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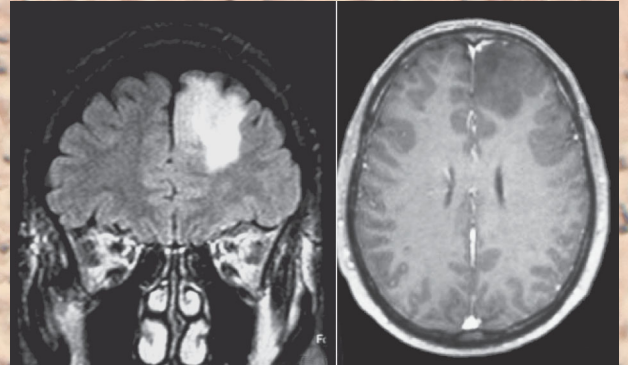
Glioblastoma Stem Cells

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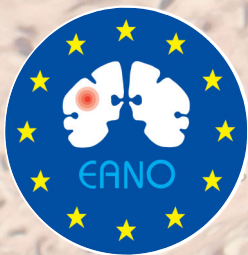
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News and Views Basic Science: Glioblastoma Stem Cells

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Abstract: Glioblastoma (GBM) are highly heterogeneous, infiltrative neoplasms with grave prognoses and five-year survival rates < 10 %. Particular challenges in GBM management include the striking therapeutic resistance of these tumours resulting in rapid and aggressive relapses, invasion of the tumour into normal brain preventing curative surgical resection, distinct intratumoural cellular heterogeneity, and limitations on delivery of therapeutics due to the blood-brain barrier. Surgical resection is beneficial but never curative and commonly followed by radiotherapy plus adjuvant chemotherapy via temozolomide, which offers

merely palliation with only modest effects on improving patient outcome. Targeted therapeutics have generally failed for glioblastomas with only modest activity from bevacizumab, an anti-VEGF antibody. Recent advances in GBM research have generated improved models, which better recapitulate the intra- and intertumoural heterogeneity found in patient tumours. These models attempt to address the complex genomic landscape as well as the presence of a cellular hierarchy with a self-renewing, tumourigenic cancer stem cell (CSC) population at the apex. These models also integrate the tumour microenvironment that

is composed of extracellular matrix components, stromal cells, vascular/hypoxic niches, and their heterotypic interactions. In this review, we provide a summary of GBM classification, their key features such as angiogenesis and therapeutic resistance, as well as the contribution of GBM CSCs and their microenvironment within the context of glioma biology and behaviour. **Eur Assoc NeuroOncol Mag 2013; 3 (2): 49–54.**

Key words: glioblastoma, cancer stem cell, angiogenesis, therapeutic resistance

■ Histopathology and Classification

Of all primary tumours, brain tumours constitute approximately 2 % and the global annual incidence reaches approximately 7 cases per 100,000 individuals [1–3]. There are > 100 types of brain tumours under the World Health Organization (WHO) classification scheme. Among these, gliomas are the most prevalent of the primary intrinsic brain tumours. Astrocytic gliomas rank among the most lethal of solid cancers and account for > 30 % of all primary brain and spinal malignancies. The World Health Organization (WHO) grades these tumours based on their histology to predict patient outcome. This classification system has been refined periodically, with the most recent changes published in 2007 [4]. WHO grade-I gliomas include several slow-growing tumours that are often located in specific anatomical locations, including juvenile pilocytic astrocytoma, pleomorphic xanthoastrocytoma, and subependymal giant cell astrocytoma. These tumours are often managed primarily through surgical resection and display specific genetic profiles with rare malignant degeneration. Diffuse gliomas are highly infiltrative tumours without discrete borders divided into 3 grades (WHO II–IV). Grade-II gliomas infiltrate into normal brain parenchyma and exhibit increased cellularity and mitotic indices. Grade-III (anaplastic gliomas) and -IV (glioblastoma [GBM]) tumours are classified as high-grade gliomas with high cellular proliferative activity, mitoses, and cellular/nuclear atypia.

The reported time to progression is approximately 2 and 5 years for WHO grade-II and -III gliomas, respectively. GBMs are distinguished by microvascular proliferation and necrosis. GBM comprises more than a half of all gliomas with a 5-year survival < 5 %. Extremely low treatment efficacy against GBM has been observed despite decades of scientific efforts. Median life expectancy of newly diagnosed patients in clinical trials (ie, a selected patient population) has improved to 12–15 months, although outcome for elderly patients is much worse. Although GBMs are generally histologically indistinguishable from one another, increasing evidence supports subgroups of GBMs that are distinct in clinical course, genetic profiles, and epigenetic features. Primary GBMs arise rapidly *de novo*, representing the most common type of GBM (90–95 %), and are seen predominantly in older patients. In contrast, secondary GBMs progress from pre-existing lesions with a lower degree of malignancy. Secondary GBMs are more frequent in younger patients and are seen in 5–10 % of all cases.

■ Molecular Pathology and TCGA Classification

Despite the significant prognostic power of the WHO classification that is based largely on morphology, individual patients within each grade can have very different outcomes that are not otherwise accounted for by established prognostic factors, including age, Karnofsky Performance Status (KPS), and therapy [5]. Even though the incidence of gliomas is relatively low, the fatal outcome and poor prognosis associated with the diagnosis of GBM have prompted extensive large-scale molecular and phenotypic profiling informing several new classification schemes discussed below. To date, GBMs have been subjected to the most extensive genomic profiling of any cancer to show major chromosomal and expression alterations – possible drivers of pathogenesis and biology of this severe malignancy. Despite the extensive characterization of genomic alterations in GBM and clinical progress across many

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cancer types, effective druggable tumour dependencies have yet to be exploited in glioma. Numerous small-scale studies with generally < 100 patients per diagnostic category have been published, identifying differentially expressed genes among morphologically defined gliomas [5]. These studies were deposited in publically available repositories (OncoPrint and Gene Expression Omnibus [GEO]) and provide a valuable “validation” tool for researchers [3]. A larger collective effort was undertaken in the National Cancer Institute’s REpository for Molecular BRAin Neoplasia DaTabase (REMBRANDT). Further, The Cancer Genome Atlas (TCGA) was established by the US National Cancer Institute and National Human Genome Research Institute in late 2005 with the mission of performing the first comprehensive cancer genome analyses applying multi-platform profiling to systematically and comprehensively analyze the gene copy number, mRNA expression, and the epigenetic state of approximately 200 tumours (today’s number has reached 600 patients), the majority being untreated primary GBMs [6]. This original study underwent accelerated publication to be published with a smaller-scale but deeper (whole exome) sequencing of a mix of patient tumours and xenografts that first identified mutations in isocitrate dehydrogenase (IDH1/2) [7], linking metabolism and tumour growth directly. A follow-on study demonstrated that IDH1 mutations occurred in low-grade tumours and secondary GBM [8]. IDH1 mutations serve a gain-of-function role to create an oncometabolite (2-hydroxygluturate) [9] and induce a methylation phenotype [10]. Other metabolic enzymes (eg, enolase) may be mutated as a passenger to cause sensitivity to specific inhibitors [11]. These studies have provided strong intellectual support for the identification for driving genetic lesions or other molecular sensitivities to inform therapeutic development.

Phenotypic gene expression profiling of glioblastomas has led to additional granularity of this disease. A seminal study by Phillips et al using unsupervised hierarchical clustering of expression profiles led to the characterization of 3 GBM tumour subgroups: proneural, proliferative, and mesenchymal [12]. A subsequent effort using the TCGA data delineated 4 subtypes: proneural, neural, classical, and mesenchymal on the basis of functional gene annotation and, prior to the Phillips study [12], linking the molecular signatures of gliomas with stages of neuroglial development. Moreover, TCGA effort utilized methylation profiling to identify a GBM CpG island methylator phenotype (G-CIMP) in a significant fraction of the proneural (29 %) subtype, secondary IDH1 mutation-positive GBM that progressed from lower-grade tumours in particular [13]. The 4 proposed subtypes were subsequently validated using previously published data and each of them was ultimately defined by a minimum list of 210 genes [14]. The proneural subtype was primarily enriched for amplifications of PDGFRA, OLIG2, CDK6, CDK4, and MET, where the first 2 represent markers highly relevant to oligodendrocyte development [15, 16]. The classical subtype can be characterized by more common epidermal growth factor receptor (EGFR) amplification/EGFRvIII mutation, PTEN and CDKN2A loss, whereas the mesenchymal subtype is enriched for mutations and/or loss of NF1, TP53, and CDKN2A. To evaluate whether these genomically identified subtypes predict biological or clinical heterogeneity in GBM, Brennan

et al [15] used a targeted proteomics approach to determine whether GBMs also segregate into the above-mentioned subtypes by activation of signalling pathways. 57 proteins or protein modifications were assessed in 20 GBM samples using unsupervised clustering and led to the identification of distinct tumour subgroups defined by EGFR-associated, PDGFR-associated signalling, or protein level changes associated with decreased NF1 expression [14]. In addition to previous studies, Sturm et al integrated paediatric GBMs to identify 6 epigenetic GBM subgroups displaying unique DNA methylation patterns, harbouring distinct hotspot mutations, DNA copy-number alterations, and transcriptomic patterns. Their findings correlate with molecular-genetic factors as well as key clinical variables such as patient age and tumour location of histologically indistinguishable GBMs [17]. Importantly, the established morphological classification system (WHO) and the evolving molecular subtyping should be considered complementary, not mutually exclusive, as the latter serves mainly as a diagnostic adjunct to more accurate classification of morphologically ambiguous tumours.

■ Angiogenesis

Robust neoangiogenesis and intratumoural heterogeneity are hallmark features of GBMs. Vascular endothelial growth factor (VEGF) expression strongly correlates with tumour aggressiveness, invasiveness, early relapse, and consequently commonly indicates poor prognosis and shorter survival of glioma patients [18, 19]. For many years, it was proposed that tumours beyond a diameter of 1 mm undergo so-called “angiogenic switch” resulting in the formation of tumour vasculature. This “switch” involves both activation of oncogenes [20] and inactivation of tumour suppressors to up-regulate pro-angiogenic pathways. To date, there are 6 principal cellular mechanisms described to take part in the process of neo-vascularization [19, 21]. These include

- vascular co-option involving formation of microvasculature surrounded by tumours cells
- vessel intussusception – formation of vessels by vascular invagination, intraluminal pillar formation, and splitting
- angiogenesis representing development of new vessels from pre-existing ones resulting in tortuous, abnormal vasculature with increased permeability
- vasculogenesis involving differentiation of circulating bone marrow-derived cells known as endothelial progenitors
- vascular mimicry – a controversial and insufficiently understood process defined as the ability of tumour cells to form functional vessel-like networks
- glioblastoma-endothelial cell transdifferentiation – the most recently reported mechanism, yet to be confirmed by further studies

VEGF is a key pro-angiogenic factor in both embryogenesis and tumour growth [18, 22]. Loss of VEGF or its 2 receptors VEGFR1 and VEGFR2 induces embryonic lethality in mouse models due to severe defects in the developing vascular system [23]. VEGF secreted by tumour cells acts through the VEGFR2 (VEGFR1 is believed to function as a “decoy” receptor to negatively regulate VEGF levels available for VEGFR2 activation) tyrosine kinase receptor, which is expressed pri-

marily in endothelial cells. Recent data, however, provide evidence of co-expression of VEGF/VEGFR2 in GBM cells – an indication of an autocrine control of VEGF signalling in gliomas in addition to the paracrine interplay of tumour and endothelial cells [24–26].

■ Therapeutic Resistance

Despite decades of concerted efforts for better understanding of the mechanisms that underlie the origin, development, and progression of GBM, currently available treatment modalities lack efficacy and these tumours remain among the most chemo- and radio-resistant solid cancers [27–29]. The current standard of care for GBM includes maximal surgical resection, followed by concurrent radio- and chemotherapy with adjuvant temozolomide (TMZ), a DNA-alkylating agent. The robust ability of GBM cells to invade normal brain parenchyma precludes surgical resection [14, 22, 30, 31]. The significant majority of GBMs (80–90 %) recur within 2–3 centimetres of the initial tumour location upon treatment with standard therapy [32, 33].

TMZ, an oral alkylating agent, has been investigated for use in primary and metastatic brain tumours for 2 decades [34]. Initially, it was approved for use in nitrosourea-resistant anaplastic astrocytomas but was extended to upfront use in glioblastoma after a seminal study in which it was administered concurrently with radiation [35]. The primary lethal adduct of TMZ involves methylation of guanine at the O6 position, a change that causes formation of DNA adducts, which if not repaired (by a mechanism known as base excision repair [BER]) results in failed replication. However, many tumours express MGMT, a suicide enzyme that can promote survival of tumour cells and hematopoietic stem cells. MGMT expression is often controlled by the methylation of the promoter and measuring MGMT expression by both promoter methylation and immunohistochemistry may identify patients likely to show limited benefit from TMZ treatment. Over the last decade, numerous phase-I and -II studies have investigated the efficacy and safety of TMZ in combination with other anti-GBM agents such as bevacizumab and interferon, as well as many other conventional chemotherapeutic agents (nitrosoureas) [36], such as irinotecan, pegylated doxorubicin, cisplatin, capecitabine, and sorafenib, for recurrent or progressive glioblastoma. MGMT promoter methylation status may serve as a prognostic factor but its use as a predictor of therapeutic response is more limited, as some patients with unmethylated MGMT promoters (thus, expected to express the gene product) do benefit from TMZ treatment [37].

EGFR amplification and EGFRvIII mutations were established as a key feature of the classical molecular GBM subtype and have long been known to be regulating intracellular signalling pathways that contribute to GBM pathogenesis including mTOR/PI3K/Akt and RAS/MAPK. The first generation of EGFR tyrosine kinase inhibitors (TKI) is represented by erlotinib and gefitinib, which were investigated in newly diagnosed and recurrent malignant gliomas, either as monotherapy or in combination with other cytotoxic agents [38]. Similarly to cetuximab, a monoclonal antibody directed at EGFR, and lapatinib that targets HER1 and HER2, these

agents were not associated with any significant treatment benefit [39–41].

Another recently evaluated inhibitor in gliomas is rapamycin and its intravenous and oral derivatives, temsirolimus and everolimus, the most widely tested mTOR inhibitors. These agents had minimal activity and no overall survival benefit in phase-II clinical trials with recurrent GBM either as monotherapy or in combination with EGFR TKIs in a smaller pilot study [38]. Additional promising strategies covering important RTK pathways in glioma development (PDGFR, VEGFR2) have only shown minimal activity in clinical trials [42–44].

The primary focus of current anti-angiogenic therapies is almost exclusively on tumour-derived endothelial cells as a constant element of tumour microenvironment and potential source of developing therapeutic resistance. Based on the response rate and presumed clinical benefit reported by 2 phase-II studies published by Vredenburgh et al [45] followed by Kreisl et al [46], bevacizumab (a humanized monoclonal antibody against VEGF; avastin, Genentech) was conditionally approved in 2009 by the US Food and Drug Administration (FDA) for the treatment of recurrent GBMs. Bevacizumab currently represents the most studied anti-angiogenic agent in the field of GBM. This agent specifically binds free VEGF ligands presumably in the systemic vasculature as a sink and so prevents its binding to putative receptors: VEGFR2 and VEGFR1, abrogating downstream signalling and resulting in decreased angiogenesis and tumour progression. Promising response rates but very limited median survival effects for recurrent GBM have been reported after bevacizumab treatment. Several concerns arose regarding the interpretation of the response rate, leading to denial of the bevacizumab registration by the European Medicines Agency because there was no inclusion of a bevacizumab-free control arm in any trial and consequently no proven effect on overall survival [36]. Ongoing phase-III trials may provide prospective data comparing bevacizumab-treated and -untreated cohorts, thereby facilitating the use of this drug in the therapy of GBM but preliminary reports suggest a modest increase in progression-free survival with significant toxicity. However, better understanding of angiogenesis on cellular as well as molecular levels is a necessity as well. It is known that anti-angiogenic agents decrease brain oedema and the requirement for corticosteroids by reducing vascular permeability [14, 47], thereby dramatically modifying the interpretation of GBM MRI scans [14, 44]. Whether these changes are a result of “vascular normalization” [48], actions on tumour cells, or effects on the blood-brain barrier remains unclear [49]. The phenomenon of so-called pseudo-response has been observed in bevacizumab-treated patients – an indication of treatment failure, development of resistance mechanisms promoting infiltrative spread of the tumour cells into healthy brain parenchyma [30, 31, 50], and/or switch in cellular metabolism and enhanced pro-survival signalling pathways.

■ Glioblastoma Stem Cells

Glioblastoma stem cells (GSC) are functionally defined by their capacity for (1) self-renewal, (2) recapitulation of a patient's phenotype when xenotransplanted into an immuno-

compromised host, and (3) multi-lineage differentiation, giving rise to a heterogeneous population of cells repopulating the tumour bulk [51–55]. These cells display severe resistance to current chemo- and radiation therapies, thus being considered one of the key determinants driving tumour recurrence [56–59]. Bao et al [56] reported elevated levels of the key components of the canonical DNA damage-response pathway in response to ionizing radiation (IR) and the ability of GSCs to escape the effect of IR by preferential activation of this pathway and lowered rates of apoptosis when compared to non-GSCs. GSCs also exhibit high levels of MGMT, thus making GSCs more sensitive to TMZ treatment [57]. However, TMZ did not affect propagation and maintenance of GSCs with “normal” levels of MGMT [60], which might be due to over-expression of certain ATP-binding cassette transporters (ABCT) such as ABCG2 responsible for active efflux of TMZ from the GSCs [58, 61].

Several prospective markers to identify and further isolate GSCs have been published so far, among those CD133 (prominin-1), CD15, Integrin $\alpha 6$, CD44, EGFR, A2B5, L1CAM are surface antigens [62–67]: Sox2, Olig2, Oct4, Mushashi-1, BMI1, NANOG represent intracellular factors involved in GSC self-renewal and maintenance [68, 69]. Despite CD133 being the most frequently used marker to enrich GSCs from the tumour bulk, 2 independent studies suggested that CD133 is not a universal GSC marker, as tumours contain CD133-negative tumour cells that showed “stem cell” properties in stem-like conditioned media *in vitro* and initiated tumours when injected orthotopically in immunocompromised hosts [70, 71]. However, some studies use extensive cultures that may affect the hierarchy.

Recent research in this area has revealed that GSCs are localized into 2 specialized niches within the tumour, perivascular and hypoxic, where the hierarchy and their maintenance are regulated by a complex network of molecular signals and cell-to-cell/cell-to-extracellular matrix interactions (Figure 1). Normal neural stem cells also exist in vascular niches, into which endothelial cells secrete factors that regulate neural stem cell function [72–74]. Calabrese et al [75] provided evidence that the brain tumour microvasculature forms a niche that is critical for the maintenance of brain tumour stem cells,

where these cells physically interact with endothelial cells and tumour vasculature. A recent study by Zhu et al [76] took into consideration cell-to-cell contact between different cell types and proposed the endothelial Notch-ligand expression Dll4 (Delta-like ligand 4) as the juxtacrine mechanism driving stem cell maintenance. Interestingly, Notch is one of the crucial factors in GSC maintenance and also contributes to the radio-resistant phenotype as described by Wang et al [59]. Moreover, GSCs have been shown to secrete elevated levels of VEGF and SDF-1 [77, 78] and to operate the pro-angiogenic VEGF/VEGFR2 signalling in an autocrine manner [24, 26], being further potentiated by interaction with other pro-angiogenic factors such as NRP-1 and c-met. Integrating the recent findings on GBM-endothelial cell transdifferentiation and vascular mimicry, the mechanisms utilized by GSCs represent a putative source of resistance to current anti-angiogenic therapies [24, 25].

Hypoxia is a well-recognized tumour microenvironmental condition linked to poor patient outcome and resistance to therapies [79–81]. Evans et al [82] analyzed normal brain and glioma tissue oxygenation and showed that physiological oxygen concentrations in healthy brains range between 12.5 % and 2.5 %, whereas GBM tumours showed mild to moderate/severe hypoxia (oxygen concentrations ranging between 2.5 % and 0.5 % for mild hypoxia and 0.5–0.1 % for moderate/severe hypoxia). Li et al [83] showed that GSCs differentially respond to hypoxia with a distinct induction of HIF2 α , and moreover that expression of HIF2 α is significantly associated with poor patient survival. Pistollato et al [84] reported that hypoxia represses the differentiation responses to bone morphogenetic proteins (BMP).

The dependence of GSCs on hypoxic and perivascular niches offers potential therapeutic strategies based on vascular targeting, which may have a higher therapeutic index to GSCs as compared with normal neural cells. As anti-angiogenic therapies continue to be developed for many cancers, including GBM, efficacy can be improved by increasing our understanding of the molecular mechanisms by which these agents function. Further work correlating cell surface makers with phenotype as well as a deeper understanding of the key survival pathways driving their resistance are indispensable for a proper definition and clinical relevance of cancer stem cells to GBM and other advanced cancers.

Conclusions

The accumulation of knowledge over the past 2 decades has revealed many challenging questions and controversies. The attempts to decipher the complex inter- and intratumoural heterogeneity of GBM is beginning to provide important information regarding tumour behaviour, and is likely to drive future contribution to the development of therapeutic approaches with the primary focus not only on the tumour itself, but also interconnected components of its complex microenvironment. The way by which GSCs and their microenvironment contributes to glioma development, progression, and responses to therapies should remain the focus of future studies. Because the efficacy of current therapies is greatly affected by these factors, future therapies will most probably

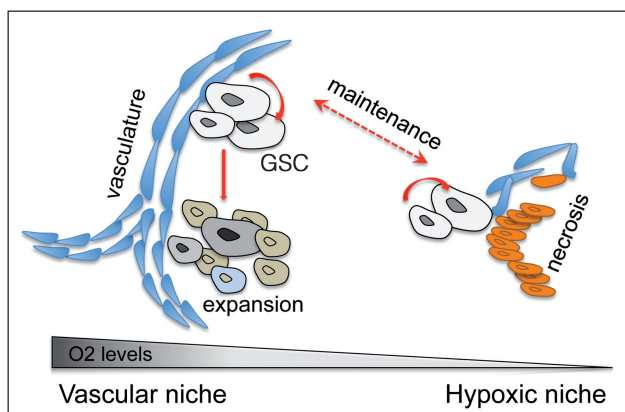


Figure 1. Glioblastoma stem cells (GSC) reside in vascular and hypoxic niches. Schematic representation of GSC residing in a vascular niche supported by blood vessels and hypoxic niche adjacent to necrotic regions. Each niche promotes the maintenance of the GSC population and supports the expansion of the tumour mass.

profit from combinational targeting of various survival mechanisms that involve niche components (such as hypoxia) that contribute to preferential selection and outgrowth of genetically unstable clones, thus being responsible for tumour recurrence and, consequently, patient death.

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Conflict of Interest

The authors state no conflict of interest.

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