-
+
-
+
Merge
γH2AX / 53BP1
C.
U87MG
GBM2
Percentage of γH2AX cells
120
90
60
30
0
ns
ns
CTL
RTX
RTX + SVZ-CM (10X)
RTX + SVZ-CM (5X)
RTX + OB-CM (5X)
RTX + CRB-CM (5X)

Figure 3.

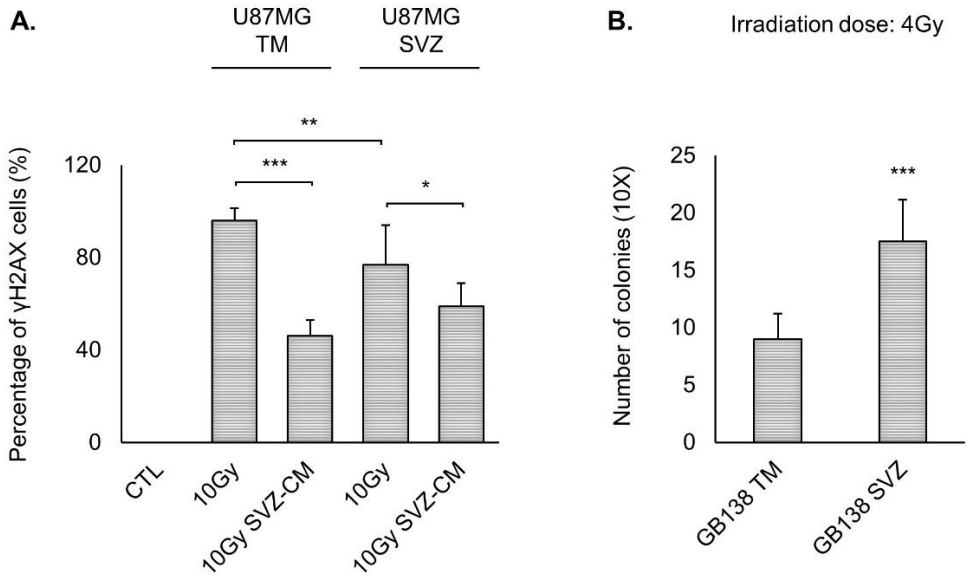

A.
U87MG TM
U87MG SVZ
**
***
*
Percentage of γH2AX cells (%)
120
80
40
0
CTL
10Gy
10Gy SVZ-CM
10Gy
10Gy SVZ-CM
B.
Irradiation dose: 4Gy
Number of colonies (10X)
25
20
15
10
5
0
***
GB138 TM
GB138 SVZ

Figure 4.

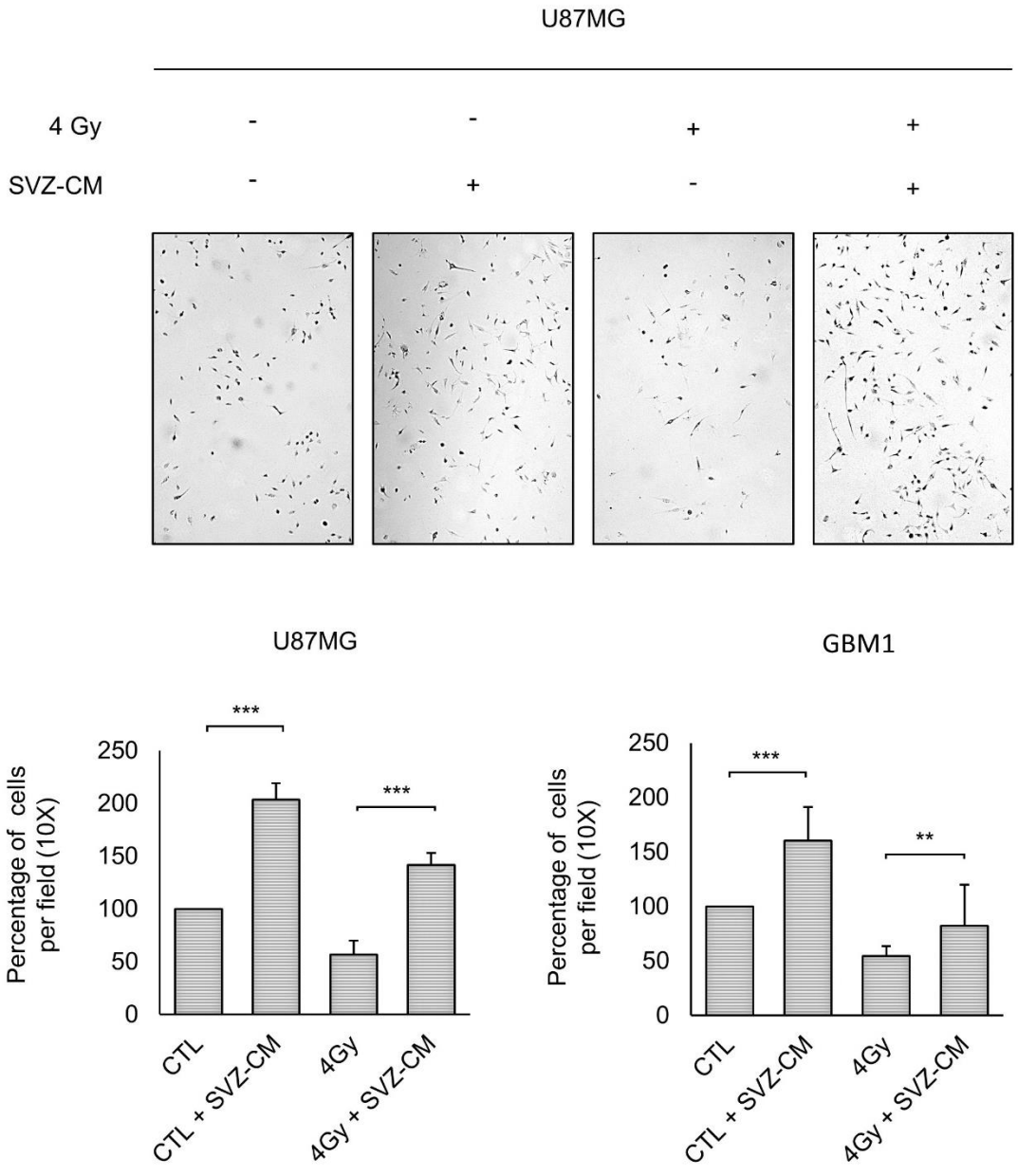

U87MG
4 Gy
SVZ-CM
U87MG
GBM1
Percentage of cells per field (10X)
250
200
150
100
50
0
***
**
CTL
CTL + SVZ-CM
4Gy
4Gy + SVZ-CM

Figure 5.

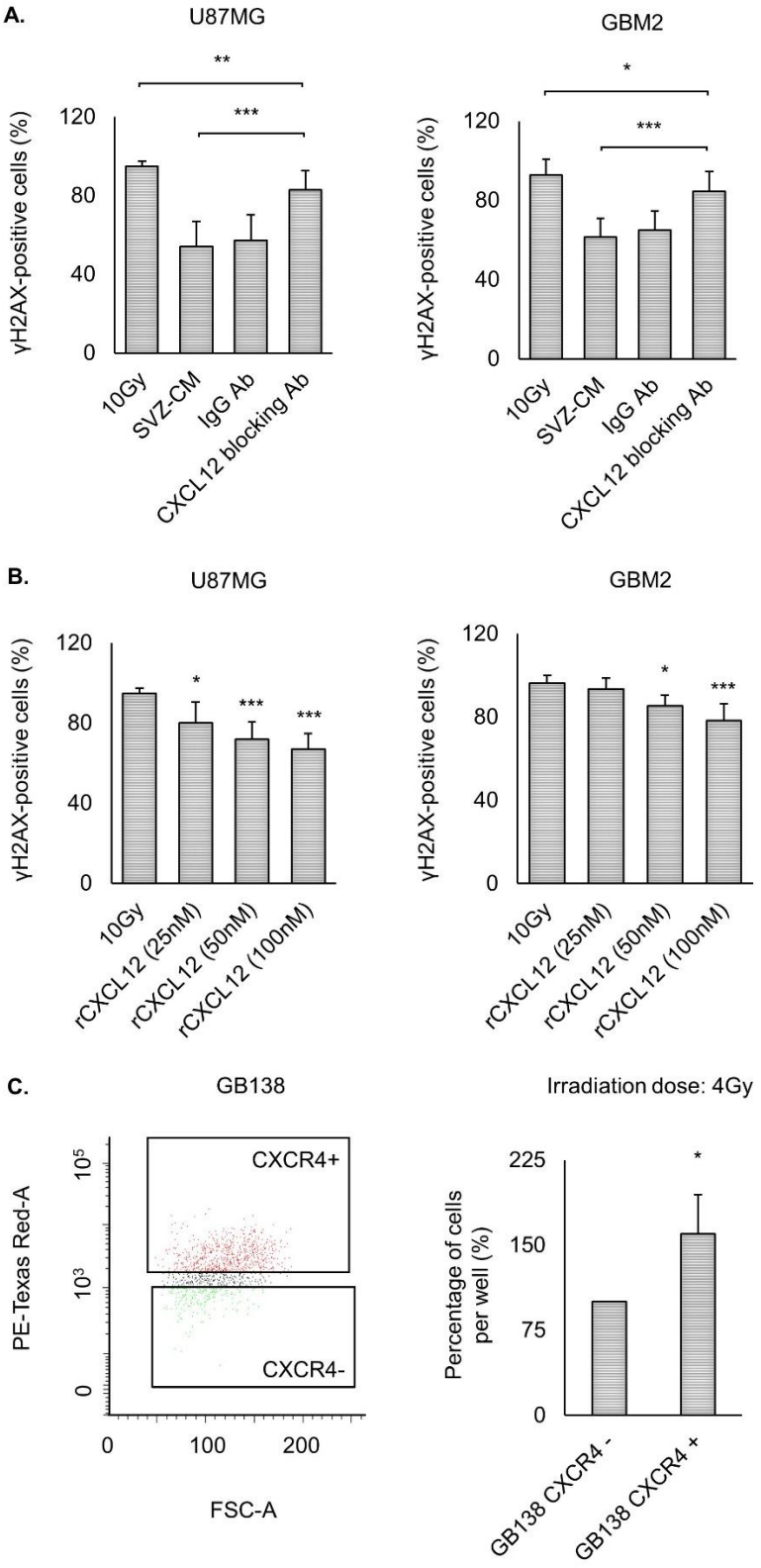

A.
U87MG
GBM2
γH2AX-positive cells (%)
120
80
40
0
**
***
*
10Gy
SVZ-CM
IgG Ab
CXCL12 blocking Ab
B.
U87MG
GBM2
γH2AX-positive cells (%)
*
***
10Gy
rCXCL12 (25nM)
rCXCL12 (50nM)
rCXCL12 (100nM)
C.
GB138
Irradiation dose: 4Gy
PE-Texas Red-A
10^5
10^3
0
CXCR4+
CXCR4-
100
200
FSC-A
Percentage of cells per well (%)
225
150
75
GB138 CXCR4 -
GB138 CXCR4 +

Figure 6.

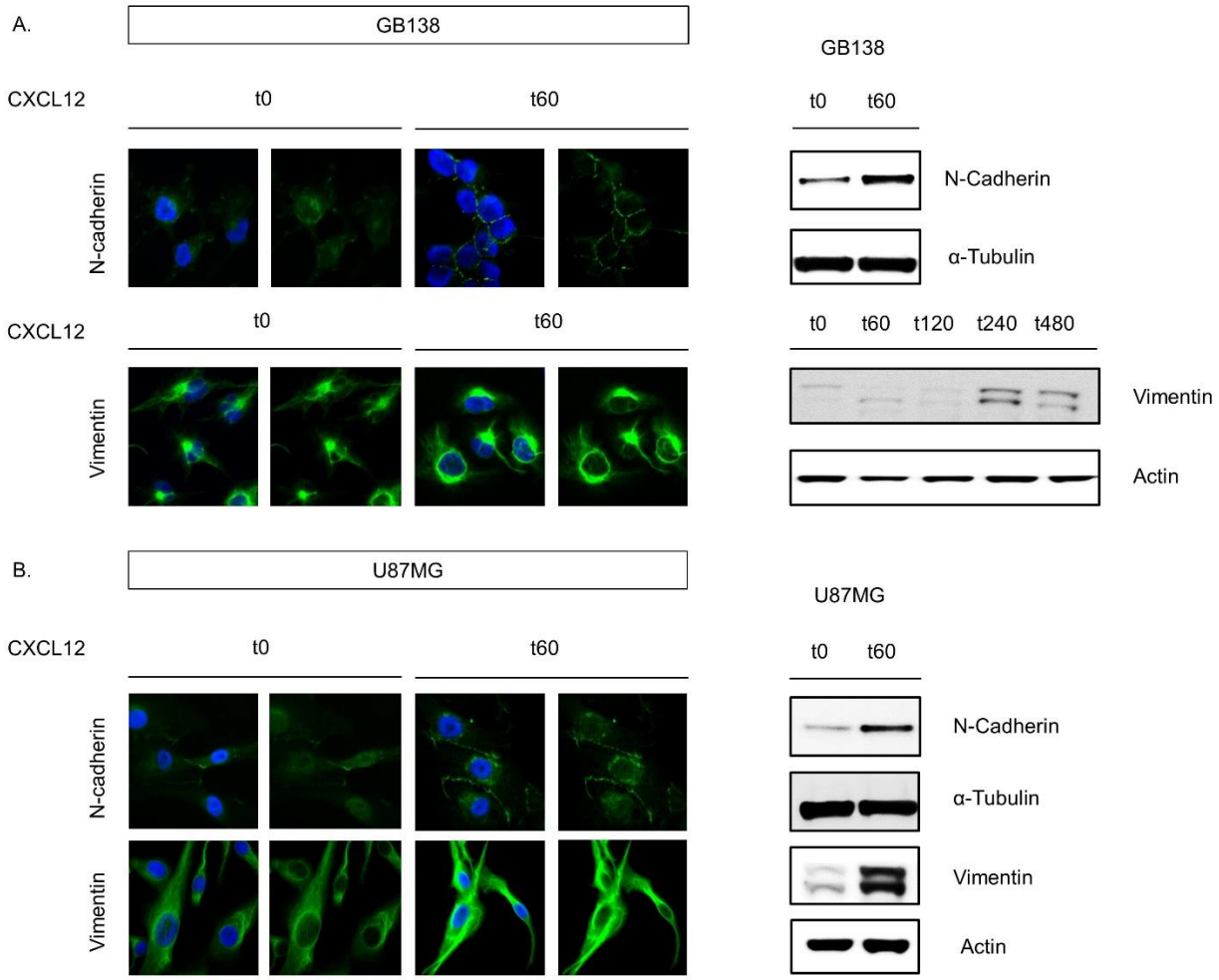
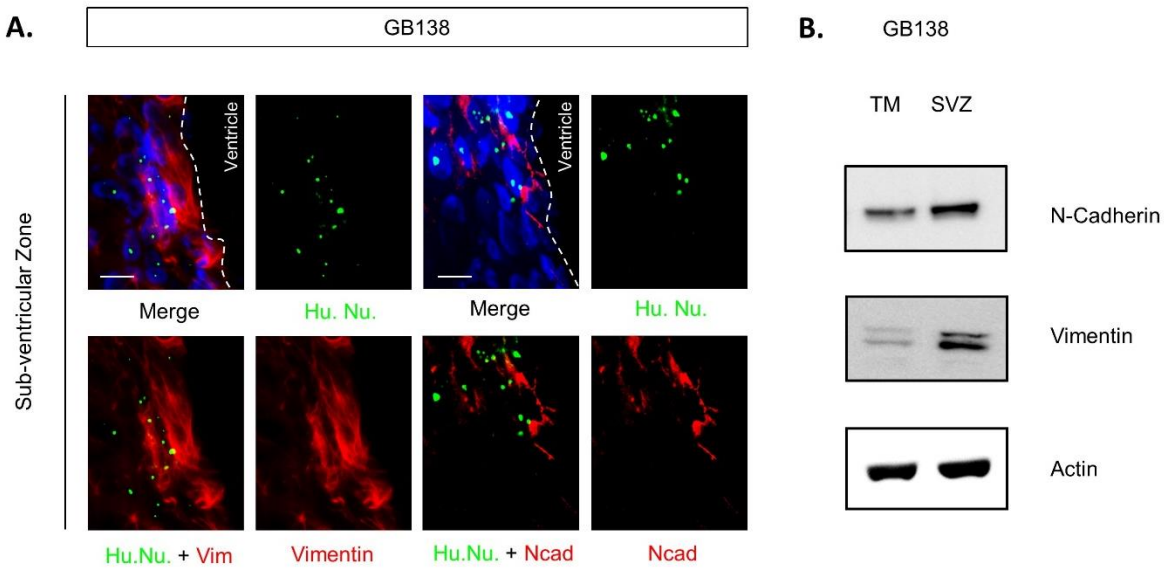

A.
GB138
CXCL12
t0
t60
N-cadherin
CXCL12
t0
t60
Vimentin
GB138
t0
t60
N-Cadherin
α-Tubulin
t0
t60
t120
t240
t480
Vimentin
Actin
B.
U87MG
CXCL12
t0
t60
N-cadherin
Vimentin
U87MG
t0
t60
N-Cadherin
α-Tubulin
Vimentin
Actin

Figure 7.


A.
GB138
Sub-ventricular Zone
Ventricle
Ventricle
Merge
Hu. Nu.
Merge
Hu. Nu.
Hu.Nu. + Vim
Vimentin
Hu.Nu. + Ncad
Ncad
B.
GB138
TM
SVZ
N-Cadherin
Vimentin
Actin

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Supplementary Data

Supplementary Experimental Procedures

Cell culture

U87MG cells and GB138 primary cells were isolated from the SVZ and the TM after dissecting both regions. The whole-mounts were then incubated in papaïn (Worthington®, Lakewood, NJ, USA) for 30 min at 37°C. Ovomucoïd (Worthington®) was next added to stop the dissociation. Cells were mechanically dissociated and plated in six-well dishes in DMEM/F12 serum-free medium containing B27 without vitamin A (Life Technologies®) and daily supplemented with recombinant human epidermal growth factor and recombinant human fibroblast growth factor 2 (EGF, 20 ng/mL and FGF-2, 10 ng/mL, Preprotech®, Rocky Hill, NJ, USA) at the density of 25.000 cells/mL. Human cells were selected with one passage allowing the selection of more than 99% of human cells. The human origin of the newborn spheres was evaluated by immunocytostainings using a specific anti-human nuclei antibody (Millipore®). Cultures were maintained at 37°C under humidified atmosphere containing 5% carbon dioxide.

Whole-Mounts Dissection

The hippocampus and septum were removed. The dissected lateral walls were cultured for 60 hours in DMEM/F12 (Life Technologies®) and supplemented with recombinant human epidermal growth factor and recombinant human fibroblast growth factor 2 (EGF, 20 ng/mL

and FGF-2, 10 ng/mL, respectively, Preprotech®) to prepare the SVZ- conditioned medium. The latter was finally centrifuged and directly frozen.

Intracranial transplantation

Crl:NU-Foxn1^{nu} mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/mL, Pfizer®, Bruxelles, Belgium)/xylazine (Sedativum 2%, Bayer®, Bruxelles, Belgium) solution (V/V). The cranium was exposed and a small hole was drilled 2.5 mm lateral and 0.5 mm anterior to the bregma with a size 34 inverted cone burr (Dremel). Mice were positioned in a stereotactic frame and 50,000 cells in 2 µl PBS were injected into the right striatum through a 27-gauge needle over 1 min at 3 mm below the *dura mater*. The incision was closed with Vetbond (3M). GB138 primary cells were grown in floating spheres culture conditions prior to injection.

Western blot analysis

Protein extracts were obtained by lysing GBM cells in lysis buffer [10mM Hepes, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT and 10% NP-40, pH 7.9] supplemented with Complete Protease Inhibitors (Roche®, Brussels, Belgium). The proteins (25 µg) were resolved with Novex 4-12% Bis-Tris gels (NuPAGE®, Life technologies) and transferred onto a PVDF membrane (Roche®) according to standard protocols. Blots were then probed with polyclonal anti-Vimentin (rabbit polyclonal IgG, 1:750, Cell Signaling®) and N-cadherin antibodies (rabbit polyclonal IgG, 1:750, Cell Signaling®). Total protein loading per lane was evaluated with an anti-β-Actin or anti α-Tubulin antibody (Abcam®). This was followed by incubation with HRP conjugated secondary antibodies (Santa Cruz Biotechnology®) and enhanced

chemiluminescent (ECL) substrate (SuperSignal West Pico, Thermo®). Blots were imaged with the ImageQuant 350 scanning system (cooled-CCD camera, GE Healthcare®).

Immunostainings

Brain coronal sections or cells on coverslips were permeabilized and unspecific binding sites were blocked for an hour at room temperature using a 10% donkey serum and 0.1% Triton X-100 PBS solution. Tissue sections or coverslipped cells were then incubated overnight at 4°C with primary antibodies diluted in PBS containing 0.1% of donkey serum and 0.1% of Triton X-100. Primary antibodies were directed against N-cadherin (rabbit polyclonal IgG, 1:400, Cell Signaling®), Vimentin (rabbit polyclonal IgG, 1:400, Cell Signaling®), Human Nuclei (mouse monoclonal IgG, 1:250, Millipore®), Ki67 (rabbit polyclonal IgG, 1:200, Abcam®), γH2AX (mouse monoclonal IgG, 1:250, Millipore®) and 53BP1 (1/100, Abcam®). Brain slides were incubated for an hour at RT with RRX- or FITC-conjugated secondary antibodies (1/500, Jackson Immunoresearch Laboratories®) and finally coverslipped in a mounting solution containing DAPI (Vectashield®).

Paper 3

Glioblastoma-Initiating Cells: Relationship with Neural Stem Cells and the Micro-Environment

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Review

Glioblastoma-Initiating Cells: Relationship with Neural Stem Cells and the Micro-Environment

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Abstract: Glioblastoma multiforme (GBM, WHO grade IV) is the most common and lethal subtype of primary brain tumor with a median overall survival of 15 months from the time of diagnosis. The presence in GBM of a cancer population displaying neural stem cell (NSC) properties as well as tumor-initiating abilities and resistance to current therapies suggests that these glioblastoma-initiating cells (GICs) play a central role in tumor development and are closely related to NSCs. However, it is nowadays still unclear whether GICs derive from NSCs, neural progenitor cells or differentiated cells such as astrocytes or oligodendrocytes. On the other hand, NSCs are located in specific regions of the adult brain called neurogenic niches that have been shown to control critical stem cell properties, to nourish NSCs and to support their self-renewal. This "seed-and-soil" relationship has also been adapted to cancer stem cell research as GICs also require a specific micro-environment to maintain their "stem cell" properties. In this review, we will discuss the controversies surrounding the origin and the identification of GBM stem cells and highlight the micro-environment impact on their biology.

Keywords: glioblastoma; subventricular zone; cancer stem cells; neural stem cells; micro-environment

1. Introduction

Malignant gliomas represent some of the greatest challenges in the management of cancer patients worldwide. Primary brain tumors are indeed considered amongst the most refractory malignancies and their most aggressive form, glioblastoma multiform (GBM, WHO grade IV), is also the most common and lethal subtype [1]. Although notable recent achievements have been made in oncology, using state-of-the-art neuroimaging techniques for surgical resections along with multimodal radio- and chemotherapy, the patients' median survival hardly reaches 15 months from the time of diagnosis [2,3]. This catastrophic survival rate mainly is the consequence of systematic relapses which reflect the failure of the current therapeutic strategies.

Over the last decade, a large number of different treatments were tested but displayed very limited efficacy. One of the most difficult problems in GBM multimodal therapy is to target the largest number of tumor cells. In this context, surgery often is ineffective given the invasive nature of the tumor, making the entire surgical resection of the tumor mass quite impossible without causing harm to the healthy brain. Moreover, particular regions of the brain are hardly amenable to surgical intervention (basal ganglia, brain stem) which makes the prognosis of the disease even worse. On the other hand, chemotherapeutic strategies are associated with several limitations as well. Various factors such as the size of the molecule, the lipophilicity of the drug, the presence of active efflux pumps and the integrity of the blood-brain barrier influence the access of the drug to the brain parenchyma and the tumor itself [4]. Recent studies have indeed demonstrated that the most forceful agents in glioma therapy achieve relatively low concentrations in the tumor surroundings due to the inability of the drug to cross the blood-brain barrier [5,6]. Finally, recent integrated genomic analysis shed the light on the tumor inter- and intra-heterogeneity. Indeed, The Cancer Genome Atlas (TCGA) classifies GBM based on PDGFRA, IDH1, EGFR and NF1 abnormalities in classical, mesenchymal, pro-neural and neural subtypes [7]. Moreover, all of these subtypes could be found in distinct areas of a single tumor as well [8]. The lack of treatment efficacy could therefore be found in this complex intra- and/or inter-tumor genetic heterogeneity.

For years, parallelisms have been made between stem cell biology and oncology, notably because of the growing evidence that genes with important roles in stem cell biology also play a role in cancer. As a result, the concept of a cancer stem cell population (CSCs) was hypothesized; concept in which a relatively small percentage of cells would share characteristics with normal stem cells and display features including maintained proliferation, self-renewal and differentiation abilities. Nowadays, the existence of such fraction of cells, referred to as cancer stem cells or tumor-initiating cells has been described in many tumors [9] including brain cancers [10–12] and raised a new hope in order to understand why glioblastomas so systematically relapse. Further down the road, glioblastoma stem cells, or initiating cells (GICs), were notably shown to be involved in experimental tumorigenesis, tumor maintenance and therapeutic resistance [13–16]. Moreover, this sub-population of cells with tumor-initiating abilities also display neural stem cell (NSC) properties which suggests that NSCs

could play a major role in tumor development and sheds the light on the kinship between GICs and NSCs [12]. However, the basic nature of GICs is nowadays still unclear, whether they derive from NSCs, neural progenitor cells or differentiated cells such as astrocytes or oligodendrocytes. On the other hand, NSCs are located in specific regions of the brain called neurogenic niches which retain the ability to produce neurons and glia throughout life, functioning as a source of stem cells and progenitors in adults [17,18]. Those niches are essential to control critical stem cell properties, to feed the NSCs and to support their self-renewal abilities. This "seed-and-soil" relationship has also been adapted to GBM stem cell research, as GICs also seem to require specific interactions with the micro-environment in order to maintain their stem-like properties and their ability to drive tumor growth [19]. Prospective identification and targeting of GICs is thus mandatory in order to fully understand their own biology, to prevent GBM relapses and to develop new powerful therapeutic strategies. In this review, we will debate over the controversies surrounding the origin and the identification of GICs and discuss the impact of the micro-environment on the biology of GICs.

2. GBM Origin(s)

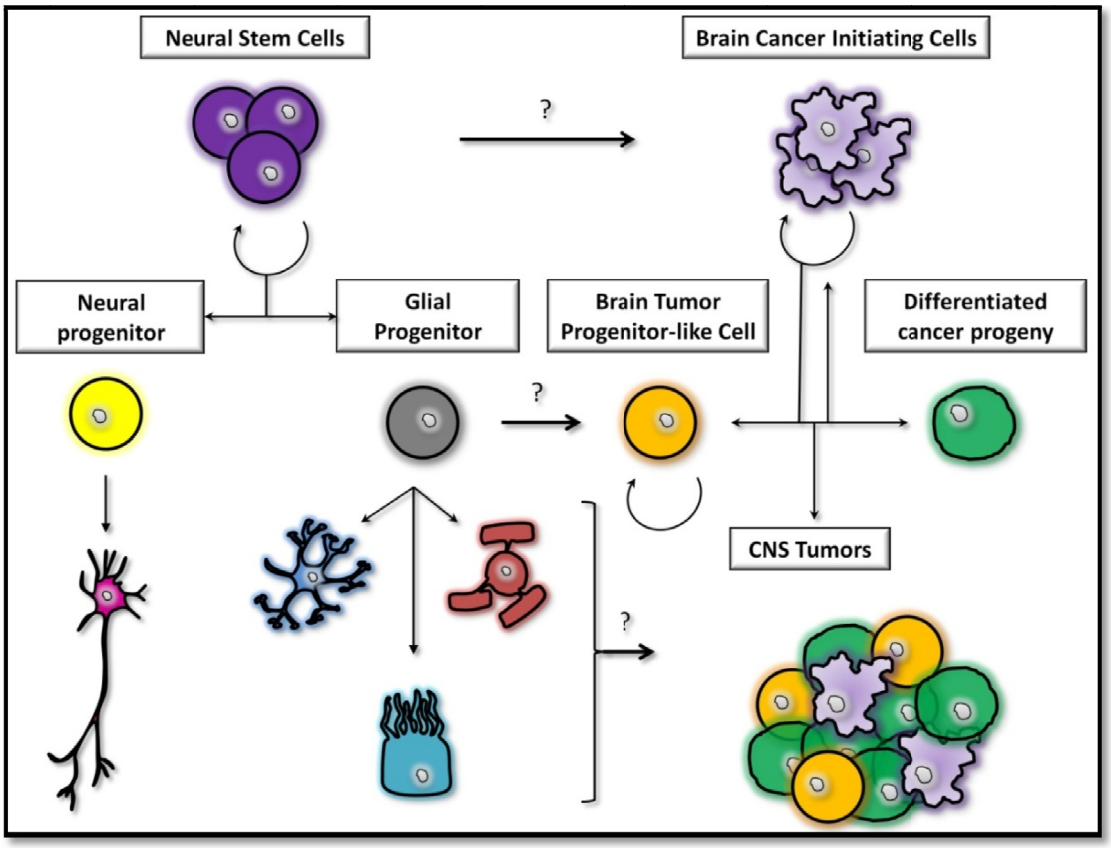
The cells responsible for the onset of malignant gliomas have been source of dissension for many years and are still under intense investigation, whether they could be astrocytes, glial precursors, or stem cells (Figure 1) [20]. In a manner consistent with the stem cell theory, growing evidences aim to demonstrate that only a limited amount of cells, exhibiting stem cell-like properties in the primary tumor, are able to trigger cancer initiation [20,21]. On the other hand, periventricular adult NSCs express high levels of glial fibrillary acidic protein (GFAP) which raised exciting questions on whether or not astrocytes could be involved in GBM initiation. Following those observations, two major hypotheses have been put forward: the astrocytes dedifferentiation theory and the glioblastoma stem cell theory.

2.1. The Dedifferentiation Theory

In this hypothetical view, tumorigenesis is regarded as a multi-step process accompanied with genetic alterations which lead to the progressive transformation of normal cells into highly malignant cells. In this case, six major alterations are required for cancer progression: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [22]. In this context, it has recently been demonstrated that the activation of specific oncogenes concomitant with the loss of tumor suppressors in cortical astrocytes trigger cancer initiation with histological features similar to GBM [12]. As an example, the loss of INK4A/Arf associated with the activation of K-Ras and Akt in mature astrocytes lead to the formation of tumors closely related to GBM morphology. In this model, loss of INK4A/Arf induces the dedifferentiation of astrocytes which consequently become more sensitive to malignant transformation via activated oncogenes such as K-Ras [23]. At the same moment, another study strengthened the idea that astrocytes might be at the origin of malignant astrocytomas. Indeed, the combined loss of tumor suppressors p16(INK4a) and p19(ARF) enabled astrocytes to dedifferentiate in response to EGFR activation. Transduction of Ink4a/Arf(−/−) astrocytes with constitutively active EGFR induced a common high-grade glioma

phenotype after xenografts experiments [24]. Last but not least, a very recent study demonstrated that the loss of p53 combined with oncogenes overexpression such as myr-Akt and c-Myc in mature astrocytes were highly tumorigenic in an *in vivo* model of brain tumor transplantation [25]. Loss of tumor suppressors and overexpression of oncogenes are not, stricto sensu, only involved in tumorigenicity since p53 and Akt have also been shown to induce the expression of stemness markers in mature astrocytes [26,27].

Figure 1. Relationship between NSCs' differentiation and cancer initiation. NSCs (purlple) are able to differentiate in glial (grey) and neural progenitors (yellow). Neural progenitors give rise to neurons (pink) whereas glial progenitors are committed to oligodendrocytes (red), ependymal cells (light blue) and astrocytes (dark blue). Brain cancer formation could arise from the transformation of NSCs into GICs (Brain Cancer Initiating Cells, light purple) which in turn give rise to a more differentiated cancer cell population (green). In the same line, glial progenitors could induce tumor development after malignant transformation of normal progenitor cells (Brain Tumor Progenitor-like Cells, orange). Traditionally, mature cells in the brain such as neurons, oligodendrocytes, ependymal cells and astrocytes are also seen as potential candidates involved in brain cancer tumorigenesis.



Loss of tumor suppressors and activation of oncogenes seem to be two mandatory criteria that both have to be met in order to trigger GBM initiation starting from astrocytes. Indeed, the only activation of oncogenes such as Ras and Akt is sufficient to induce GBM formation in nestine-positive progenitor cells but not in mature astrocytes [28]. In parallel, low grade gliomas can develop as a result of the

inhibition of tumor suppressor Ink4a/Arf in nestine-positive progenitor cells but not in GFAP positive progenitors [29].

2.2. The Stem Cells Theory

Rudolf Virchow has described this second theory for the first time in 1863. Based on histological similarities between embryonic stem cells and cancer cells, Virchow proposed that tumors originally develop from "dormant" or quiescent cells located in the host tissue. From then on, the existence of such a fraction of cells has been described in many types of cancers [9] including brain tumors [10–12]. As a matter of fact, astrocytic gliomas contain a sub-population of cells which exhibits stem cell-like properties such as multipotentiality, the ability to self-renew or to form neurospheres *in vitro* [30–32]. Interestingly, growth properties of glioma-derived neurospheres *in vitro* were found to be significant predictors of tumor progression and clinical outcome [33].

In the same line, several genetic studies using murine glioma models and imaging analyses from clinical studies provided the evidence that GBM may arise from the SVZ stem cell niche (SVZ) [34–36]. This region notably maintains the ability to produce neurons and glia throughout life, functioning as a source of stem cells and progenitors in adults [17,18]. At this level, NSCs are hierarchically organized. Quiescent type B cells give rise to highly proliferative cells, also known as transit-amplifying progenitor cells (type C cells), which then differentiate into two lineage-restricted progenitor cells; neuroblasts (type A cells) and oligodendrocyte precursor cells (OPCs) [37,38]. In this context, tumor-initiating cells are thought to arise from quiescent type B cells located in the SVZ. Indeed, those cells were demonstrated to pile up the largest number of genetic mutations in a transgenic hGFAP-Cre/p53$^{flox/flox}$ mouse model. Conversely, this study also showed that transit amplifying type C cells were able to accumulate strings of alterations which finally lead to tumor initiation and that Olig2-positive type C cells were notably involved in the early stages of gliomagenesis [39]. Additionally, another study recently showed that intraventricular infusion of PDGF was able to induce PDGFR alpha-positive type B cells to proliferate, contributing in this way to the generation of large hyperplasias exhibiting some GBM features [40]. In parallel, various studies have demonstrated the presence of human cytomegalovirus (HCMV) in GBM. This virus is now accepted as a tumor promoter in malignant brain tumor [41]. It has also been shown that HCMV preferentially infects NSCs. In this context, it has been hypothesized that NSCs' modulation by HCMV may contribute to the brain tumor genesis [42]. However, there are no reports so far on how HCMV modulates the pre-tumorigenic environment of the brain.

Although the SVZ is usually considered to be the stem cell compartment for glioma formation in mice following the introduction of genetic alterations observed in adult malignant brain tumors [34,39,43], several other germinal zones in the brain could potentially be at the origin of brain tumorigenesis as well, including the third and the fourth ventricle [44,45]. For instance, it has been shown that pediatric gliomas are more likely to arise from NSCs located in the third ventricle [46]. This observation notably allowed us to shed the light on the crucial role of innate brain region NSCs' heterogeneity in the patterning of gliomagenesis both in children and adults.

In 2009, the first example of a donor-derived brain tumor was reported. A boy with ataxia telangiectasia was treated with intracerebellar injection of human NSCs and was then diagnosed with a multifocal brain tumor four years after the treatment. Molecular and cytogenetic studies revealed that the

tumor was derived from at least two donors, suggesting in this case the implication of NSCs in gliomagenesis [47]. This work was the first report of a human brain tumor complicating neural stem cell therapy but has also been nuanced by other studies which, nevertheless, do not minimize the role of stem-cell-like astrocytes during reactive neurogenesis after brain injury or disease and during brain tumorigenesis [48].

Finally, a very important study recently underlined the crucial role of NSCs in brain tumors and the relevance of initial genetic mutations in the pathogenesis. While recombination of PTEN/p53 in NSCs gave rise to gliomas, the deletion of either Rb/p53 or Rb/p53/PTEN generated primitive neuroectodermal tumors (PNET), indicating the significant role of the initial Rb loss in driving the PNET phenotype [43]. Futhermore p53, Rb and RTK were shown to be core-signaling pathways commonly activated in GBM [49].

2.3. The Midway Theory

However, despite the plethora of examples showing that both astrocytes and NSCs seem to be strong contenders involved in malignant brain tumor formation, the GBM cell of origin remains largely elusive. Recent studies have indeed shown that other non-stem cells, including NG2+ cells [50] and oligodendrocyte precursors (OPCs) [51–53], can also be viewed as potential cells for the origin of malignant glioma [54]. As a matter of fact, OPCs are the most dividing cells in the adult brain. Whether this means those progenitors are more susceptible to tumorigenicity enhancement is yet to be determined. However, their proliferative ability and their broad distribution in the white matter as well as the grey matter make those cells potential suspects in gliomagenesis. Favorable indications supporting this hypothesis can be found in the literature. OPCs are plastic cells that can be converted *in vitro* to immature multipotent cells able to give rise to neurons, astrocytes and oligodendrocytes [55]. Glioblastomas freely express NG2 and PDGFR, two markers closely associated with OPCs [56]. Moreover, PDGFRα signaling pathway, controlling proliferation and migration of OPCs, is commonly altered in GBM [7,49,57].

Very recently, mosaic analysis with double markers confirmed that malignant transformation generating GBM only occurred in OPCs in a mouse model where NSCs are homozygously mutated for p53 and NF1 [51]. Interestingly, the authors also reported for the first time that the GBM "cell of origin" could be distinct from the cell of mutation. It is therefore of major interest to find reliable candidate in order to promote quiescence and differentiation of OPCs. As a first recent example, treatment of primary murine GBM cells with agonists of Grp17 resulted in a decreased number of neurospheres [58]. Grp17 is a 7TM receptor involved in the differentiation of OPCs which can be activated by two classes of molecules such as uracil-nucleotides and cysteinyl-leukotrienes [59].

Following this controversy, it is thus of great importance to gather major attention on the GIC population in order to better understand their biology and origin(s) (Figure 1) to improve or develop new groundbreaking therapeutic strategies. The induction of GICs' differentiation into less proliferative cells [60] along with the inhibition of signaling pathways involved in GICs' proliferation [61] or even the disruption of the GICs' relationship with their micro-environment [19] are as many hints which are given for further investigations.

3. The Tumor Micro-Environment

Glioblastomas are made of heterogeneous cell populations which do not only catch external signals from the environment but also respond to the latter in order to take advantage of it. It is commonly accepted that tumor-associated parenchymal cells such as vascular cells, microglia, peripheral immune cells and neural precursor cells directly interact with GBM cells and play a crucial role in controlling the course of the pathology. In the following paragraphs, we will try to describe the multiple interactions between the GIC population and the parenchymal cells in order to highlight the pathological impact of the tumor micro-environment on malignant brain tumors (Figure 2).

3.1. Involvement of Microglia

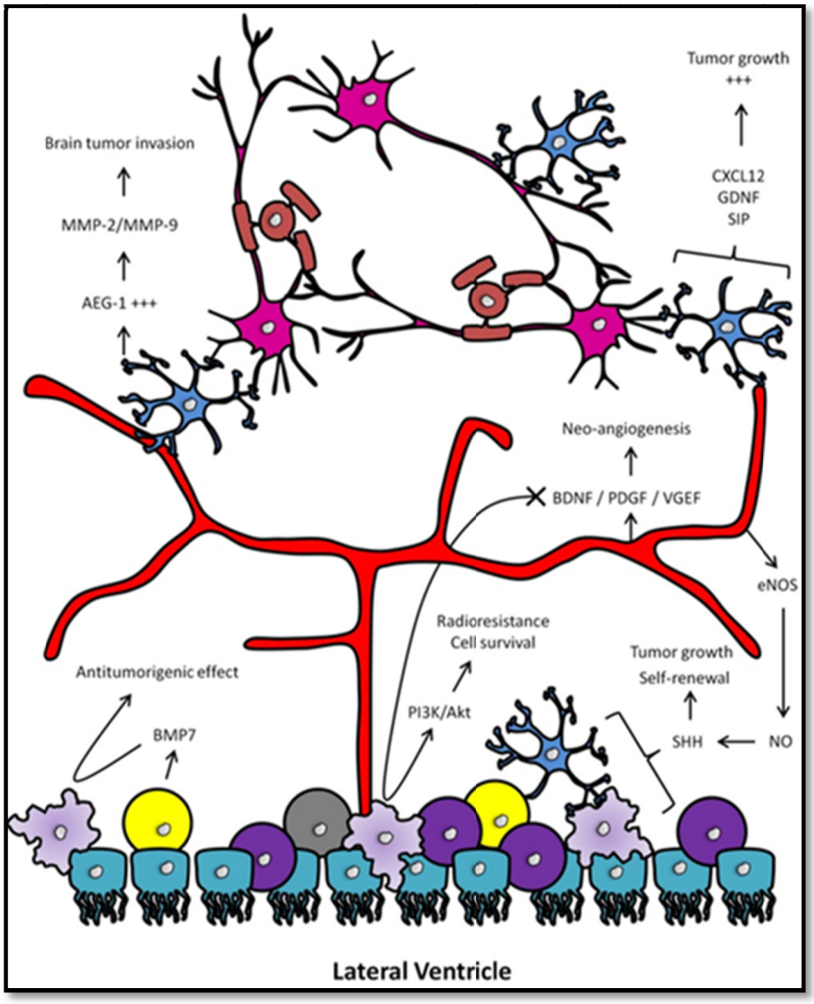
Tumor-associated macrophages are the most predominant inflammatory cell type which infiltrate GBM [62] and account for the major non transformed cell population in GBM biopsies [63,64]. Tumor-associated microglia notably break into the tumor mass in response to chemo-attractive cytokines released by the tumor it-self such as monocyte chemotactic protein-3 (MCP-3), colony-stimulating factor 1 (CSF-1) and granulocyte-colony stimulatory factor (G-CSF) [65,66]. Malignant brain tumors take advantage of this situation since tumor-associated macrophages were shown to infiltrate the tumor in order to enhance GBM cells' invasion by degrading the extracellular matrix. Indeed, microglia is able to trigger the release of membrane Type 1 metalloprotease (MT1-MMP) in response to soluble factors secreted by GBM cells which, in turn, release matrix metalloprotease 2 (MMP-2) that will be fully activated by the microglia MT1-MMP [67]. Matrix metalloprotease 2 is notably upregulated in microglia following the activation of the CX3CL1/CX3CR1 signaling pathway. Interestingly, chemokine receptor CX3CR1 was also shown to be upregulated in glioma associated microglia [68]. Moreover, a recent study showed that the common CX3CR1 allelic variant, termed V249I, was associated with increased GBM survival and reduced microglial cell infiltration in primary tumor biopsies as well [69]. All these findings definitely demonstrated the importance of microglia in GBM invasive properties and the necessity for developing more reliable *in vivo* models. We are convinced that better *in vivo* models would definitely improve our knowledge on those invasive tumor (initiating) cells which escape neurosurgery and radiotherapy modalities by leaving the tumor bulk.

3.2. Involvement of the Immune System

Following the example of tumor-associated macrophages, lymphocytes were also reported to infiltrate human gliomas. It has recently been suggested that a specific subtype of lymphocytes, regulatory T cells or Tregs, play an important role in the regulation of the immune response. In 2007, El Andaloussi and Lesniak described a positive correlation between the progression of the disease and the presence of Tregs in tumors with high malignancy [70]. Once again, chemokines such as CCL2 and CCL22 were incriminated for the attraction of Tregs towards the tumor site [71]. This specific infiltration was correlated with an increase in TGF-β1 mRNA and protein expression in a model of intracranial xenografts. The crucial role of the brain environment was markedly put to light in this study since this correlation was not found in gliomas injected subcutaneously [72]. For those reasons,

Tregs-associated glioma have been the center of attention for decades and represent nowadays innovative target in glioma therapy.

Figure 2. The tumor (SVZ) micro-environment. GICs (light purple) are regulated by the activation of oncogenes or by environmental conditions such as hypoxia or acidosis. GICs are able to express pro-angiogenic factors, such as VEGF (vascular endothelial growth factor), able to stimulate the outgrowth of new blood vessels (red) which will in the end help to maintain tumorigenicity. Endothelial cells are also able to produce nitric oxide in the endothelium through the activation of eNOS. Activated Notch signaling by nitric oxide in GICs is notably known to accelerate tumor progression. Defects in growth factor signaling pathways usually result in the activation of downstream growth factor signaling such as the PI3K/Akt signaling pathway which has been shown to be an important regulator of glioma cell survival and GICs' radioresistant abilities. Neural progenitor cells (yellow) display antitumor effects by secreting tumor suppressors in the niche such as bone morphogenetic protein-7 (BMP7), inducing GICs' differentiation. Astrocytes (dark blue) are closely associated with the vascular endothelium of the niche and were shown to secrete neurotrophic factors capable of driving the invasive properties of GBM cells (CXCL12, SIP, and GDNF). Astrocytes also express high levels AEG-1. Reports suggest that AEG-1 impacts on brain tumor invasion notably through the activation of MMP-2 and MMP-9.



3.3. Involvement of Neural Progenitors

Throughout the last decade evidence is accumulating that glioblastomas also interact with neural progenitor cells (NPCs) in the micro-environment. It has indeed been widely demonstrated that endogenous NPCs, from the subventricular zone or from the *corpus callosum*, preferentially home to experimentally induced brain tumors [73–76] probably in a CXCR4 dependent-manner [77]. Those tumor-associated NPCs are in fact diverted from their physiological migratory path in order to end up their journey in cellular layers surrounding the tumor mass [76]. There, they display important antitumorigenic effects by releasing soluble factors which interfere with GBM cell proliferation [78,79], causing GBM cell death [76] and promoting GICs' differentiation [73]. Moreover, significant survival improvements were observed *in vivo* using orthotopic coinjection of NPCs together with glioblastoma cells. This study also demonstrated that the tumor-suppressor effect of NPCs is largely related to aging and neurogenic abilities since younger mice significantly outlived older ones. Strikingly, this survival default was sealed by inoculating GBM cells along with NPCs in older mice brains suggesting the close relationship between NPCs' antitumorigenic properties and neurogenic aptitudes [76]. Let's keep in mind that the antitumorigenic capacity of NPCs has only been described in rodent models. It seems therefore required to check if human NPCs also display antitumorigenic properties similar to what has been described in rodents so far, especially since aging is considered as one of the most important prognostic factor for the disease. Moreover, the fact that neurogenesis declines with aging in humans [80] strengthens the link between GBM and this prognostic factor even more, notably by potentially decreasing the amount of NPCs and their related antitumorigenic effects throughout lifespan.

3.4. Involvement of the Vascular Niche

In physiological context, NSCs are located in specific regions of the brain called neurogenic niches [81,82]. Those niches, usually defined by a large vasculature network, have been the center of attention for many years since these anatomical structures were demonstrated to be the stem cell niches for normal and malignant neural tissue as well. Indeed, just like the adult NSCs, GICs also rely on vascular niches in order to control the balance between self renewal capacities and differentiation [19,83,84]. Moreover, let's just not forget that high grades glioma are mainly characterized by hallmarks such as endothelial hyperplasia and microvascular proliferation which are associated with a transition to a more aggressive phenotype, making malignant gliomas among the most vascularized tumors [85].

The vascular niches have been shown to be the primary location for cancer cells with stem cell-like characteristics [86]. In parallel, it has recently been shown that GICs can acquire a specific endothelial phenotype in order to create an early bound between the vasculature network and the tumor mass [87]. Moreover, GICs preferentially associate with endothelial cells which, in turn, accelerate their tumorigenic capacities. In fact, endothelial cells were shown to selectively interact with the GIC population in culture and supply them with secreted factors which maintain these cells in a self-renewing and undifferentiated state. Moreover, increasing the number of endothelial cells or blood vessels in orthotopic brain tumor xenografts expanded the pool of self-renewing cancer stem cells and accelerated the initiation and growth of tumors [19]. Interestingly, protein ligands that are found within the vascular niche have been demonstrated to regulate both stem cell self-renewal and angiogenesis, putting forward the idea that

these two processes are related. For instance, KIT ligand, also known to be a stem cell factor, was shown to be a powerful GBM-derived proangiogenic factor also involved in migration, survival and proliferation of NPCs [88,89]. In parallel, pigment epithelium-derived factor (PEDF) has also been demonstrated to play a crucial role in angiogenesis and to be involved in NSC self-renewal [90,91]. More recently, SVZ blood vessels and the ependymal cell layer of the vascular niche were shown to secrete CXCL12, creating in this way a u-shaped gradient in the niche [92]. Besides, chemokine receptor CXCR4 is known to be preferentially expressed by GICs [93] as well as the entire SVZ lineage [92]. In this study, the authors notably speculated that high levels of CXCL12 in the ependymal layer could help to promote quiescence. Indeed, high levels of CXCL12 were shown to result in receptor internalization, desensitization, and quiescence of hematopoietic stem cells, whereas lower concentrations resulted in proliferation and differentiation [94].

Following these observations, it has been suggested that the molecular crosstalk between GICs and the vascular network of the niches plays a critical role in tumor progression. A better understanding of these lines of communication will definitely provide new insights to improve the actual therapeutic means and develop new therapies which better target the micro-environment.

As a clinical example, there are considerable paracrine interactions between endothelial cells and the brain tumor cells in the micro-environment notably through the release of endothelial-derived soluble factors such as VEGF [95]. This factor has been shown to mediate the intercellular crosstalk between GICs and the tumor endothelium in order to induce angiogenesis [96]. Interestingly, neo-angiogenesis in astrocytomas reflects the tumor grade and is therefore often correlated with the poor prognosis or the aggressive phenotype of the disease. Furthermore, the increased amount of VEGF in the tumor micro-environment has been demonstrated to enhance the ability of GICs to promote angiogenesis compared to the non tumor-initiating cell populations [96,97]. Although the molecular mechanisms underlying this increase of VEGF production remain unclear, environmental factors such as hypoxia or acidosis have been proposed to play an important role in this process [98,99]. Activation of oncogenes such as EGFR or loss of PTEN can also lead to higher levels of VEGF in malignant gliomas [100]. As a result, the use of bevacizumab, an anti-VEGF antibody, allowed to significantly reduce tumor angiogenesis both *in vitro* and *in vivo* [101]. It is possible that this drug directly disrupts the maintenance of GICs and, in this way, effectively eliminates the roots of tumor progression. Although data on randomized phase III clinical trials with anti-angiogenic molecules are not yet available, this treatment regimen is already applied in several clinical centers at the time of recurrence (NCT00671970 and NCT00350727, [102]). Our opinion is that future anti-angiogenic therapies will have to rely on strategies combining chemotherapy and drugs which target invasive GBM cells. Indeed, those cells, sometimes located far away from the highly vascularized tumor core, are notably not targeted by anti-angiogenic therapies.

4. The Human SVZ and Its Clinical Implications in GBM

The discovery by Eriksson *et al*., in 1998, of neural progenitor cells (NPCs) capable of becoming mature neurons in the human brain, thought for decades to be a quiescent organ, has brought the brain's plasticity into sharp focus [103]. However, researches about stem cells implication in neurological disorder repair have met little success so far and their capacity to regenerate neurons after a lesion is,

for now on, very limited. Indeed, NPCs were only found to replace interneurons in specific regions of the brain such as the olfactory bulbs or the *dentatus gyrus*. Human NPCs, which look like glial cells but with stem cell features, remain in the adult brain in two restricted regions after that the hippocampal sulcus has become the subgranular zone (SGZ) of the hippocampus and the lateral ganglionic eminences turned into the SVZ [104,105]. Because neurogenesis in the SGZ is rigidly fixed by the age of 30 and is composed of a very small number of cells and nor could a link be established between the hippocampus and brain tumors, we will only focus on the SVZ environment in this review. As a matter of fact, the SVZ is the region that borders the ependymal layer on the lateral wall of the lateral ventricle and is separated from the caudate nucleus by a layer of myelin [106]. In the late 90s, specific culture conditions, using neurosphere formation, were used in order to isolate cells from the lateral wall of the temporal lobe in epileptic patients. These experiments already suggested at that time the presence of human NSCs in the adult brain [107–109]. As shown in rodent, progenitor cells located in this specific area are able to produce neuroblasts which migrate and integrate the olfactory bulbs. However, it seems that there is a considerably less activity in the human SVZ compared to rodents. Nevertheless, those human progenitors have the ability to proliferate and migrate towards injured regions close to the SVZ. This should be taken into consideration for the development of new treatment in neurological disorders and for our basic understanding of GBM (Figure 3).

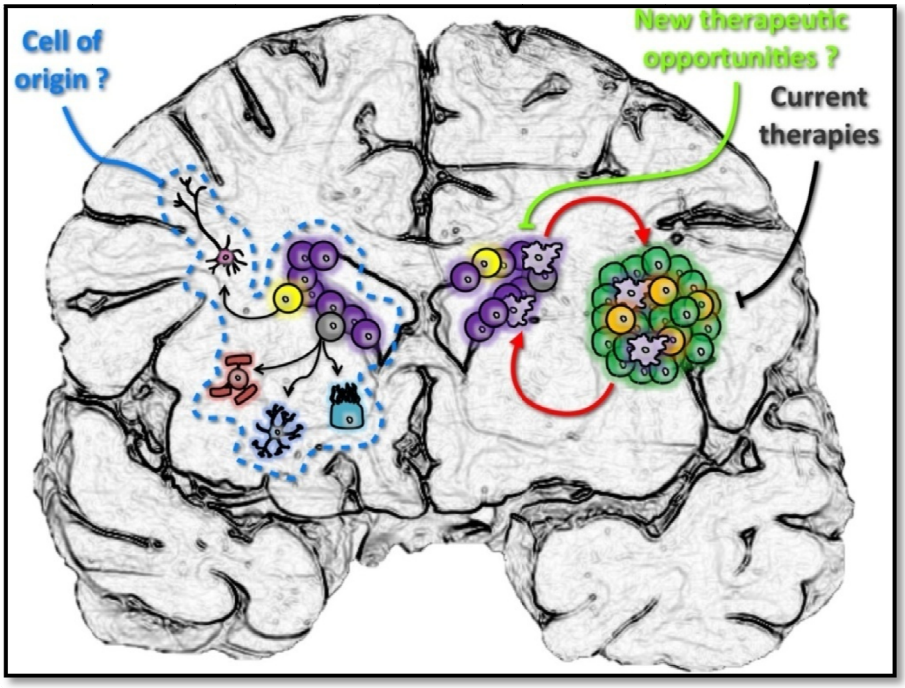
The adult human SVZ hosts three types of cell harboring progenitor properties (A, B and C) as already mentioned in the previous section [110]. Type C cells are found in the deepest layer (regarding the ventricle's wall) close to the myelin compartment. Type B cells are located in a well-defined cell-rich region. Type A cells staid in the cell-poor layer immediately beneath the ependymal layer. The ratio of cell types between rodent and human differs with the particularity that type A cells, or the migrating neuroblasts, are the most abundant in rodents while type B cells, identified as the most quiescent primary progenitors in rodents, are the major type in human [111,112]. Type C cells were shown to be less numerous in both species.

In rodents, the migration of type A cells to the olfactory bulb to replace interneurons is well established [113–115]. Recently, Curtis and collaborators have extended this knowledge to humans and discovered that human neuroblasts are also able to leave the SVZ and reach the olfactory bulbs trough the rostral migratory stream (RMS), a vestigial lumen that connects the lateral ventricle to the olfactory bulb [116,117]. The human RMS harbor neurogenic properties with a large number of cells proliferating found on the road to the olfactory bulbs [118,119].

To date, many studies in rodents have currently supported the idea that the "cell of originin" malignant brain tumors could derive from SVZ progenitors. Unfortunately the biology and the precise contribution of neural progenitors to normal human brain functions remain to be addressed and the understanding of their roles in neurological diseases has just started. Beyond the hypothetic role of the SVZ in generating GBM, it could be that GICs do not originate from NPCs. Using bio-mathematical models, Bohnam and collaborators discovered that 50% of GBM are actually located away from the SVZ environment and that their SVZ origin would therefore be doubtful [120]. No matter what this study shows, let's not forget that the SVZ offers a specific environment for GICs, as described in the previous section, which could directly or indirectly be involved in GBM growth and help to escape conventional treatment which finally account for tumor recurrence. Moreover, evidence accumulating

from clinical observations push in that direction as well and tend to evidence bit by bit the relationship between the SVZ and malignant brain tumors (Figure 3).

Figure 3. Relationship between the adult neurogenic niches and glioblastoma-initiating cells. GICs (light purple) have been found to play an important role in GBM aggressiveness and the resistance of the tumor to current treatments, making them an attractive target for therapeutic interventions. However, the design of new GIC inhibitors has proven difficult because their definition is nowadays still confused by the twilight zone of their origin. Indeed, whether GICs are derived from NPCs (yellow) or differentiated/mature cells remains uncertain. In any case, the vascular niche offered by the brain SVZ might be of interest to maintain GICs' features, even if they are not indigenous. Undoubtedly, particular characteristics of this specific niche and its localization away from the tumor mass make it a significant resource for tumor recurrence. It is expected that future therapies targeting GBM, GICs and their specific environment will help to increase the dramatic survival rate of GBM patients.



In 2007, Lim and collaborators reported for the first time the association between lesions surrounding neurogenic niches and the tumor phenotype [35]. Based on GBM MRI classification, they showed that tumors connecting the SVZ (almost 50%) are more frequently characterized as multi-focal tumors. At recurrence, tumors connecting neurogenic niches also favor new lesions at greater distance. It is of course tempting to speculate that those SVZ contacting tumors derived from NPCs. However there is no evidence so far that SVZ-related tumors are more likely to derive from stem cells, using for instance GBM genetic analysis [121]. In contrast, immune system genes were significantly over-expressed in tumor connected to the SVZ. This study by Kappadakunnel and collaborators failed to confirme that tumors which reach the SVZ display a more invasive phenotype at recurrence. The small size of the cohort was proposed as an explanation for this lack of reproducibility.

The SVZ involvement has been assessed as a potential independent prognostic factor for the overall survival (OS) and the progression free survival (PFS) in GBM patients [121–124]. Radiological observations of an intimate contact with the SVZ demonstrated an association with poor survival rates. An interesting study reported a significant decrease of survival in patients bearing tumors connected to the lateral ventricle [122]. However, the observed median survival difference (8 *vs.* 11 months) did not show statistical signification after that patients had undergone surgery (11 *vs*. 14 months). In a same line, SVZ connexion profile, analyzed in a cohort of 47 GBM patients, failed to demonstrate a significant correlation with survival [121]. However, a trend to a shorter survival rate was once again observed when GBM cells invaded the SVZ environment (median OS of 358 *vs.* 644 months). Recently, Kaplan-Meier analyses on a cohort of 91 GBM patients demonstrated shorter PFS at 6 months (47% *vs.* 69% survivors) as well as shorter OS at 2 years (23% *vs.* 48% survivors) in the group of patients whose tumors were connected to the SVZ compared with patients harboring no SVZ lesions [124]. This study also explored the impact of a cortical involvement and reported that such a relation does not exist. Nevertheless, conclusions reached by independent studies differ widely and the precise meaning of this phenotype is not clear yet. Cohort homogeneity may have contributed to this controversy. Tumor sizes were shown to be different between tumors classified according to the SVZ contact [125] but tumor volume did not impact survival [126]. However, the type of surgery performed, temolozomide adjuvant chemotherapy protocol and Karnofsky performance status score (KPS) are well-established independent prognostic factors of the disease [127–129]. Be that as it may, but the human SVZ has to be taken into consideration speaking about GBM.

Radiotherapeutic data also support the claim that GBM are related to the SVZ. A group from the University of California Los Angeles (UCLA) tested the hypothesis that targeting adult neurogenic niches could be of benefit for GBM patients in a retrospective study of 55 patients, including 17 patients with grade III and 38 patients with grade IV histology [130]. Aside the small size of the study and the lack of crucial information, the authors reported that a >43Gy irradiation dose of the ipsi- and contralateral SVZ increased the median progression free survival (15.0 *vs.* 7.2 months). Another retrospective study measured the SVZ dose-volume parameters and found a correlation with the survival outcome of 40 patients with GBM [131]. Multivariate Cox regression analyses for important prognostic factors (age, KPS, surgery type) revealed that higher ipsilateral SVZ irradiation doses were not found to be independent predictors for PFS but for OS. Furthermore, higher irradiation of the contralateral SVZ (>57.9 Gy) was associated with worse prognosis. Likewise, it has also been shown by another study that the SVZ involvement during radiotherapy is an independent predictive factor for shorter PFS and OS [123]. More recently, the UCLA group confirmed their previous data about the impact of SVZ irradiations on PFS and OS on a larger cohort of 173 patients by Cox regression analyses including five covariates (ipsilateral and contralateral SVZ doses, clinical target dose, age and extent of resection) [132]. Again, a significant correlation was found between high ipsilateral SVZ irradiation and improved survival, both for PFS and OS. Multivariate analysis only confirmed this advantage for PFS. Last but not least, a recent retrospective study confirmed these trends and specified that patients with GBM are more likely to benefit from SVZ irradiation when gross total resection was performed [133]. In this case, PFS was significantly higher in patients receiving ipsilateral SVZ doses of 40 Gy or above (15.1 *vs.* 10.3 months). Interestingly, OS was also significantly improved in patients receiving ipsilateral SVZ doses of 40 Gy or above (17.5 *vs.* 15.6 months). Such benefits could not be

observed in patients treated with biopsy and subtotal surgery. Obviously, those retrospective studies do not seem to be sufficiently robust to provide assurance that there is a consistent role of the SVZ contact in the GBM tumorigenicity.

5. Conclusions

While it is apparent that targeting GICs and neurogenic niches, given their particular architectures, should be seen as a great opportunity to improve the survival of GBM patients, critical data are nowadays still lacking. Indeed, further studies inquiring the origin(s) and the exact definition of GICs as well as robust prospective clinical trials are mandatory. We are convinced that a better understanding of the relationship between GICs and the so called neurogenic niches will provide new insights in order to improve or to set up new therapeutic strategies for highly malignant brain tumors. Indeed, even if the percentage of patients who survive two years from diagnosis of GBM has more than tripled in the last five years, largely because of the use of temozolomide plus radiation in addition to progress made with bevacizumab, research on treatment options for GBM is way more exciting now than ever before. Nowadays, the development of state-of-the-art neuroimaging techniques for improved surgical resections, vaccines and therapies aiming molecular targets as well as signaling pathways are bit by bit bending the tail end of the curve and raise great hope of making major improvements for GBM patients.

Conflicts of Interest

The authors declare no conflict of interest.

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Paper 4

Glioblastoma Stem Cells: New Insights in Therapeutic Strategies

REVIEW

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Future NEUROLOGY

Glioblastoma stem cells: new insights in therapeutic strategies

Nicolas Goffart[‡,1], Matthias Dedobbeleer[‡,1] & Bernard Rogister[*,1,2,3]

ABSTRACT Despite notable achievements in glioblastoma diagnosis and treatment, the prognosis of glioblastoma patients remains poor and reflects the failure of current therapeutic modalities. In this context, innovative therapeutic strategies have recently been developed to specifically target glioblastoma stem cells, a subpopulation of tumor cells involved in experimental tumorigenesis and known to be critical for tumor recurrence and therapeutic resistance. The current review summarizes the different trails which make glioblastoma stem cells resistant to treatments, mainly focusing on radio-, chemo- and immunotherapy. This broad overview might actually help to set up new bases for glioblastoma therapy in order to better fight tumor relapses and to improve the patients' prognosis.

KEYWORDS
• chemotherapy
• glioblastoma
• glioblastoma stem cells • immunotherapy
• radiotherapy • surgery

Brain tumors could arise from the CNS or as a result of metastasis coming from primary tumors growing in distant organs. WHO classifies primary brain tumors according to different features including the tumor localization, the cell types that are involved and the degree of malignancy. Glioblastoma multiforme (GBM; WHO grade IV) are considered among the most refractory and most aggressive malignancies of the CNS accounting for approximately 80% of malignant brain tumors [1]. High-grade gliomas (grade III and IV) also include anaplastic ependymoma, astrocytoma, oligodendroglioma or oligoastrocytoma and are much more aggressive than grade I and II tumors. GBM appear histologically similar to glial cells, including astrocytes and oligodendrocytes [2]. However, the exact origin of GBM remains nowadays still unclear, whether it is deriving from neural stem cells, neural progenitor cells or glial cells.

Cancers are commonly characterized by six biological abilities acquired during the multistep development of human tumors. These hallmarks include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis [3]. GBM are no exception to the rule and particularly display a large area of necrosis, a high level of tissue infiltration and resistance to radio- and chemo-therapy [4–6]. Integrated genomic analysis recently described high levels of tumor inter- and intra-heterogeneity highlighting conserved and individual mutations that make targeted therapies hard to design. The Cancer Genome Atlas research effort and independent genomic profiling studies indeed classified GBM based on *PDGFRA*, *IDH1*, *EGFR* and *NF1* abnormalities in classical, mesenchymal, proneural and neural subtypes [7]. Moreover, all of these subtypes could be found in distinct areas of the same tumor revealing the genome-wide architecture of intratumor variability [8]. The lack of

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treatment efficacy is to be found in this complex intra- and/or inter-tumor genetic heterogeneity.

Current treatment regimens are up to now only palliative and involve tumor resection in conjunction with radiation, chemotherapy or other experimental treatments, such as targeted immunotherapies [9,10]. However, as we already mentioned, patients systematically relapse within a year from the time of diagnosis, reflecting the failure of current therapeutic strategies. With these considerations in mind, a paradigm shift in the way we treat GBM must occur. Tumors should indeed be viewed as aberrant entities characterized by a really complex network of cell-to-cell interactions more than distinct entities residing in the normal environment. Furthermore, tumors should no longer be thought of as a homogeneous population of cells possessing equal tumorigenic potential but as a hierarchical organization in which a stem-like population of cells would be responsible for tumor progression and therapeutic resistance [11].

Nowadays, the existence of such fraction of cells, referred to as cancer stem cells or tumor-initiating cells has been described in different tumor types [12] including brain cancers [13,14] and raised a new hope in order to better understand the origin of GBM relapse. Glioblastoma stem cells, or initiating cells (GSCs or GICs), have been shown to share characteristics with normal neural stem cells including maintained proliferation, self-renewal and differentiation abilities. More importantly, they have been largely described as key mediators in experimental tumorigenesis, tumor maintenance and therapeutic resistance [15,16]. GSCs are now seen as a tumor subpopulation able to self-renew, generate diversified neuron-like and glia-like postmitotic progeny and, most importantly, perpetuate a tumor in orthotopic transplants [17]. Given the high mortality and the challenges associated with GBM treatment, this review will mainly focus on different therapeutic modalities recently developed in order to specifically target GSCs within the tumor.

Surgery

One of the most challenging problems in GBM therapy is to target the largest amount of tumor cells, including GSCs, without causing harm to the healthy brain. In this context, surgery often is ineffective given the invasive and infiltrating nature of the tumor, making the entire surgical resection of the tumor mass almost impossible [18]. GBM surgical methods have nevertheless significantly improved over the last years due to major progress in brain imaging. As examples, intraoperative MRI, robot- and fluorescence-guided surgery represent three major breakthroughs which now allow a reliable histological diagnosis, rapid palliation of symptoms and more accurate tumor resections. Intraoperative MRI (iMRI) is used during tumor resection to constantly update neuronavigation (robot-guided surgery) information in order to prevent distortion of the brain parenchyma, referred to as 'brain shift' [19]. More than guiding neurosurgeons during tumor resection, iMRI has notably been shown to be effective in increasing the extent of tumor resection and prolonging survival of GBM patients [20].

Fluorescence-guided surgery (FGS) of malignant gliomas based on 5-aminolevulinic acid (ALA) allows to specifically determine the borders of the tumor, making radical resection easier [21]. Interestingly, malignant gliomas are, to our knowledge, the only application for which FGS has been examined using a randomized controlled multicenter Phase III clinical trial [22]. Further testing and research are needed to keep on developing high-tech tools which will in the end finely improve tumor resection. Neurosurgery is nowadays an ever-changing field which recourses on never-before-conducted research to discover and create new methods of surgery, new technology and new treatments to help patients live longer.

The biggest challenge of the 21st century will probably be the improvement of surgical procedures with the aim to target GSCs. Indeed, surgical resections do not specifically tackle GSCs, which are known to display high invasive capabilities and which could in this way migrate away from the tumor core and infiltrate the normal brain parenchyma as lonely cells. We strongly believe that a better detection of GSCs during surgery could definitely help to resect this subpopulation of tumor cells more accurately and therefore improve the patients' survival as well as the efficacy of adjunct/adjuvant therapies.

Chemotherapy

• DNA repair & cell cycle modulators

The standard of care in GBM treatment is the administration of temozolomide (TMZ), an alkylation agent causing DNA damage by methylating O^6-position of guanine. This methylation will first result in mismatches with

thymine in dsDNA and will then activate the mismatch repair system, impairing the cancer cells to repair the DNA break. At the end, after multiple futile cell cycles and the accumulation of double strand breaks, replication is blocked leading to cell death [23]. To avoid cellular death, cells are able to promote the expression of the MGMT enzyme which, in turn, will withdraw the methyl group from the O^6-methylguanine. However, if the MGMT promoter is methylated, cells will not be able to face the mismatches in their own DNA and this will inevitably lead to cell death. The methylation status of the MGMT promoter has risen a lot of questions over the years since GBM patients do not display the same MGMT pattern of expression, leading to discrepencies in TMZ treatment sensitivity [24]. Despite the fact that the methylation status of the MGMT promoter indicates increased efficacy of current standard of care [25], taking it as a robust and reliable predictive biomarker that can be used for stratification of treatment regimes seems risky due to the diversity of TMZ responses in GBM patients [26]. Regarding the specific GSC response to TMZ, Biere and collaborators have shown that this agent induces a dose- and time-dependent decline in stem cell subpopulation. Moreover, higher doses of TMZ can dramatically change its effect on GSCs expressing MGMT [27]. In the same line, Okada and colleagues recently reported JNK as a rational therapeutic target promoting the expression of MGMT and TMZ resistance of GSCs [28]. JNK is a cytoplasmic tyrosine kinase that regulates cell growth, cell differentiation and apoptosis. They showed that inhibition of JNK, either pharmacologically or by RNA interference, in stem-like glioblastoma cells derived directly from glioblastoma tissues reduces their MGMT expression and TMZ resistance. Nonetheless, controversy remains regarding the association between poor survival rates and the specific expression of MGMT in GSCs [10,29]. Further investigations are thus mandatory in order to first clarify whether or not the methylation status of the MGMT promoter could be a reliable prognostic marker in the evolution of the tumor. Second, further research need to reconsider the time between the administration and the concentration of the drug as potential factors in treatment resistance. Finally, coupling TMZ with other drugs in order to sensitize GSCs to TMZ activity is nowadays thought as a promising gold standard in GBM treatment.

The expression of MGMT is not the only cause of resistance to TMZ. Indeed, it has recently been shown that the expression of several growth factors such as IGF1 may also confer resistance to TMZ via activation of Hedgehog/Gli pathway [30]. In addition to growth factors, environmental factors such as hypoxia could also confer TMZ resistance to cancer stem cells [31]. Hypoxia conditions may also trigger the rebooting of non-GBM stem cells into cells displaying a stem-like cell phenotype notably through the expression of stem cell factors such as Oct4, NANOG and c-MYC [32].

In parallel to the MGMT repair system, TMZ alkylation can also target a N3/7-methylpurine that will induce the blockage of DNA replication, DNA strand breaks leading to apoptosis. To counteract with cell death, GSCs have found a way to trigger the base excision repair pathway through the activation of PARP-1, recruiting DNA repair enzymes at the break site. Recently, overexpression of PARP-1 in GSCs has been shown to induce either radio- or chemo-resistance [33]. PARP-1 inhibitors like ABT-888 or Veliparib, have then been developed. These inhibitors are supposed to block the catalytic domain of the enzyme preventing in this way the synthesis of ADP ribose polymers and avoiding the reparation of the N-methylpurine. Barrazuol and collaborators recently showed the benefit of this inhibitor against GSCs and preferentially for patients carrying an MGMT-unmethylated promoter [34]. As reported by Tentori and collaborators, PARP inhibition specifically sensitizes GSCs to TMZ which results in lower administered doses and increases TMZ efficacy [35]. Several clinical trials are now ongoing to test the efficacy of these inhibitors together with administration of TMZ. In example, side effects and best dose of ABT-888 has already been assessed in a Phase I/II clinical trial involving newly diagnosed GBM patients (NTC00770471 [36]).

Altogether, accumulating data from last decades have shown that TMZ does not display full efficacy on GSCs, efficacy which is not reproducible from one cohort patient to another. It is thus urgent to clarify the situation and highlight the resistant pathways limiting the efficacy of the drug to fulfill loopholes remaining in the field. From then on, development of multimodal therapies including TMZ in combination with other chemotherapeutic agents seems mandatory in order to better fight GSCs.

• Antiapoptotic mechanisms

Recent research has demonstrated minder sensitivity of GSCs to apoptosis through greater methylation of the caspase-8 promoter that typically initiate apoptosis [37]. Following this, mechanisms to enhance apoptosis have been investigated and inhibition of the proteasome by bortezomib proved to be an effective way to target GSCs. This drug acts specifically by blocking the proteasomal chemotrypsin-like activity in mammalian cells, inhibiting the renewal of the protein pool. By inhibiting the destruction of specific factors like DR4 and DR5, two death receptors involved in the transduction of death signals, cells will be more sensitive to death cytokines such as TRAIL. This activation will be followed by a downregulation of inhibitors of the apoptosis intrinsic pathway such as XIAP and Akt, notably via the activation of PKCεand the stabilization of tBid [38,39]. Proteasomal activity includes degradation of death signal proteins as well as other factors such as HIF1α, a key regulator of hypoxia which promotes neoangiogenesis via VEGF production. As a result, accumulation of HIF1α and VEGF in the nearby environment following bortezomid treatment leads to the formation of new blood vessels surrounding the tumor. This issue has notably been solved by the administration of bevacizumab [40], a monoclonal antibody blocking VEGF and inhibiting the formation of blood vessels. Despite this interesting observation, bevacizumab, or Avastin, has largely been criticized because of a lack of specificity and the fact that GSCs could differentiate into endothelial cells. Indeed, exposure to bevacizumab does not inhibit the differentiation of CD133-positive GSCs into endothelial progenitors [41–43]. However, clinical trials are currently ongoing to identify the potential benefits of bevacizumab alone or together with other chemo-agents such as Bortezomib as previously described (NTC00611325 [44]) or Plerixafor, also known as AMD3100, in order to prevent the growth of recurrent high-grade gliomas (NCT01339039 [45]). AMD3100 is a specific antagonist of CXCR4 which has been found to be a prognostic marker in various types of cancer. CXCR4 is also involved in cancer cell chemotaxis, stemness and drug resistance [46,47]. Interestingly, Gatti and collaborators recently demonstrated that CXCL12/CXCR4 interactions specifically mediate GSC survival and self-renewal abilities. These observations could then be hampered with a high selectivity by the use of AMD3100, highlighting CXCR4 and CXCL12 as candidates responsible for the maintenance of cancer progenitors and providing survival benefits to the tumor [48]. In the same context, a recent study demonstrated that AMD3100 could prevent GSCs from specifically invading the subventricular zone, one of the two neurogenic niches of the adult brain [49].

The transcription factor NF-κB has also been described as a key mediator involved in cancer stem cell growth [50] and its regulator, A20, was found highly expressed in GBM cells [51]. The exact role of A20 remains nowadays still unclear whether it acts as an antioncogenic factor like in non-Hodgkin's lymphoma [52] or as a prooncogenic factor. Indeed, while high levels of *A20* mRNA are correlated with a poor prognosis in GBM patients (Repository for Molecular Brain Neoplasia Data [REMBRANDT]), Hjelmeland and colleagues pointed that A20 was overexpressed in GSCs and suggested that A20 may function as a tumor enhancer in glioma through promotion of GSC survival [53]. On the other hand, low levels of A20 in GBM were correlated with increased chemoresistance against alkylating agents like TMZ. Further investigations are thus necessary to clarify the exact role of A20 in GSCs since anticancer therapies targeting A20 seem to widely vary depending on the tumor type.

• Differentiation of GSCs

A key characteristic of GSCs is to stay in a quiescent state. This being said, many therapeutic concepts have been developed in order to promote GSC differentiation. In this context, the role of metformin, an antidiabetic drug, has been assessed in oncology with substantial GSC therapeutic effects. More specifically, metformin has been proven to first potentiate TMZ pro-apoptotic effects via the modulation of the AMPK signaling pathway [54] and to affect both survival and proliferation of GSCs [55]. In parallel, Sato and colleagues administered metformin to mice in order to study its potential therapeutic effects on GSCs. This study highlighted a substantial survival benefit by promoting the differentiation of GSCs via the activation of the AMPK-FOXO3 axis [56]. Metformin was shown to exert anticancer effects by blocking the LKB1/AMPK/mTOR/S6K1 pathway inducing selective lethal effects on GSCs. The potential association of metformin with arsenic trioxide (ATO) in GBM therapy has recently

been assessed. Indeed, both drugs promote differentiation of GSCs into nontumorigenic cells. As already mentioned, metformin acts via activation of the AMPK-FOXO3 axis whereas ATO blocks the IL-6-induced promotion of STAT3 phosphorylation. A prompt clinical assessment of metformin and ATO in GBM patients would definitely represent a valid attempt to improve their survival [57]. In this context, clinical trials are currently ongoing in order to check whether or not metformin could be used to improve the outcome of GBM patients (NCT01430351 [58] and NCT02149459 [59]).

Several other signaling pathways have been identified for their implication in GSC differentiation into mature cells such as astrocyte- or neuron-like cells. Among these pathways, bone morphogenetic protein family is known to inhibit GSC proliferation and to induce their differentiation. Paradoxically, GSCs are also known to express high levels of bone morphogenic proteins (BMPs) [60]. Three different proteins have been highlighted in this family for their role in GSC differentiation including BMP2, BMP4 and BMP7 [61]. BMP4 was first described by Piccrillo and collaborators [60] and was shown to reduce the proliferation of GSCs and to specifically increase the expression of neural differentiation markers. Another study recently demonstrated that human adipose derived mesenchymal stem cells overexpressing BMP4 were able to induce differentiation of GSCs while decreasing both proliferation and migration of GBM [62]. Moreover, BMP7 has also been described as a key factor in GSC differentiation into astroglial lineage [63]. Implantable microsphere system optimized for the controlled release of BMP7 was engineered as a therapeutic device against GSCs. This BMP7 microsphere delivery showed a remarkable capacity to stop tumor formation in a GSC culture model by reprogramming tumorigenic cells into nontumorigenic astroglial lineage [64]. Finally, Persano and colleagues showed that BMP2 induced a strong differentiation of GSCs and subsequent addition of TMZ caused a dramatic increase of apoptosis. Importantly, the authors correlated these effects to a BMP2-induced downregulation of both HIF-1α and MGMT [65]. In addition, they also described the key role played by BMP2 as a differentiating factor of GSCs. Still in the same line, a recently discovered protein, specifically expressed in GSCs, was shown to play a key role in the maintenance of the stem cell phenotype by inhibiting the BMP family. Gremlin1 plays indeed an important role in self-renewal, growth and proliferation of GSCs by inhibiting the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ [66]. This study demonstrated that Gremlin1 is specifically expressed by GSCs and protects these cells from BMP-mediated differentiation effects. Overexpression of Gremlin1 in non-GSCs decreased their endogenous BMP signaling, promoted stem cell-like features and increased growth and tumor formation abilities. GSC-derived Gremlin1 is thus considered as a driving force in maintaining GBM proliferation and GBM hierarchies through the modulation of endogenous differentiation promoting signals.

To end up the story on differentiation factors, several studies highlighted the role of SOX11 as a tumor suppressor by inhibiting *Plagl1*. *Plagl1* is known as an oncogenic gene that regulates the expression of other genes involved in tumorigenesis and early development, including *IGF2* and *DLK1* [67]. Moreover, according to the REMBRANDT repository, GBM patients displaying low *Plagl1* mRNA levels have an increased survival rate compared with those with an intermediate level of expression. Hide and colleagues clearly made the link between SOX11 and *Plagl1* and showed that SOX11 prevents gliomagenesis by blocking the expression of *Plagl1* and inducing neuronal differentiation [68]. Interestingly, a similar role in promoting GSCs differentiation has been described for *Plagl2* upon the activation of the Wnt and β-catenin axis [69].

• Growth factors & the maintenance of the stem cell phenotype

Let's now focus on factors involved in the maintenance of the stem cell phenotype. In this line, the role of TGF-β has been investigated and linked to immunosuppression, migration and invasion of different types of malignancies including GBM [70]. In correlation with high levels of TGF-β2 in GSCs, Penuelas and colleagues highlighted the crucial role of LIF in self-renewal and prevention of differentiation via the Jak/STAT pathway [71]. Moreover, TGF-β inhibitors, currently under clinical development, were shown to specifically target GSCs in GBM patients [72]. Gene responses to TGF-β inhibition were recently determined and included inhibitors of ID-1 and ID-3 transcription factors which are preferentially enriched in GSCs that also express high levels of CD44. Inhibition

of the TGF-β pathway decreases the number of CD44(high)/ID1(high) GSCs through repression of ID1 and ID3 levels, therefore inhibiting the capacity of these cells to initiate tumors. Interestingly enough, high levels of CD44 and ID1 were found to confer poor prognosis in GBM patients strengthening the importance of TGF-β pathway. Trying to target the self-renewal potential of GSCs via TGF-β axis, Ikushima and colleagues, questioned the role of SOX2 and SOX4 in the maintenance of GSC stemness [73]. This study demonstrated that TGF-β induces the expression of SOX2, a stemness gene, which is itself mediated by SOX4, a direct TGF-β target gene. Inhibition of TGF-β signaling deprived GSCs from tumorigenicity whereas these effects were attenuated in GSCs transduced with SOX2 or SOX4. In addition, Oct4 was shown to play a crucial role in retention of stemness properties of GSCs through positive regulation of SOX2 expression. In GSCs, Oct4 was found associated with SOX4 to form a complex which will cooperatively activate the enhancer activity of the *SOX2* gene [74]. Creating a bridge with Capper's observation who detected a preferential hypermethylation of the caspase-8 promoter in GSCs [37], Held-Feindt and colleagues questioned the eventual relationship between the expression of TGF-β1 and the adhesion molecule L1CAM. They showed indeed that TGF-β1 and L1CAM expression increased during GSC differentiation. Differentiated GSCs then showed a reduced apoptotic response after TMZ treatment. Accordingly, siRNA-mediated knock-down of L1CAM in differentiated GSCs increased chemosensitivity whereas overexpression of L1CAM in GBM spheroids reduced the apoptotic response. Interestingly enough, elevated L1CAM expression was also at the origin of a decreased expression of caspase-8 in differentiated GSCs [75]. Altogether, these observations highlight TGF-β as a robust potential target that makes GSCs less resistant to common treatment, blocks the maintenance of the stemness phenotype and enhances apoptotic death.

Since the Wnt/β-catenin axis is involved in proliferation and self-renewal capacities of neural stem cells, deeper investigations have been made in order to unravel the eventual role of this pathway in GSC resistance to treatment. Recently, FoxM1 was described as a key downstream component of the Wnt signaling allowing β-catenin transcriptional function in GSCs [76]. Moreover, FoxM1 has been shown to be partially responsible for TMZ resistance in GSCs via the expression of the DNA repair gene *Rad51* [77]. Indeed, FoxM1 knockdown inhibited *Rad51* expression and sensitized recurrent GBM cells to TMZ. A recently published study demonstrated that FoxM1 is able to form a protein complex with the mitotic kinase MELK in GSCs, leading to phosphorylation and activation of FoxM1 in an MELK kinase-dependent manner which finally results in a subsequent increase in mitotic regulatory genes in GSCs [78]. The MELK-mediated FoxM1 signaling can be disrupted using Siomycin A. Surprisingly, addition of Siomycin A to TMZ treatment in mice harboring GSC-derived intracranial tumors enhanced the effects of the latter. In the same line, Compound 1 (C1), a multikinase inhibitor, was recently described as a promising therapeutic target, causing GSC death after inhibiting MELK *in vitro* [79].

The regulation of protein expression always begins with a tiny regulation of gene expression. Last decades of research in the GBM field revealed the importance of the transcription factor STAT3 in malignant brain tumors [80]. As soon as GBM recurrence has been linked to GSCs, emerging data on the role of STAT3 involved in GSC proliferation or maintenance of the stemness phenotype started to pile up [81,82]. One of the most important signaling pathways leading to STAT3 activation is the Notch pathway [83]. It has indeed been described that Notch promotes both survival and self-renewal abilities in GSCs and that its downstream effectors including STAT3 and AKT are related to Notch signaling activation via their phosphorylation [84]. Recently, relying on the importance of neoangiogenesis in GBM, Guichet and colleagues linked Notch1 to the regulation of GSC plasticity and angiogenic properties [85]. Notch1-stimulated GSCs indeed expressed pericyte cell markers and were then closely associated with endothelial cells. Other investigations on the methylation of EZH2, already described as a crucial mediator in cancer stem cell maintenance [86], showed that EZH2 could trigger the activation of STAT3, leading to GSC-enhanced tumorigenicity [87].

The Hedgehog (HH) pathway leading to the activation of transcription factor Gli1, has also been broadly described notably for its role in self-renewal [88], proliferation and GSC malignancy [89]. In addition, Zbinden and collaborators have recently described the relationship between

NANOG in human GBM and the HH-Gli1 activity. They notably found that NANOG modulates gliomasphere formation and clonogenicity (two functional characteristic of GSC behavior) as well as their proliferation. NANOG was also found essential for GBM tumourigenicity in orthotopic xenografts [90]. Inactivation of the HH-Gli1 axis using Cyclopamine was shown to specifically deplete stem-like cancer cells in glioblastoma [91,92]. On a broader spectrum, the association of Guggulsterone, an inhibitor of the NF-κB and STAT3 pathways, together with SANT-1, an inhibitor of the HH pathway has been proposed in GBM treatment [93]. Guggulsterone-induced ERK activation also contributes to caspase-9 activation. Since SANT-1 and Guggulsterone differentially target GSCs and nonstem GBM cells, respectively, this combination warrants investigation as an effective antiglioma therapy.

On the same topic, arsenic trioxyde (ATO), a US FDA-approved drug for the treatment of promyelocytic leukemia, was described as an inactivator of Notch and HH pathways leading to the inhibition of stem cell properties in GBM neurospheres [94,95]. Keeping in mind that the inactivation of Notch and HH pathways enhances the sensitivity of GSCs to TMZ treatment [96], the choice of ATO as a future therapeutic perspectives may also carry great hope for future GBM therapy.

Relying on the importance of hypoxia in the tumor microenvironment, the enzyme ALDH1 has been described as a key modulator in GSC maintenance. ALDH1 is indeed highly expressed in GSCs and hypoxia was shown to upregulate its expression [97,98]. Moreover, ALDH1A1 overexpression in GSCs predicted TMZ resistance *in vitro*. In this study, ALDH1A1 was characterized as a new mediator for GBM resistance to TMZ and a reliable predictor of clinical outcome [99]. A recent study investigated the effect of disulfiram, an aldehyde dehydrogenase inhibitor, in combination with gemcitabine on GSCs. The treatment was shown to abolish the stem-like cell population in GBM cell lines by induction of ROS and inhibition of both ALDH and the NF-κB pathways [100].

To conclude this GSC chemoresistance part of the manuscript, we would like to emphasize the fact that targeting a single pathway or a molecule alone does not make sense in the context of multimodal therapy. We believe that the combination of multiple therapeutic agents targeting a variety of proteins/signaling pathways could be much more efficient in terms of GBM treatment than trying to gather the effort on a specific target.

Radiotherapy

Current therapies for malignant brain tumors are as already mentioned only palliative. Nowadays, increasing evidence suggests that GSCs are to be incriminated for brain tumor relapses. GSCs have indeed been demonstrated to be involved in experimental tumorigenesis and tumor maintenance but also in radioresistance [4]. γ-radiation induces DNA double-strand breaks (DSB) that will be identified by the auto-activated ATM kinase and NBS1 [101]. Following radiation, NBS1 will form a complex called MRN constituted of Mre11 and Rad50. This MRN complex will then be recruited at the DNA break and is required for efficient phosphorylation and recruitment of ATM to the break [102]. MRN and ATM will finally phosphorylate H2AX to generate γH2AX, which represents the first mark of DNA damage sensing. Following DNA damage recognition, mediator proteins such as BRCA1, MDC1 and 53BP1 will amplify the signal which will be sent out to downstream effectors involved in the regulation of chromatin remodeling and DSB repair [103,104]. In this context, Bao and collaborators have shown that GSCs preferentially activate DNA damage checkpoint proteins including ATM or the cell cycle checkpoint protein Rad17 in response to radiation. The authors also showed in the same study that GSCs are able to repair radiation-induced DNA damage more effectively and more rapidly than non-GSCs [105]. In addition, the capacity of GSCs to be radioresistant could be overturned with specific inhibitors of Chk1 and Chk2 checkpoint kinases, suggesting a new potential therapeutic target for malignant brain cancers. Checkpoint kinases 1 and 2 have been shown to activate the DNA repair machinery in response to radiation after phosphorylation by L1CAM which, in turn, specifically regulates NBS1, the core component of the MRN complex [106]. Since L1CAM is preferentially expressed in GSCs (as previously described), this protein therefore represents a potential molecular target that may be beneficial in attenuating GSC radioresistance.

Nowadays it is commonly accepted that preferential activation of DNA damage response in GSCs contributes to their radio-resistance

although the molecular mechanisms underlying this efficient activation of DNA damage response remain elusive. In this context, it has recently been shown that RNA-binding protein RBM14 stimulates DNA repair by controlling the DNA-PK-dependent nonhomologous end-joining (NHEJ) pathway [107]. NHEJ is considered as one of the two major pathways engaged during DNA repair. It is typically initiated during the G_1-phase of the cell cycle and relies on ligases and excision repair enzymes to adhere broken DNA ends that can introduce spontaneous mutations [108].

The second repair mechanism, called homologous recombination, is an error-free method of repair which occurs during late S- and G_2-phases and which uses sister chromatids as templates to replace the damaged DNA [109]. Homologous recombination and cell cycle checkpoint abnormalities have recently been reported to contribute to GSC radioresistance. Lim and collaborators have indeed compared the DNA damage response to ionizing radiation in neural progenitor cells (NPCs) and GSCs. They found that NHEJ in GSCs was equivalent or, in some cases, even reduced as compared with NPCs. There was also evidence for more efficient homologous recombination repair in GSCs. Finally, the study revealed attenuated checkpoint kinase activation in GSCs suggesting inadequate cell cycle arrest at G_1-S and allowing a portion of G_1 damaged cells to enter S-phase [110]. These data suggest that homologous recombination and cell cycle checkpoint abnormalities may contribute to GSC radioresistance and that both processes may be suitable for future therapies targeting GSCs in comparison with established non-GBM stem cells. Moreover, the population doubling time was shown to be significantly increased in GSCs and the basal activation of the checkpoint kinases Chk1 and Chk2 was more pronounced in GSCs compared with non-GSCs [111]. This observation could determine the observed cell cycle delay in GSCs and contribute to their radioresistance by providing an extended time for DNA-damage repair.

In the same line, an additional mechanism of radioresistance through the DNA damage response may involve the polycomb group protein, BMI1. Following radiation, BMI1 preferentially copurifies with ATM, γH2AX and NHEJ proteins such as DNA-PF, PARP-1, hnRNP U and histone H1 in GSCs. BMI1 deficiency was also shown to severely impair DNA DSB responses resulting in increased sensitivity to radiation [112]. Interestingly, other studies supporting GSC radioresistance have shown that BMI1 was highly enriched in GSCs and is required in order to sustain cancer initiating stem cells renewal [113]. This observation really emphasizes the role of BMI1 in cancer stem cells resistance to radiation since stem cell maintenance pathways also seem to play a critical role in promoting radioresistance in GSCs. Indeed, relying on the fact that Wnt/β-catenin signaling mediates radioresistance in mammary progenitor cells through survivin upregulation [114], Kim and colleagues generated an orthotopic GBM model to show that GBM cell lines and patient-derived freshly dissociated GBM specimen were enriched with cells positive for both ABC and SOX2 after radiation treatment [115]. Interestingly, this subpopulation of ABC/SOX2-positive cells was further increased after additional *in vitro* radiation treatments, suggesting that radiation resistance of GBM is mediated by the activation of stem cell associated pathways including Wnt.

Notch signaling has also been demonstrated to be essential for maintaining stemness properties and tumorigenic potential of GSCs [84]. Notch signaling notably promotes radioresistance by upregulating the PI3K/AKT signaling and increasing levels of prosurvival Bcl-2 family members such as MCL1. Recent studies have shown that blocking Notch activation using γ-secretase inhibitors impairs GSC survival and enhances radiation-induced cell death [116]. Interestingly, constitutive activation of intracellular domains of Notch1 and Notch2 attenuates the radiosensitizing effects of γ-secretase inhibitors in GSCs. In the same line, Notch1 and/or Notch2 knockdown sensitizes GSC radiation therapy and impairs tumorigenic capacity, indicating a critical role of Notch/PI3K/Akt signaling in GSC radioresistance [116]. Recently, the radioprotective effect of Akt signaling was suggested in response to the activation of IGF-1 receptors on GSCs while treatment of tumors with specific IGF receptor blockers increases GSC sensitivity to ionizing radiation [117].

Similar to Notch receptors, CD44, a glycoprotein transmembrane receptor, is also subject to proteolytic activation: an extracellular cleavage followed by a γ-secretase-dependent release of C-terminal intracellular domain (CD44ICD) [118,119]. The CD44 ligand OPN has recently been described to promote glioma

stem cell-like phenotypes via the γ-secretase-regulated CD44ICD, which promoted aggressive GBM growth *in vivo* and, first and foremost, radiation resistance in GSCs via activation of CD44 signaling [120]. Interestingly enough, the role of OPN in radioresistance has already been suggested in other types of malignancies such as lung cancer by controlling autophagy [121].

Immunotherapy

In the context of cancer and more specifically in GBM, cells have developed mechanisms to suppress or bypass the immune system giving the opportunity to tumor cells to freely proliferate. Cancer stem cells are notably able to interfere with specific signaling pathways in order to promote immunosuppression, avoiding in this way any kind of immune reaction in the near microenvironment. Recently, the first immunobiological characterization of different GSCs isolated from patients stated that these cells display lower immunogenicity and higher suppressive activity than non-GSCs. The immunogenicity could however be rescued by immune modulation leading to anti-GBM T-cell-mediated immune response [122]. Furthermore, GSCs have been demonstrated to contribute to tumor evasion of the immunosurveillance by markedly inhibiting T-cell proliferation and activation and inducing T-cell apoptosis notably mediated by soluble factors such as Galectin-3 [123]. Interestingly enough, another study strengthened the link between malignant brain tumors and immunosuppression even more. In this paper, the authors showed that the ability of GBM infiltrated macrophages to be stimulated via Toll-like receptors, to secrete cytokines and to upregulate costimulatory molecules which in turn activate antitumor effector T cells is not sufficient to initiate an immune response [124]. GSCs were later incriminated to recruit circulating monocytes into GBM through the secretion of chemokine CCL2 and CSF-1. As we already mentioned, GSCs also secrete TGF-β1 which, together with CSF-1 will polarize the monocytes toward the immunosuppressive M2 macrophage phenotype with an upregulation of STAT3 and a downmodulation of the pro-inflammatory STAT1 [125]. Finally, GSC-exposed macrophages were also shown to increase the secretion of immune suppressive cytokines such as IL-10, TGF-β1 and IL-23 and to increase their capacity to inhibit T-cell proliferation [125]. Surprisingly, the key role played by GSCs in immunosuppression seems to be mediated by the STAT3 pathway. As we already described, STAT3 is upregulated in GSCs and plays a crucial role in GSC growth and self-renewal [81]. Once activated, STAT3 is shown to regulate the transcription of IL-10 which, in turn, suppresses Th1-mediated cytotoxic immune responses essential for Treg function [126,127]. The STAT3 pathway has also been described as a key mediator in tumor immunosuppression by inhibiting macrophage activation [128,129], inhibiting polarization to the effector M1 phenotype and promoting polarization to the immunosuppressive M2 phenotype [130]. In addition, GSC-mediated immunosuppression can be blocked by inhibitors of activated STAT3 [125,131]. It has indeed been demonstrated that p-STAT3 inhibition by WP1066 in GSCs blocks the JAK2/STAT3 interaction and subsequent phosphorylation of STAT3 resulting in the upregulation of costimulatory molecules (CD80 and CD86), secretion of pro-inflammatory cytokines essential for T-effector responses and activation and proliferation of T cells [125,132]. A similar study demonstrated that GSC immunosuppressive properties were markedly diminished when the STAT3 pathway was blocked using specific STAT3 siRNA in GBM cancer-initiating cells [131]. Taken together, these data indicate that the STAT3 signaling pathway is a central regulatory factor that mediates multiple mechanisms of immunosuppression and that GSCs promote immunosuppression by activating p-STAT3.

In the same field, different studies have tried to highlight specific GSC markers in order to develop innovative cancer immunotherapeutic approaches. Given the fact that GSCs induce immunosuppression by inhibiting the antigen presentation, targeting GSC surface antigens could serve as a strategy to improve the outcome of cancer therapy via more effective destruction of tumor-initiating cells. In this context, central immune effectors in cancer cell destruction are $CD8^+$ T cells, also called cytotoxic T lymphocytes (CTLs) [133]. $CD8^+$ T cells are able to identify antigenic peptides presented by HLA class I molecules on the surface of cancer cells with their antigen-specific T-cell receptor [134]. The interaction of T-cell receptor with the tumor antigen, together with costimulatory molecules will result in the targeted release of CTL effector molecules such as perforin and granzyme, inducing apoptosis, as well as cytokines such as IFN-γ and TNF-α/β. SOX6, a transcriptional factor specifically expressed in the developing

CNS, has recently been proposed as an interesting candidate in order to immuno-target GBM [135]. Moreover, SOX6-derived peptides have been identified as specific targets for effective and safe T-cell-mediated immunotherapy targeting SOX6-positive glioma and GSCs [136]. This study reveals that SOX6 peptides are potentially immunogenic in HLA-A24- or -A2-positive glioma patients and could be considered as a promising strategy for effective T-cell-based immunotherapy. On the same basis, a recent study investigated the role of HER2-targeted T cells as a potential therapeutic agent for GBM. The study showed that T cells from GBM patients can readily be genetically engineered into autologous HER2-positive tumors including their putative stem cells, with potent antitumor activity in an orthotopic xenograft model [137]. These results notably open up new perspectives to rationally design new immunotherapies against GSCs.

Finally, dendritic cell (DC) vaccines have recently been in the spotlight. The major objective of DC vaccination is to activate lymphocytes against tumor antigens. In this line, Pellegatta and colleagues demonstrated that DC loaded with GL261-neurosphere lysates protected mice against tumors from both GL261 neurospheres and GL261 adherent cells [138]. These results suggested that DC vaccination against neurospheres can restrain the growth of highly infiltrating GBM and may have implications for the design of novel, more effective immunotherapy trials. Interestingly, a Phase I/II clinical trial of vaccine therapy specifically targeting GSCs expanded as neurospheres has recently been completed. The mRNA of these isolated GSCs was first amplified and transfected into monocyte-derived autologous DC which were then used as vaccines. During the trial, patients did not develop any adverse autoimmune events or other side effects and, more importantly, the progression-free survival of vaccinated patients was 2.9-times longer compared with matched controls [139]. Owing to the fact that conventional therapies are quite limited due to nonspecific damage to the normal tissue, DC vaccines now act as a strong innovative approach to brain tumor treatment by targeting GSCs.

Conclusion

Since growing evidence shows that GSCs escape from conventional therapy, targeting GBM cancer stem cells with different therapeutic strategies would provide new hope to better fight malignant brain tumors. In this line, current innovative therapies specifically targeting GSCs achieved promising results. However, due to high GSC genetic instability [140], further research is desperately needed in order to better understand the biology of these GSCs. Identification of cancer stem cell regulators that are less critical in normal stem cell biology as well as resistance mechanisms within cancer stem cells is thus mandatory in order to improve the efficacy of GSC-targeted therapies. The national motto of Belgium is 'United we stand, divided we fall'. Relying on this quote, we strongly believe that greater success will be achieved with the elaboration of innovative GSC-targeted therapies in combination with cytotoxic therapies or with other targeted therapies that may finally prevent GBM deadly relapses and improve the patients' survival.

Future perspective

Recent findings into the drivers of human primary brain tumors and the mechanisms of GSC resistance to treatments have led to the elaboration of innovative clinical trials. Despite only small improvements in overall survival for GBM patients so far, these advances have however resulted in significantly improved outcomes for a subset of patients, providing evidence we are aiming in the right direction. As our knowledge constantly increases on malignant brain tumor hallmarks and heterogeneity, novel technologies, such as nanotechnology, together with specific targeted therapies will probably allow to personalize the treatment for each patient. However, in order to do so, further investigations are to be conducted to notably highlight predictive biomarkers. Personalized treatments will only be possible when tumor heterogeneity and identification of malignant brain tumor biomarkers will suit with progress in the understanding predictive factors and clinical outcomes. This will, in turn, provide each individual the greatest chance to win battles in the war against malignant brain tumors.

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EXECUTIVE SUMMARY

- Glioblastoma multiforme (GBM) are considered among the most refractory and most aggressive malignancies of the CNS.
- GBM patients systematically relapse despite therapeutic modalities which involve surgery, radiation and chemotherapy.
- Glioblastoma stem cells (GSCs) have been described as key mediators in experimental tumorigenesis and therapeutic resistance. They are seen as evident candidates responsible for GBM relapses.
- Surgical resections do not specifically tackle GSCs. A better detection of GSCs during surgery could help to resect these tumor cells more accurately. This would improve the patients' survival as well as the efficacy of adjuvant therapies.
- The quest for GSC-targeted treatment has rapidly led to a large number of promising therapeutic strategies and different clinical trials that are currently ongoing.
- A better understanding of the molecular mechanisms involved in GSC radioresistance should sensitize this subpopulation of cancer cells to radiotherapy.
- GSCs are able to bypass the immune system allowing these cells to freely proliferate. In this context, different studies are currently trying to highlight specific GSC markers in order to develop innovative immunotherapeutic approaches.
- Additional research in order to elucidate the molecular mechanisms underlying GSC resistance to treatment will be important in refining and improving current therapies.

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Paper 5

Glioblastoma Circulating Cells: Reality, Trap or Illusion?

(In Press)

Glioblastoma Circulating Cells: Reality, Trap or Illusion?

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Abstract

Metastases are the hallmark of cancer. This event is in direct relationship with the ability of cancer cells to leave the tumor mass and travel long distances within the bloodstream and/or lymphatic vessels. Glioblastoma multiforme (GBM), the most frequent primary brain neoplasm, is mainly characterized by a dismal prognosis. The usual fatal issue for GBM patients is a consequence of local recurrence that is observed most of the time without any distant metastases. However, it has recently been documented that GBM cells could be isolated from the bloodstream in several studies. This observation raises the question of a possible involvement of glioblastoma-circulating cells in GBM deadly recurrence by a "homing metastasis" process. Therefore, we think it is important to review the already known molecular mechanisms underlying circulating tumor cells (CTC) specific properties, emphasizing their Epithelial to Mesenchymal Transition (EMT) abilities and their possible involvement in tumor initiation. The idea is here to review these mechanisms and speculate on how relevant they could be applied in the forthcoming battles against GBM.

Key words: Glioblastoma, Epithelial to Mesenchymal transition (EMT), Dormancy, Circulating Tumor Cell (CTC), Circulating Tumor Stem Cell (CTSC)

Introduction

Circulating tumor cells (CTC) are the main required substrate for cancer to spread and extend metastases. These cells originally come from the primary tumor and reach the vascular compartment. CTC are then able to leave the circulation, migrate through the conjunctive tissue of different organs and proliferate to form metastases. It remains unclear whether CTC are able to go back to the primary tumor site, specifically after therapeutic treatment, and therefore to participate to tumor recurrence.

In fact, it has been suggested that a very small proportion of CTC can form metastases. This sub-population of cells are called Circulating Tumor Stem Cells (CTSC). Indeed, this subpopulation is thought to be self-renewing, multipotent and capable of tumor initiation [1]. Up to now, different hypotheses try to explain their presence in the peripheral blood, involving several mechanisms to cross the vascular barrier. Because of their properties, these cells are of high interest to counteract the evolution of the disease and metastases formation. This review aims to better understand the biology of these CTSC with a particular focus on glioblastoma multiforme, a grade IV malignant brain tumor characterized by a dead-end prognosis, systematic relapses and rare metastases.

Origins, Circulation and Destinations of Circulating Tumor Stem Cells (CTSC)

CTC come from the initial tumor or from eventual metastases. In the tumor mass, less than 5% of malignant cells [2] is known to preserve a self-renewal potential through multiple generations and able to create a new tumor. These are called Cancer Stem Cells (CSC). Classically, CSC are defined by three major *in vitro* properties: formation of spherical colonies in culture suspension, differential levels and patterns of surface markers and increased survival

after radiation or chemotherapeutic treatment [3-7]. Moreover, in experimental models those CSC are the only tumor cells able to initiate the development of new tumors in heterotopic or homotopic xenotransplantation experiments. These CSC present high tolerance to the lethal environment, host defense and growth-suppression factors thanks to immune mediators, cell cycle checkpoints and DNA damage control pathways [8].

From this, different hypotheses attempted to elucidate the presence of CSC in the blood or Circulating Tumor Stem Cells (CTSC). CSC can use a normal morphogenetic process, called Epithelial Mesenchymal Transition (EMT) [9] to modify their features in order to escape the tissue of origin and to migrate towards the vascular compartment [10]. Liu and collaborators recently demonstrated that differentiated tumor cells acquire migratory abilities due to the development of EMT pathways [11] (Figure 1A). The intravasation is finally possible by the secretion of enzymes, such as serine/cysteine proteases, matrix metalloproteases (MMP) or disentegrin and other metalloproteases (ADAMS), in order to degrade the basal membrane of blood vessels [12]. The presence of tumor-associated macrophages (TAM), especially in hypoxic region of tumor [13], seems indeed to facilitate the intravasation process, maybe via secretion of MMP-9 [14].

Once in the bloodstream, most of the CTC including CSC, undergo an important selection by shear forces or natural killer (NK) cells from the immune system [15]. However, CTC can aggregate to cellular elements [16] or platelets [17] and express several receptor tyrosine kinases (RTK), anti-apoptotic molecules and invasion signaling components [16, 18]. CTC are in this way able to avoid the immune response but also anoikis [18]. To extravasate, CTC use diapedesis to escape the vascular compartment [19]. Then, CTC that present mesenchymal features can inverse their transition and then recover their epithelial phenotype of origin via a process called Mesenchymal to Epithelial Transition (MET). Some CTC finally

become quiescent in a new and favorable environment and can later on fully participate to cancer relapses (Figure 1E).

EMT Conditions and Molecular Regulation

If CTC are the substrate, EMT might be a necessary condition for cancer dissemination. EMT is indeed thought to be the program that cancer cells follow to acquire metastatic features. This substantially simplifies our conception of the metastatic cascade, even if EMT is certainly not sufficient. EMT/MET is a normal embryologic reversible program that allows the conversion of epithelial cells to mesenchymal cells and inversely during development. Its embryonic implication, especially in gastrulation, neural crest delamination, organ formation and development is well described [20]. Later, in response to injuries, EMT was shown to be induced by EGF [21] and used by keratinocytes in healing process [22].

The corner stone of EMT/MET processes is the down/up-regulation of E-Cadherin (E-Cad), an integral membrane protein but also a component of adherent junctions and an important mediator of cell-cell adhesion. The *CDH1* gene encodes E-Cad. It can be repressed in two ways, depending on the effect on the E-cadherin promoter. First, transcriptional repressors including Snail, Slug, Zeb1 and Zeb2 (zinc finger proteins) and basic Helix-Loop-Helix (bHLH) such as E47 transcription factor, bind directly to E-boxes of the *CDH1* promoter region [23-26]. Kruppel-like Factor 8 also represses E-Cad expression by fixing *CDH1* promoter in an E-box independent way [27]. Second, the bHLH Twist1 factor, E2-2 factors and the embryonic transcription factor Goosecoid indirectly repress the *CDH1* transcription [28, 29]. Interestingly, Snail and Twist appear to control positively Zeb1 expression [30].

Many EMT inducers are currently known. Nuclear factor kappa-B (NF-κB) has a putative binding site on the *Snail* promoter, inducing Snail protein and preventing its

phosphorylation by Glycogen Synthase Kinase-3 (GSK-3) and its subsequent degradation [31]. It has been shown that Tumor Necrosis Factor α (TNF-α) induces and stabilizes Snail protein via NF-κB [32]. Transforming growth factor-β (TGF-β) is a well-known EMT inducer. The binding of TGF-β to its receptor leads to phosphorylation of Smad transcription factors, which strongly induce Snail and Twist expression, particularly in presence of High-Mobility group protein HMGA 2 [33]. Protein Kinase A (PKA), Signal Transducer and Activator of Transcription 3 (STAT3) and Protein Kinase D (PKD) are involved in TGF-β-induced EMT [34, 35].

Local conditions could also modulate the EMT process. This is the case of hypoxia, a local condition frequently encounter in the tumor mass. Indeed, during hypoxia, Notch pathway is activated, resulting in Notch Intracellular Domain (NICD) liberation. NICD acts then as a transcription factor that interacts with DNA-binding protein CSL to regulate gene expression. NICD particularly upregulates Snail expression by direct binding to its promoter [36]. Similarly, still in hypoxic condition, Hypoxia-inducible factor-1 (HIF-1), potentiated by Notch, is able to stabilize Snail by recruiting Lysyl-Oxydase (LOX) [36]. However, HIF-1 can also induce Twist expression by binding directly to the Hypoxia-Response Element (HRE) to the *Twist* promoter sequence [37]. As another factor upregulated during hypoxia or inflammation, Vascular-Endothelial Growth Factor or VEGF can induce Twist and Snail expression by GSK-3 inhibition [38, 39]. The same regulation of Twist and Snail expression is observed with EGF as it can particularly act in cooperation with α5β1 integrin [40]. Sonic-Hedgehog pathway is also related to Snail expression, probably induced by Gli1 [41] and contributes to TGF-β-induced EMT [42]. Hyperactive Wnt signaling occurs with the progression of different carcinomas and it has been shown that Wnt stabilizes Snail (and therefore EMT) by GSK-3B inhibition via Axin-2 [43]. Thus, EMT appears to be the result of E-Cad repressors activities,

especially Snail factors, in response to inflammation and hypoxic conditions [44], both features that are met in cancer.

On the other side, another pathway including miRNAs is well known for its rule in epithelial transition. Bone Morphogenetic Protein (BMP) pathway, especially via BMP7, induces miR-205 and miR-200 family of microRNAs, which induce *CDH1* promoter and suppress Zeb1 and Zeb2 expression [45], and thus promotes MET [46] (Figure 1E).

EMT-related Changes

During EMT, epithelial cancer cells, which lean bit by bit towards the mesenchymal state, loose epithelial features and change their protein expression. Hence, it is possible to characterize the CTC epithelial or mesenchymal phenotype and specifically the degree of transition. Epithelial cellular adhesion molecule (EpCAM) [47], cytokeratins [48], *zonula occludens [49]* or epithelial splicing regulator 1 (ESPR1) [50] expression characterizes an epithelial phenotype, while N-Cadherin [51] or Vimentin [52] are expressed in mesenchymal phenotype. Activation of biochemical pathways, such as Twist-1 or the Akt-PI3K pathway [53] can also be specific hallmarks of the mesenchymal state. EMT is associated with the acquisition of several properties that are critical for cancer dissemination including first repression of the epithelial cell polarity and proliferation, and second, promotion of cell resistance to therapy, migration and invasion [20]. Inversely, MET promotes cell proliferation and metastasis formation.

E-Cad repressors as well as EMT inducers are involved in this acquisition. For example, Snail factors induce MMP-9 expression that is then able to degrade the basement membrane of blood vessels, a prerequisite step to intravasation. Conversely, some metalloproteases, such as MMP-3 and MMP-13, can induce EMT [54, 55]. Additionally, TGF-β confers resistance to

cell death and DNA damage [56]. In fact, Snail and Slug factors repress pro-apoptotic genes expression, in particular PUMA, ATM and PTEN that are usually up-regulated in the p53-mediated apoptotic pathway [57]. In the same line, Twist1 and Twist2 were shown to be overexpressed in a large fraction of human cancers and are thus able to override the oncogene-induced premature senescence by abrogating key regulators of the p53- and Rb-dependent pathways. In epithelial cells, the oncogenic cooperation between Twist proteins and activated mitogenic oncoproteins lead to complete EMT. Taken together, these data underlined an unexpected link between early escape from failsafe programs and the acquisition of invasive features by cancer cells [58]. EMT is also associated with chemo- and radio-resistance. Snail indeed inactivates p53-mediated apoptosis [57] whereas Twist up-regulates the serine/threonine kinase AKT2 [59]. Finally, Kubo-Saito et al. showed in melanoma, that Snail positive tumor cells have recourse to Thrombospondin-1 (TSP-1) in order to impair dendritic cells, resulting in CD4+ regulatory T cells induction with immunosuppressive capacity, hence promoting immunoresistance, immunosuppression and/or escape of immune surveillance [60].

Mesenchymal transition also appears to confer or enhance stem cell properties by activation of Ras/MAPK pathway [61] (Figure 1A-1B). Snail factors can indeed promote the Wnt pathway (known for its regulation in self-renewal and differentiation in stem cells) by E-Cad repression [62]. Zeb1 and Zeb2 factors down-regulate some specific members of the microRNAs 200 family (miR-200), particularly miR-200c, which targets the polycomb group member BMI1, an essential regulator of stem-cell renewal, acting as a repressor of various genes by modulating the chromatin status [45, 63, 64]. More and more reports highlight the importance of the miR200/ZEB feedback loop in determining epithelial and mesenchymal future of tumor cells [64]. In the same way, Lu et al. used the loop to define three different states in the continuum between the epithelial and mesenchymal differentiation; epithelial (high miR-200/low ZEB), mesenchymal (low miR-200/ high ZEB) and partial EMT (medium miR-

200/medium ZEB) [65]. The acquisition of stem cell properties could explain the possible various origins of CSC.

No matter the epithelial or mesenchymal state, some cell markers suggest stemness character in CTC. In breast cancer for instance, Aldehyde Deshydrogenase-1 (ALDH1) allows to detect CTC with CSC properties [53]. The expression of cell surface markers CD44+/CD24- is also associated with CSC in breast carcinoma and with CTSC in colon carcinomas [66]. Gangliosides (GD2, GD3 and GD1A) in breast cancer and ABC proteins (ABCG2) in lung cancer are also useful for stemness detection [67, 68] but their utility for CTSC detection remains uncertain.

Dormancy

Tumor cells that are physically separated from the primary tumor mass and have spread to other anatomical locations through circulation are called Disseminated Tumor Cells (DTC). They can be classified as a sub-group of CTC. Metastasis formation is one option that DTC can follow but part of them are also able to become quiescent, a process that is different from senescence and consists in a non-proliferative state consequent to cell cycle arrest in phase G0/G1 [69]. Quiescence results from mitogenic signaling reduction and implies autophagy [70], reduced PI3K-AKT signaling [71] and activation of stress signaling pathways [72]. Interestingly, dormancy is significantly influenced by the microenvironment which can be permissive or restrictive [73]. In the bone marrow compartment, the presence of proteins such as GAS6, BMP4, BMP7 and TGF-β2 confers an adequate environment for dormancy [74-76], whereas VCAM1, periostin and extracellular matrix stiffness, with high density of type I collagen, appear to induce escape of dormancy [77-79]. Many key players modulate tumor cells dormancy. Among them, the balance of two prominent pathways, p38 mitogen-activated

protein kinase (MAPK) and extracellular signal-regulated kinase (ERK), might be key determining factors [75]. A high ratio of ERK/p38 is observed in metastatic lesions [80], while low ratio of ERK/p38 is associated with dormancy [81]. In addition, inactivation of Myc oncogene also leads to senescence [82].

Multiple actors are involved in quiescence process. For example, fibroblasts express periostin, which recruits Wnt pathway ligands and increases Wnt signaling in cancer stem cells, resulting in metastatic colonization [83]. In the bone marrow stem cell niche, stromal cells, such as osteoblasts, via TGF-β2, induce low radio of ERK/p-38 and p27 expression, inhibit CDK4 and in this way induce cancer cell quiescence [81]. In the same way, bone morphogenetic protein 7 (BMP-7) binds to BMPR2 that activates p38 and increases the expression of cell cycle inhibitor p21 and metastasis suppressor gene NDRG1 (N-myc Downstream-Regulated Gene 1) [75]. Macrophages, CD4+ and CD8+ T Cells, founded in immune niche, use Tumor Necrosis Factor Receptor 1 (TNFR1) and Interferon-γ (IFN-γ) in order to induce antiangiogenic chemokines and prevent proliferation and carcinogenesis [84]. More specifically, CD4+ T cells product CXCL9 and CXCL10, which were described inhibit angiogenesis [85]. Endothelial cells from bone marrow vascular niches can also induce quiescence via TSP-1 or perlecan production [78, 86].

Dormancy appears as an important phenomenon in the cancer relapse as it implies higher resistance against targeted and conventional therapies, after long period, sometimes decades, tumor cells can quit this specific dormancy state and develop regrowth capacities [87]. A strong link between dormancy state and tumor stem cells is suspected [88]. Indeed, both dormant tumor cells and tumor stem cells show a high resistance to current treatments [89, 90] and can undergo cell cycle arrest in response to various form of therapy [91, 92]. In glioblastoma for example, the CSC pool in tumors is enriched after ionizing radiation. This situation seems to be in direct consequence with the activation of DNA damage repair pathways

coupled to a reduction of proliferation and apoptosis via DNA checkpoint kinases [93]. In fact, a subpopulation of CSC is thought to be quiescent [94]. This view is supported by the fact that dormant cells and CSC use same pathways such as Shh, Notch and Wnt [95]. The overlap between dormancy state and ability of tumor-initiation could help to determinate the subpopulation of tumor cells, which are highly involved in relapses.

CTSC and Glioblastoma

1) Clinical Evidence

GBM is the most frequent primary brain tumor and is well known for its poor prognosis despite multimodal therapies. The rapid relapse of tumor in GBM patients has indeed been regarded for years as the major cause of the lack of GBM spread out of the central nervous system. However, there are several clinical descriptions of glioblastoma metastasis. In 1928, Davis and colleagues described the first case ever reported of glioblastoma metastasis in a 31year-old woman. Since then, a growing body of evidences has shown the capacity of GBM to spread via the cerebro-spinal fluid (CSF) but also via blood or lymphatic vessels [96, 97] (Figure 1E). Interestingly, the number of GBM metastatic reports increases progressively [98]. This could be explained by a higher rate of diagnosis due to imaging improvement but also to the modest but real increase of patient survival and outcomes. Interestingly, the incidence of glioma metastases on post-mortem examinations ranges from 6 to 25% for supratentorial tumors [99, 100]. The actual delay between the initial tumor diagnosis and metastases found in the literature is 1 to 60 month [101]. Thus clinical evidences allow to asser the existence of CTC and DTC in GBM.

2) CSC in Glioblastoma

Ignatova et al. first highlighted the presence of CSC in GBM [102] (Figure 1A). Many similarities exist between GSC and normal stem cells in the adult brain, also termed Neural Stem Cells (NSC). These populations indeed share particular resemblances in gene expression and signaling pathways including Notch, Wnt or TGF-β signaling [103-105]. CD133 or Prominin-1 was proposed as a biomarker of tumor progression/initiation cells described in glioblastoma [106] but it appeared later to be insufficient as CD133-negative cells were also able to initiate tumors [107]. Interestingly, Sox2 (a transcription factor) but also nestin (an intermediate filament protein) and integrin α6 expression are highly expressed in GSC population [108, 109]. EGFR, whose amplification and mutations are well known in GBM, also promotes stemness in GBM cells [110]. Although it is unclear whether GSC result from cancerous transformation of NSC, they have been demonstrated to preferentially locate in specific niches, more specifically in neurogenic niches, such as subventricular zone [111]. Evidences also consider their presence in necrotic niches [112] or in tumor edge niches [113].

3) Defective Brain-Blood Barrier (BBB) and GBM-circulating Cells

The Blood Brain Barrier (BBB) consists basically of endothelial cells connected by tight junctions, surrounded by astrocytic endfeet with pericytes embedded in the vessel basal membrane. Nevertheless, neurons and microglia are also implicated in the BBB cyto-architecture [114]. In fact, a double interaction exists between endothelial cells and astrocytes, called gliovascular coupling. While endothelial cell can stimulate astrocytic growth and differentiation, astrocytes also modulate tight junctions formation and angiogenesis via the src-

suppressed C-kinase substrate (SSeCKS) [115, 116]. Moreover, astrocytic endfeet use aquaporins (AQP) to maintain the BBB integrity [117].

GBM is the most vascularized tumor in humans [118]. Among others, this can be explained by high levels of vascular endothelial growth factor (VEGF), particularly in necrotic core, resulting in endothelial proliferation [119]. Nevertheless, glioblastoma-induced angiogenesis is imperfect, leading to vessel formation with variable diameter and permeability, heterogeneous distribution and basal lamina irregularities [120] (Figure 1C-1D). In 1975, Hirano et al. had already shown fenestrations and tight junctions disruption in GBM vessels [121]. At the beginning, GBM cells use host vessels as pathways of invasion [122] and then, co-opt to these vessels [123]. These interactions of GBM cells with vessels become more and more prominent as the disease progresses. Indeed, new-generated vessels by angiogenesis can support tumor growth, with a tone controlled by glioma cells [124]. Watkins et al. also showed that glioma cells displace, or even eliminate, astrocytic endfeet and make direct contacts with endothelial cells (Figure 1C-1D). The result is first the cessation of endothelial/astrocytic interaction and second the breach of BBB, by reduction of tight junctions [124]. Thus, glioblastoma progression seems to tightly associate with an altered BBB permeability, which also constitutes the first condition to intravasate.

4) Glioblastoma Subtypes and EMT: the Mesenchymal Link

Based on gene expression signatures, four GBM subtypes have been described: Proneural, Neural, Classical and Mesenchymal [125]. In particular, the mesenchymal subtype is characterized by high expression of CHI3L1 and MET, wild-type IDH1, mutation/deletion of *NF1*, Schwann-like features and important presence of necrosis/inflammation [125-127]. This subtype is usually associated with worse prognosis and most of the time, appears *de novo*

[128, 129]. Fibronectin and collagen 5α1 are used as markers of mesenchymal GBM subtype [125]. Some regulators of mesenchymal status have also been highlighted in this subtype, such as C/EBP-B and STAT3 transcription factors or the transcriptional co-activator TAZ [130, 131].

In this context, Bhat and colleagues have recently shown that microglia is able to induce the mesenchymal status via the TNF-α/NF-κB pathway, notably resulting in radioresistance [132]. Moreover, it has been shown that the mesenchymal phenotype is associated with higher migratory capacities of GBM cells. In fact, TGF-β, which is well present in the GBM environment and secreted by microglia, stromal and tumor cells [133], is able to induce the mesenchymal transition, via a SMAD2 phosphorylation and a recruitment of Zeb1, especially in GBM with a low or absent expression of mesenchymal markers [134]. This mesenchymal differentiation can be effectively blocked by A8301, an inhibitor of the TGF-β type 1 receptors [134]. Hypoxia, via HIF-1α and Zeb1, is also able to induce a mesenchymal switch in GBM [135]. Moreover, Twist overexpression enhances GBM invasion [136]. Snail is also up-regulated in glioma cells compared to normal brain cells and was shown to promote invasion [137]. Indeed, its inactivation inhibits glioma progression and migration [138]. Finally, stromal cell-derived factor (SDF-1) or CXCL12 and its receptor CXCR4 can induce EMT in GBM via activation PI3K/Akt and ERK pathways [139]. Interestingly another recent study reported the involvement of the CXCL12/CXCR4 axis in EMT transition via up-regulation of survivin, a protein involved in apoptosis inhibition [140]. Moreover survivin-mediated EMT was shown to promote resistance to γ-radiation, suggesting a potential role of EMT in GBM therapeutic resistance [141].

As mesenchymal transition is associated with the acquisition of stem cell properties, hypoxia seems to increase stem-cell markers in GBM cells, via HIF-1α and Notch inductions

[142]. Speaking about new properties, mesenchymal transition in GBM was shown to confer tumor resistance to anti-VEGF therapy [143].

GBM metastases are not *stricto sensu* associated with the mesenchymal subtype. This is no surprise as it has been demonstrated that different subtypes of GBM can co-exist within the same tumor [144]. Moreover, Ozawa et al. showed that GBM could derive from a common proneural-like precursor and that additional NF1 loss can convert this proneural subtype to a mesenchymal subtype [145]. Thus, mesenchymal transition can be understood as a late phenomenon in GBM, leading to more aggressive, invasive and recurrent tumor. This idea is support by the fact that mesenchymal subtype is frequently found in glioblastoma metastases and recurrences [146].

5) CTSC in Glioblastoma

Recently, CTC have been found in GBM patients' blood, highlighted by GFAP detection, EGFR amplification or increased telomerase activity [147, 148]. The phenotypes of these CTC in GBM patients were closed to the mesenchymal or proneural subtypes. However, recent studies have not found stemness features in those cells yet but it doesn't rule out that part of these CTC are also indeed true CTSC. Of course, this hypothesis is sustained by clinical evidences and the existence of GBM metastases [98]. Recently, Song et al. showed that MMP-9 is required to cross the BBB, especially the parenchymal barrier [149]. Interestingly, as Snail also induces MMP-9 expression, the mesenchymal transition therefore seems a necessary condition to intravasate (Figure 1C). Besides, as dormancy is also a reality in GBM [150], we can speculate that some of these CTC remain quiescent in other tissue and could later on initiate relapses. Moreover, circulating endothelial cells and circulating hematopoietic progenitor cells also appear to be present in GBM [151]. Interestingly, GSC have the ability to differentiate into

endothelial cells and show the ability to generate new tumors when grafted in immunodeficient mice [152] (Figure 1D). This reinforces even more the hypothesis according to which CTSC are an underrated reality in GBM.

Conclusion

For many years, GBM was thought to be restricted to the central nervous system but a growing body of evidence indicates that, like many other cancers, hematogenic dissemination is a reality. CTC characterization is needed to confirm the presence of CTSC. The question of a possible CTC role in GBM relapses remains open. We think this is a crucial question to address as its response could significantly modify actual therapeutic protocols and have an important impact on patient outcomes.

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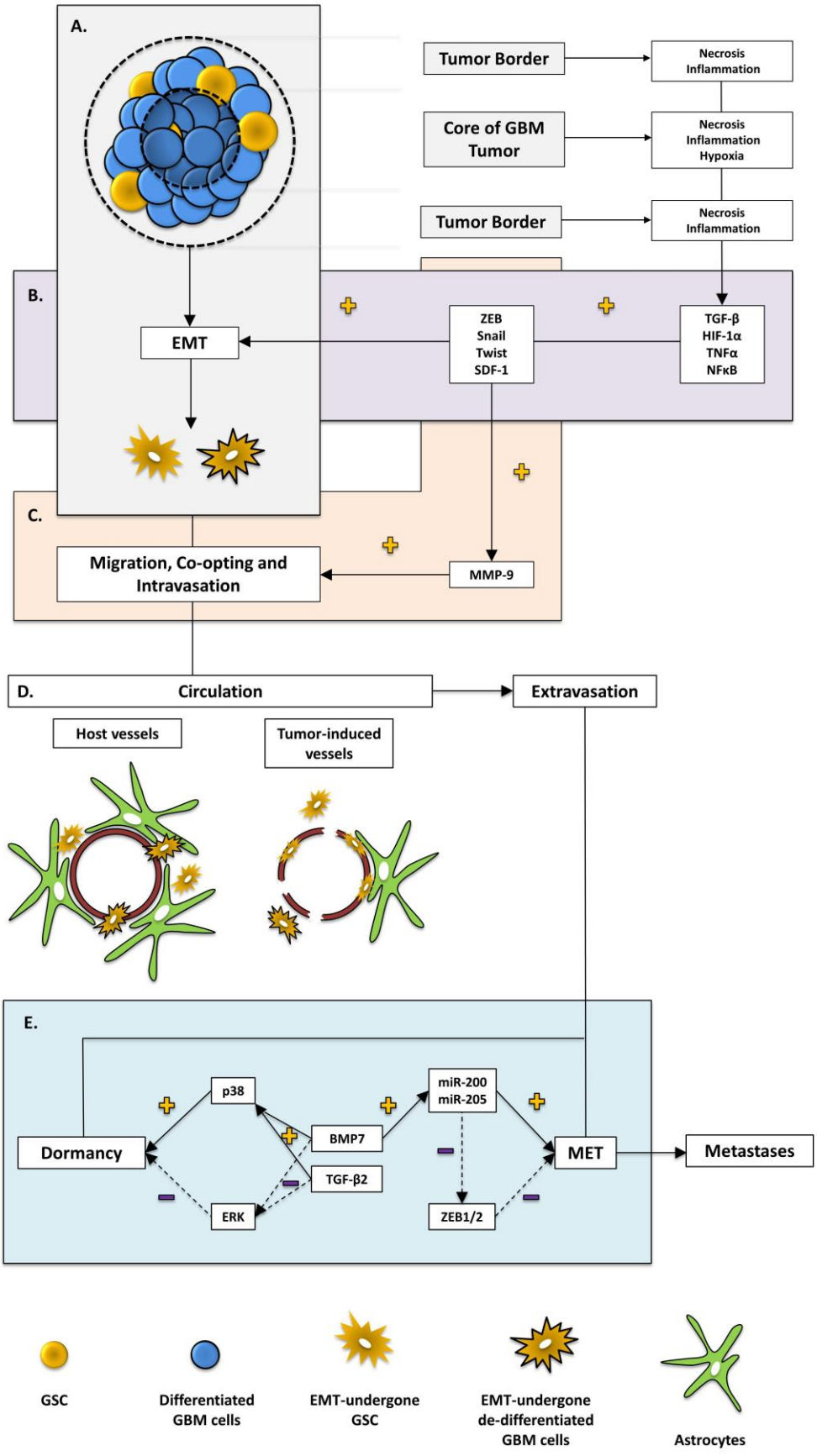
Conflict of interest

The authors declare they have no conflicts of interest.

Figure Legend

Figure 1. Insights on GBM dissemination process. Both GSC and differentiated cells can undergo EMT in order to invade the brain parenchyma. This process is regulated by different transcription factors including ZEB, SNAIL, Twist or NF-κB that are activated upon several environmental conditions (Inflammation, necrosis, hypoxia) (A and B). This consequently results in the acquisition of mesenchymal properties and the expression of ECM degrading enzymes in order to favor tumor spread. This process also sustains intravasation, leading to systemic dissemination (C and D). Tumor blood vessels are usually incomplete and leaky, therefore favoring intra/extravasation (D). In pathological conditions, the BBB is often disrupted, facilitating GBM cells to jump in the blood flow as well (D). When tumor cells extravasate, they may either become quiescent or develop metastases. This balance is tightly regulated by environmental conditions and factors including BMP7 or TGFβ2 among many others which may either induce dormancy or a switch toward MET and metastases (E)

Figure


A.
Tumor Border
Necrosis
Inflammation
Core of GBM
Tumor
Necrosis
Inflammation
Hypoxia
Tumor Border
Necrosis
Inflammation
B.
EMT
ZEB
Snail
Twist
SDF-1
TGF-β
HIF-1α
TNFα
NFκB
C.
Migration, Co-opting and
Intravasation
MMP-9
D.
Circulation
Extravasation
Host vessels
Tumor-induced
vessels
E.
p38
BMP7
TGF-β2
ERK
miR-200
miR-205
ZEB1/2
Dormancy
MET
Metastases
GSC
Differentiated
GBM cells
EMT-undergone
GSC
EMT-undergone
de-differentiated
GBM cells
Astrocytes

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