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Review

REVIEW

Glioblastoma stem-like cells: at the root of tumor recurrence and a therapeutic target

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Abstract

Glioblastoma is the most common and most aggressive primary brain malignancy. The current initial standard of care consists of maximal safe surgical resection followed by radical radiotherapy and adjuvant temozolomide. Despite optimal therapy, median survival is ~15 months from diagnosis in molecularly unselected patients, and <6 months for patients with recurrent disease. Therefore, clinical treatments are currently palliative, not curative. Collectively, current knowledge suggests that the continued tumor growth and recurrence is in part due to the presence of glioma stem-like cells, which display self-renewal and tumorigenic potential. They differ from their more differentiated progeny, as they are more resistant to current treatments. Recurrent disease may be a consequence of the enhancement and/or gain of stem cell-like characteristics during disease progression, together with preferential death of more differentiated tumor cells during treatment, signifying that the cancer stem cell phenotype is a crucial therapeutic target. The limited knowledge of the characteristics of these cells and their response to current clinical treatments warrants intensive investigation with the aim to improve patient survival and/or develop a cure for this disease.

Introduction

Glioblastoma (GBM) is the most aggressive primary brain malignancy. They constitute ~70% of all gliomas, are currently incurable and confer a poor prognosis. One variant of GBM, gliosarcoma (GSM) are tumors with a biphasic growth pattern that contain both glial and mesenchymal components. GSM are thought to either occur *de novo* as a primary tumor or as a progression from a primary GBM. The greater molecular heterogeneity of GSM together with reported cases of GBM that have recurred as secondary GSM suggests that GSM may be a more progressive form of GBM that has transformed to include multiple cell lineages (1). Although secondary GSM is not commonly seen, this may be due to the low frequency of subsequent tumor sampling in patients with GBM.

The reported epidemiology and outcomes of GBM and GSM are similar. However, there are larger proportions of long-term GBM survivors. A recent analysis of data from the Australian Genomics and Outcomes of Glioma (AGOG) database showed that the median survival of patients with GSM was 9.7 months, versus 12.2 months for GBM. While 25% of patients with GBM survived 2 years or more, only 10% of those with GSM survived this long (1). It is now clear that tumors characterized histologically as GBM constitute at least three, and up to six different groups of genetic and epigenetic characteristics (2,3). The difference in tumor phenotype and outcomes suggests that we are dealing with multiple subtypes of GBM, and potentially a different cancer in GSM.

In the last decade, evidence has accumulated in support of the stem cell theory of carcinogenesis (4). Cancer stem-like cells (CSCs) or tumor-initiating/seeding cells are functionally defined as a subpopulation of cells within a tumor that can self-renew, have tumorigenic potential and can recapitulate the original tumor (5). Although the CSC subpopulation may initially constitute a small minority of the tumor, the ability of these cells to self-renew and resist standard therapies enables

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Abbreviations

CSCs	cancer stem-like cells
EGFR	epidermal growth factor receptor
GBM	glioblastoma
GSCs	glioma stem-like cell
GSM	gliosarcoma
HIF	hypoxia inducible factors
L1CAM	L1 cell adhesion molecule
NSCs	neural stem cells
TF	transcription factor.

them to persist, contributing to post-treatment recurrence (6). Additionally, some anticancer treatments may enhance the CSC subpopulation by switching the cellular hierarchy of the tumor towards stem-like cells.

Glioblastoma or glioma stem-like cells (GSCs) were initially recognized in 2003 (7). CSCs can be prospectively identified in various cancers by selection for stem cell markers (8), and considerable work has been devoted to the detection and characterization of these markers. To date, most studies have focused on cell surface markers including CD133, CD44 and L1 cell adhesion molecule (L1CAM), with the aim of isolating GSCs for further characterization. Unfortunately, most markers have limitations, and current techniques cannot clearly define the profile nor purify GSCs. The ability to accurately identify GSCs will provide insight into the molecular mechanisms sustaining the tumorigenic process of GBM and will set the foundations for the development of treatments that specifically target tumor-seeding cells.

GBM: an incurable disease

The prevailing standard treatment for GBM is maximal safe surgical debulking, subsequent adjuvant conventional fractionated radiotherapy with concomitant temozolomide, followed by temozolomide for six cycles (9). In the last decade, major progress has been made on the molecular characterization of adult gliomas, leading to the identification of strong diagnostic and prognostic markers. These include the methylation status of methyl guanine methyltransferase (*MGMT* (10)) and mutations in isocitrate dehydrogenase-1 (*IDH1* (11)). However, these classifications do not account for all the differences in patient outcome and presently do not influence treatment choices. Furthermore, although clinical and genetic indicators suggest that GSM may be a different disease, these patients are also treated with the same protocol. Therefore, there is a compelling need to further explore molecular transformations for identification of a clinically useful model.

Work by The Cancer Genome Atlas project (TCGA) and others on the molecular characteristics of GBM has led to the understanding that GBM comprises several tumor subtypes displaying distinct molecular and epigenetic characteristics (3,4,12,13). The main subtypes proposed are the classical, proneural and mesenchymal (3,12,14). The classical subtype of GBM harbors frequent amplifications or mutations in the gene encoding the epidermal growth factor receptor (EGFR (12)). The proneural subtype is characterized by frequent mutations in the tumor suppressor *TP53*, platelet-derived growth factor receptor A (*PDGFRA*) and *IDH1* and can be further classified by G-CIMP status (3). In contrast, the mesenchymal subtype demonstrates frequent mutations in the neurofibromatosis type 1 (*NF1*) gene (3). Although the clinical significance of these subtypes is unclear, they may run different clinical courses and display distinct responses to treatment (3,12). Although a number of gene fusions have been described by Shah *et al*(15), most of these were not present in greater than one sample, that is, they were not recurrent. However, rare clinically relevant fusions of receptor tyrosine kinases do occur and may be druggable (15). This tumor heterogeneity in molecular traits and biological behavior highlights the significance that treatment needs to be patient-tailored to the underlying molecular circuitry.

The CSC theory in glioma and GBM

In normal tissues, mature cell types originate from a common multipotent stem cell through intermediate progenitors. The heterogeneity of normal cells arises from a hierarchical program of differentiation. Cancer, however, was thought to originate from a multistep process initiated by progressive genetic alterations. Intratumoral heterogeneity was initially described as following a stochastic model (16). According to this model, the strongest clone continues to grow and surpass the others, while heterogeneity was attributed to the presence of residual weaker clones (16).

The more recent CSC model of oncogenesis follows a hierarchical organization similar to that of normal tissue regeneration and turnover, in which self-sustaining CSCs give rise to more differentiated progeny (17). In the meantime, the CSC population remains as a small subpopulation that is capable of reseeding the tumor. There is ongoing controversy regarding the term 'cancer stem cell' as it infers that CSCs originate through the mutation of normal stem cells. However, this is not necessarily true, since CSCs might also arise from dedifferentiation of tumor cells that acquire alterations imparting stem-like features (Figure 1; (18,19)).

Moreover, the term CSC implies multipotency, hence the preference of the term 'cancer-seeding cells' by some authors. There is also debate as to whether stemness of CSCs is a phenotypic property of some cancer cells at a certain time versus a defined cell subpopulation (20). Dieter *et al*.(21) have observed that CSCs are composed of a heterogeneous population of cells that can influence tumor growth, which further supports the notion that there is more than one proliferating cell subpopulation with differing properties within the CSC pool. They proposed three distinct CSC types, including transiently amplifying cells contributing to tumor growth but being unable to metastasize, long-lived tumor-initiating cells, which can initiate metastasis and delayed contributing tumor-initiating cells, which do not appear to influence tumor growth (21).

Studies using breast cancer models demonstrated augmentation of stem-like $CD44^{+}/CD24^{low}$ cells following administration of chemotherapy (22). A small population of these breast cancer stem-like cells survives after chemotherapy (23), and can recapitulate the tumor. The existence of quiescent and active stem cell subpopulations within normal tissues (24) together with the knowledge that chemotherapy and irradiation both target proliferating cells (25), propose the following hypotheses:

1. CSCs that are less proliferative (or in a quiescent slow-cycling state) survive current treatments, which kill the bulk of the more differentiated actively cycling tumor cells, but in turn switch some of the remaining quiescent CSCs into a transient active state of self-renewal. Symmetric and asymmetric division of CSCs then recapitulates the heterogeneous tumor.
2. Less proliferative (quiescent) CSCs that survive current treatments give rise via asymmetric division to more proliferative progenitor cells, which then recapitulate the tumor (26). Those progenitor cells may also sustain the ability to dedif-

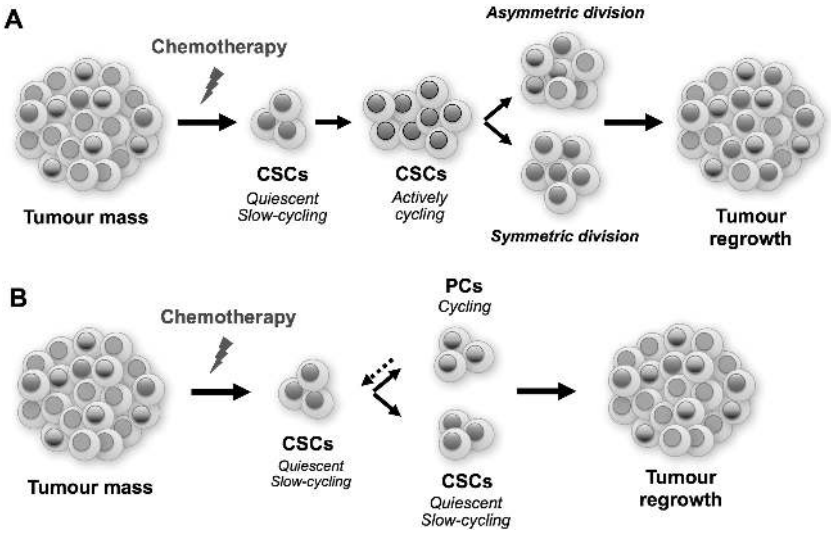

Figure 1. CSC hypotheses of tumor recapitulation post-treatment. (**A**) Quiescent slow-cycling CSCs persist following treatment and may transform into actively cycling cells that can self-renew and recapitulates the original tumor. (**B**) Quiescent CSCs that persist following treatment give rise to more proliferative progenitors that are capable of recapitulating the original tumor. Additionally, these progenitor cells (PC) can dedifferentiate into CSCs.

ferentiate towards CSCs, a feature reminiscent of the plasticity associated with CSCs and their immediate progeny (27).

The two hypotheses above do not contradict one another, and may both occur, either each in different tumor types or both within the same tumors.

There are reports demonstrating that multiple cell signaling pathways including Hedgehog, Wnt and Notch, trigger the formation of CSCs; thus, agents that inhibit these pathways have emerged as promising cancer therapies (28). For example, it has been reported that breast CSC numbers are reduced when Hedgehog activity is suppressed by cyclopamine (29). Direct inhibition of overexpressed oncogenic transcription factors (TF) is also a promising option. Several agents have been developed targeting various levels of transcriptional regulation, including DNA binding by TF, protein–protein interactions and epigenetic alterations (30).

GSCs were among the first self-renewing, multipotent tumor-initiating CSCs to be isolated from solid tumors (7,31,32). As few as 100 $CD133^+$ cells had tumorigenic potential and could recapitulate the parent tumor in immunodeficient mice (31). Notably, as many as 1 000 000 $CD133^-$ tumor cells could not form tumors (31). These results led to GSCs being initially defined by the expression of the cell surface marker CD133. However, subsequent studies showed that subpopulations of $CD133^-$ cells are also able to form tumors in immunocompromized mice, highlighting the heterogeneity of glioma and the need for additional markers (14).

Another factor that may contribute to GBM heterogeneity is the underlying cell of origin. Initially, astrocytes were thought to be the cell of origin of gliomas (5). This is supported by the fact that mature astrocytes can be genetically reprogrammed to gain stem-like properties (33). However, recent findings that both neural stem cells (NSCs) and glial progenitors are present in the adult brain (34) and that glial fibrillary acidic protein, a mature glial marker, is also expressed by adult NSCs (35) suggest that gliomas may originate from a less differentiated cell. Although normal neural progenitors have limited self-renewal capacity an oncogenic insult may induce proliferation in premalignant neural progenitors, and confer multilineage properties (36). 'Niche' cells may also be the initial targets for oncogenic insults (37). These are tissue-specific stem cells localized within a tissue niche that harbors a special microenvironment acting as a repository for stem cells and controlling stem cell function (38). Niche stem cells remain dormant and preserve their potential to differentiate until the need arises, such as during tissue injury (39). When tumorigenesis occurs, the stem cell niche is transformed into a tumor microenvironment, which triggers the dysregulation of stem cell growth. This in turn can interfere with signal transduction pathways and gene expression profiles and favor tumor invasion (40).

Several studies have shown that high-grade gliomas tend to develop in areas adjacent to ventricles which are rich in NSCs (41). Liu *et al.*(42)also demonstrated that NSCs give rise to oligodendrocyte progenitor cells, which in turn generate glioma. Indeed, different cell types may be responsible for the different glioma types (Figure 2). Identification of the glioma cell of origin has become more complicated since genetically distinct but related CSCs have been isolated from different areas of the same GBM (43). This suggests coexistence of CSC subclones within a tumor (44). These genetic pathways may differ between patients, as well as change during the disease course of a single patient. It is becoming clear that we are dealing with different diseases within the GBM tumor family, and identifying the key molecular characteristics may facilitate development of personalized treatments.

Similar to NSCs, GSCs are located close to the vascular niche in tumors (45). The vascular niche protects NSCs from apoptotic stimuli and preserves a balance of self-renewal and differentiation (46). GSCs exert their *in vitro* tumorigenic properties through the release of exosomes containing mRNA, miRNA and angiogenic proteins that promote a microenvironment supporting tumor growth (47). In turn, GSCs may be protected from external factors via specific survival signals they receive from the tumor niche (46). For example, hypoxia induces vascular endothelial growth factor (*VEGF*) expression, which promotes angiogenesis and supports the GSC tumor-initiating capacity (48).

The GSC cell surface markers controversy

Several molecules including CD133 and CD44 have been classified as cell surface markers of GSCs. However, there is uncertainty as

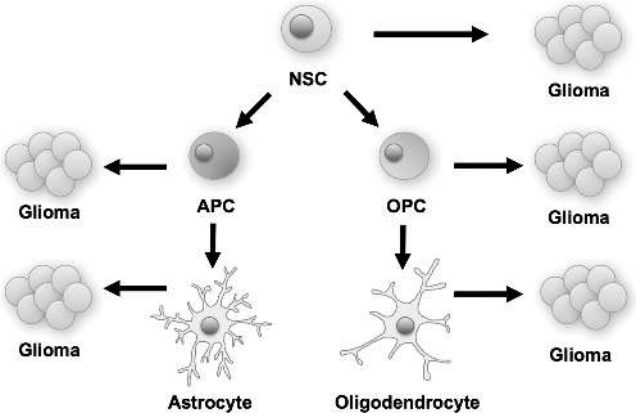

Figure 2. Potential cells of origin of GBM. It is not clear whether the glioma-initiating cells (or GSCs) are derived from adult stem cells, mature committed progenitors and/or even terminally differentiated cells that have dedifferentiated, or a combination of the above. APC and OPC are astrocyte progenitor cells and oligodendrocyte progenitor cells, respectively.

to their specificity to GSCs and thus far, the GSC cannot be confidently identified (49). Although Singh *et al.*7 detected expression of CD133 in fresh GBM samples, subsequently CD133 has not been found in other series of fresh GBM specimens (14,50), highlighting the heterogeneity of the disease and indicating the need for testing more tumor samples.

Aside from potential methodological differences among studies, a source of variability in results may be the inclusion of different subtypes of GBM with varying transcriptional profiles. $CD133^-$ GSCs may be associated with the more aggressive mesenchymal or proliferative gliomas (51). This is supported by the observation that when tumors recur, their expression profile commonly shifts towards the mesenchymal subtype (12). On the other hand, $CD133^+$ glioma cells exhibit transcriptional profiles resembling the better prognosis proneural subtype (50,51). As $CD133^-$ GSCs may predominate in the mesenchymal GBM subtype, it is possible that $CD133^-$ GSCs are even more resistant to radiotherapy and chemotherapy compared to $CD133^+$ GSCs (50). CD133 expression may be downregulated in GSM, given the mesenchymal characteristics of the former and its poorer prognosis. We also propose that $CD133^+$ cells represent CSCs, whereas $CD133^-$ include proliferative but more differentiated progenitors, which can readily dedifferentiate when they are isolated from their parent $CD133^+$ cells.

High expression of CD133 may be associated with poor prognosis (52). CD133 expression is more commonly seen in grade IV versus grade II gliomas (52). This indicates either a lower frequency of $CD133^+$ CSCs, and thus fewer aggressive cells in lower grade gliomas, or lower CD133 expression in $CD133^+$ CSCs, and thus fewer cells of proliferative potential, if we accept that CD133 expression per cell correlates with proliferation capacity. However, this remains controversial as others found no correlation between CD133 expression and either tumor grade or outcomes (53). This may in part be due to methodological differences between studies (52,53).

Further to CD133, recent studies demonstrate subpopulations of glioma cells distinguished by high expression of the cell adhesion molecule CD44 and the transcriptional regulator Id-1, displaying a stem-like cell phenotype (54–56). CD44 is a glycoprotein commonly expressed in numerous malignancies (57,58). In GBM xenograft models, knockdown of CD44 inhibited cell growth and improved response to chemotherapy (55). Furthermore, CD44 is highly expressed in mesenchymal GSCs (59). Collectively, these data suggest that CD44 may be useful as a GSC marker. This is supported by the coexpression of CD44 with CD133 in GBM spheres (60). More work is warranted on the significance of CD44 in GBM and particularly in GSM, given the mesenchymal characteristics of the latter. At the same time, Id-1 is a protein that regulates cell growth and differentiation in adult and embryonic tissues (61), and has been shown to control GBM invasiveness both *in vitro* and *in vivo* (56). In addition to positive correlations of Id-1 expression with histopathological grade in glioma patients, *in vitro* knockdown of Id-1 reduced GBM invasiveness, and GSC markers (56). Moreover, in an orthotopic model of human GBM, knockdown of Id-1 increased survival (56). These data provided important evidence that Id-1 regulates multiple tumor-promoting pathways, and may be a useful therapeutic target.

L1CAM is another cell surface marker related to CD133 expression and GSCs. It is a neuronal cell adhesion molecule regulating neural cell growth, survival and migration during central nervous system development (62). L1CAM is overexpressed in gliomas and other solid malignancies (63,64). Bao *et al.*(65) demonstrated that most $CD133^+$ glioma cells were also positive for L1CAM, and in contrast, $CD133^-$ cells were $L1CAM^-$. Interestingly, inhibiting L1CAM by lentiviral-mediated short hairpin RNA interference reduced the growth and survival of $CD133^+$ cells in murine xenografts of human glioma (65). These findings suggest a L1CAM-mediated interplay between different cell subpopulations within a tumor. However, the use of L1CAM to identify GSCs or glioma grade has not yet been validated, meriting further investigation.

Tyrosine kinase receptors as GBM targets and their relationship to GSC

Alterations of several tyrosine kinase receptor genes have been described in GBM including the MET tyrosine kinase receptor gene (*MET*) and *EGFR* (66). MET regulates cell growth and motility and has a role in embryogenesis, wound healing, degenerative disease and response to organ damage (67). However, overexpression of the MET ligand hepatocyte growth factor promotes the acquisition of stem-like properties in tumor cells and the formation and malignant progression of gliomas (6,68). More recently Met^{high} cells were shown to be highly clonogenic *in vitro* (67). Additionally, MET expression appears to be associated with genetic features such as wild-type *EGFR*, phosphatase and tensin homolog inactivation and the mesenchymal/proneural

subtype (66,69). Patients with upregulated MET displayed a more aggressive disease course following radiotherapy (67). This suggests that some treatments in current clinical use may activate genetic pathways that enhance the GSC phenotype in certain patients, resulting in unfavorable outcomes. This is an area that warrants immediate attention.

Mutation of *EGFR* and overexpression of EGFR proteins are more frequently seen in classical or neural GBM subtypes, compared with proneural or mesenchymal subtypes (3,69). Approximately 20–40% of patients with GBM express *EGFRvIII*, a variant of the EGFR gene (70). Importantly, it is uncommonly found in normal tissue (71) making it a good therapeutic target. Preclinical data on a vaccine targeting the *EGFRvIII* antigen have demonstrated tumor inhibition in mouse glioma models (72). A number of phase I and phase II studies (72) have prefaced a phase III clinical trial of an EGFRvIII vaccination strategy in GBM which has recently completed recruitment (the ACT IV study; ClinicalTrials.gov Identifier: NCT01480479). However, a recent mouse study showed that EGFR inhibition induces increased MET expression and associated proliferation of GSCs expressing pluripotency TFs and displaying multilineage potential (6). This has now raised serious concerns about the long-term safety of anti-EGFR treatments, which may in fact induce rather than suppress MET-driven GSC populations. However, it suggests the possibility of combining EGFRvIII and GSC targeting as a therapeutic strategy.

Compared to $CD133^-$ cells, $CD133^+$ cells express higher levels of MET (68). However, the tumorigenicity of $MET^{low/-}$ cells shows that MET is not a specific marker of cancer-seeding cells or GSCs (66). The reported frequency of MET expression in primary GBM specimens is also variable, ranging 30–100% (66). $MET^{low/-}$ cells probably represent GBM cells which harbor an *EGFR* gene amplification or mutation instead (66). These findings suggest separate GBM entities and describe criteria that might also be utilized to guide therapy.

TF associated with GSCs

Like other CSCs, GSCs share common signaling pathways such as Wnt, Notch and Hedgehog with embryonic stem cells (ESCs), and display similar multilineage features (73). ESC gene signatures are present aberrantly in various types of cancer. Particular attention has been focused on pluripotency TFs SOX2, NANOG and OCT4, which constitute the core transcriptional circuitry controlling self-renewal and multilineage differentiation of ESCs (74). An ESC-like signature characterized by activation of targets of these TFs is associated with aggressive tumor behavior (75). MET signaling also enhances expression of pluripotency TFs. This supports the theory that ESC genes contribute to the stem-like cell phenotype observed in many tumors, including GSCs, to sustain their proliferation and potential multilineage plasticity (73,74).

SOX2 is a key TF controlling the undifferentiated state of normal NSCs (76) and its upregulation in glioma cells has given rise to the concept that gliomas originate from the molecular transformation of NSCs (32) (Figure 3). Moreover, SOX2 silencing in GBM tumor-initiating cells has been shown to halt their proliferation and reduce their tumorigenicity (77). NANOG has been proposed as an independent prognostic factor for GBM (78). However, expression of these genes has been reported inconsistently among specimens and studies (73,78).

Clearly, more work is needed to elucidate the variability in expression as well as the significance of these TFs in GBM and more specifically in GSM. Based on the current data, it can be hypothesized that these TFs may not be restricted to GSCs, but they may also be expressed to some extent by more differentiated glioma progenitor cells that display plasticity as well as tumorigenic potential, yet potentially in lower levels. Further work on elucidating the cellular hierarchy of GBM and GSM based on the expression and function of these TFs is warranted

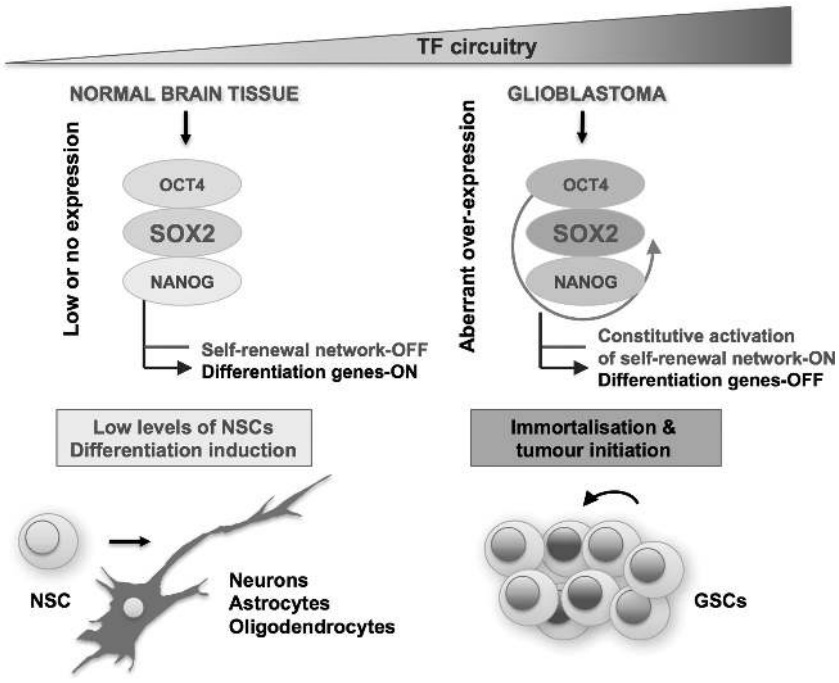

Figure 3. Proposed model of the function of pluripotency TFs in glioma. Normal brain is characterized by minimal expression of the pluripotency TFs, which allows maintenance of quiescence and low self-renewal activity in NSCs. Uncontrolled overexpression of one or more of these TFs leads to attainment of a glioma-initiating cells (GSC) phenotype via oncogenic activation, transformation and aberrant expansion of the target cell, which may be a NSC or a more differentiated cell.

and may allow identification of novel targets for future glioma therapy (78).

In addition to pluripotency TFs, other TFs have been linked to GSCs. STAT3 participates in transcriptional activation of apoptosis and cell cycle progression, and may be activated via EGFR to promote tumor formation (79). STAT3 is implicated in tumorigenicity through the production of antiapoptotic and GSC maintenance factors, proinvasive enzymes and angiogenic elements such as vascular endothelial growth factor (80). It has also emerged as a key initiator and regulator of mesenchymal transformation in malignant gliomas (81). STAT3 is a potential target for molecular therapy, either through direct targeting of its gene or its downstream effects to inhibit glioma growth. Prospective drugs include triterpenoid oleanolic acid, which suppresses the JAK-STAT3 activation in human macrophages and GBM cells (82). Additionally, small molecule inhibitors such as LLL3 increase survival in GBM-bearing mice (83), and these drugs remain prospective agents for the management of human GBM.

Cancer stem cell theory and treatment resistance

The exact mechanisms of GSC chemo- and radio-resistance are unknown, but probably include a combination of slow cell cycle kinetics, resistance to DNA and oxidative damage, avoiding cell death, hypoxia and multidrug resistance (84–87). CSCs probably exist in a quiescent state (88) and may be more treatment resistant compared to rapidly dividing cells. This notion has been well documented in treatment-resistant leukemia stem cells (88). However, whether this can be extrapolated to solid tumors remains controversial. Al-Hajj *et al.* (57) found that cell cycle profiles of human breast CSCs and non-tumorigenic cells were identical. However, a later study showed that $CD44^{high}$ epithelial cells with stem-like properties were more resistant to induction of apoptosis compared with $CD44^{low}$ cells, and such differences were directly associated with extended G2 phase of cell cycle in cells exhibiting the CSC phenotype (89).

CSC radioresistance can in part be attributed to proficient DNA damage repair (87). Specifically in GBM, $CD133^{+}$ GSCs are more resistant to radiation compared to $CD133^{-}$ cell populations, due to preferential activation of DNA repair checkpoints by phosphorylation of CHK1/CHK2 (85) producing more efficient DNA damage repair. Hence, targeting checkpoint kinases CHK1/2 may block DNA repair and circumvent radioresistance.

Hypoxia is acknowledged as a contributor to treatment resistance in solid tumors (90). CSCs have a molecular phenotype similar to that of cells exposed to hypoxic conditions (91). Effects on CSCs are primarily mediated by hypoxia inducible factors (HIF), particularly HIF2α (92). HIF2α is highly expressed in CSCs in gliomas and neuroblastomas, with loss of HIF2α leading to significant decrease in CSC proliferation (93). These findings establish a means through which oxygen tension and the specific microenvironment affect cancer development. Indeed, it has been demonstrated that targeting HIF levels in GSC inhibits self-renewal, survival and tumor initiation (94)

CSCs also express multidrug resistance genes including breast cancer resistance protein 1 (*BCRP1*) and ATP-binding cassette subfamily G member 2 (*ABCG2*), whose function aids CSC survival (95). Specifically, $CD133^{+}$ cells highly express the *BCRP1* drug resistance gene, the *MGMT* DNA-mismatch repair gene and antiapoptotic genes including FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (*FLIP*), *BCL-2* and B-cell lymphoma extra large (*BCL-XL*), compared with autologous $CD133^{-}$ cells (60). $CD133^{+}$ cancer cells are also resistant to cytotoxics such as temozolomide, carboplatin, VP16 and Taxol when compared with autologous $CD133^{-}$ cells.

Consequently, CSCs are probably treatment-resistant through a number of potential mechanisms which may vary between both individuals and cancer types. A better understanding of these mechanisms will improve our knowledge of how CSCs resist treatment and will guide towards future designs of chemotherapy and radiosensitization (Table 1).

Identifying therapeutic targets for GSM

The pathogenesis of GSM is controversial. GBM can recur as secondary GSM, suggesting that GSM may be a more progressive form of GBM (1). Alternatively, a small GSM component may persist through clonal selection and become dominant. Similar genetic anomalies have been recognized in both glial and mesenchymal components of GSM, implying a shared origin from a multilineage stem-like cell (96). Reis *et al.*(96) showed identical genetic alterations in both components through a tissue microdissection study and DNA sequencing. Alterations included p53 mutation, phosphatase and tensin homolog mutation, p16 deletion and amplification of CDK4 and MDM2 (96). However, it is not known whether the mesenchymal differentiation in GSM reflects the genomic instability of GBMs and/or whether mesenchymal differentiation is induced similar to the epithelial–mesenchymal transition phenomenon seen in epithelial neoplasms. One study demonstrated similar genome-wide chromosomal gain/loss patterns between glial and mesenchymal tumor areas in 12 of 13 cases of GSM. In one specimen, there was a significant gain at chromosomal segment 13q13.3-q14.1 in mesenchymal parts of the tumor. The respective genes from this locus were also amplified. They included stomatin (EPB72)-like 3 (*STOML3*), FRAS1-related extracellular matrix protein 2 (*FREM2*) and lipoma HMGOC fusion partner (*LHFP*) genes in 11–20% of mesenchymal areas but not in glial areas (97). Another study revealed that Slug, Twist, matrix metalloproteinase-2 and matrix metalloproteinase-9 were expressed in the majority of GSM mesenchymal areas, although they are rarely seen in GBM (98). These two studies confirm a possible effect of epithelial–mesenchymal transition in GSM.

Table 1. Summary of targets that merit further investigation from a therapeutic point of view

Mechanism of treatment resistance	Potential targets	Examples
Activation of stem cell phenotype	Transcriptional network controlling stemness	ESC TFs (SOX2, NANOG, OCT4), downstream targets
Variation in cell cycle kinetics	G2 checkpoint proteins	Cyclin A
Efficient DNA repair	Checkpoint kinases	CHK1, CHK2 kinases
Expression of antiapoptotic molecules	—	Bcl-2, Akt, FLIP, BCL-XL
Hypoxia	Hypoxia inducible factors	HIF2α
Multidrug resistance	Multidrug resistance genes	BCRP1, ABCG2

In the scenario of common cellular origin, the GSC of origin would acquire higher plasticity during progression towards GSM to allow for the generation of mesenchymal components. This could be mediated via altering expression of pluripotency TFs, selected downstream targets, which control plasticity, and multilineage and/or mesodermal differentiation in ESCs. More disturbingly, the demonstration of transformation from GBM to GSM raises the possibility that pluripotency may be induced by treatment. This was supported by a recent report demonstrating the potential of GSCs exposed to anti-EGFR therapy to differentiate into multiple lineages and express OCT4 and NANOG (6). This highlights the importance of investigating the effect of current and new treatments on GSCs and their transcriptional regulators.

Information about the treatment responsiveness of GSM may also be gained by studying other tumors with mesenchymal features. Like GSM, osteosarcomas have histological variability, with both osteoblastic and chondroblastic regions (99), indicating that the cell of origin may have multipotentiality. Mesenchymal stem cells (MSCs) are multipotent stem cells in adult bone marrow which can differentiate into osteoblasts, cartilage, fat, tendon, muscle and marrow stroma (100). Under specific conditions, bone marrow-derived stem cells can also exert pluripotency (101,102). A similar picture has been seen in mesenchymally derived cancers. Under the control of OCT4, Levings *et al.*(103) generated a transgenic osteosarcoma cell line (OS521Oct-4p) that stably expressed a human OCT4 promoter-driven GFP reporter. This supports the hypothesis that a subpopulation of osteosarcoma cells has CSC characteristics mediated by overexpression of pluripotency TFs. Local MSCs have also been successfully isolated from brain tissues (104), suggesting that, as for osteosarcomas, tumors arising from MSCs could display the varied histological phenotype of GSM.

Supporting the theory of a different cell of origin for both GBM and GSM, NSCs can differentiate into both mesenchymal and endothelial lineages (105). However, this plasticity is lost in the transition from stem to progenitor cells in the normal brain (36). The fact that most GBM tumors comprise malignant astrocyte phenotypes without mesenchymal components suggests that the cell of origin is the neural progenitor cell, as it cannot generate mesenchymal cells. In this case, GSM would originate from either a NSC with multilineage properties or a GBM progenitor cell that has dedifferentiated and/or acquired greater plasticity. It is possible that pluripotency TF and associated genes are overexpressed by GSM cells and have a role in the plasticity and increased aggressiveness of the GSM cell population. Indeed, the aberrant expression of pluripotency genes in other tumors has been linked to aggressiveness, proliferation and poorer prognosis, all features of GSM (73,106).

Outlook and future directions

Despite the lack of extensively validated GSC markers, studies continue to support the theory that gliomas follow a CSC model of initiation and progression. Because GSCs can recapitulate the parent tumor following treatment, and have intrinsic potential for treatment resistance, they are key targets for novel therapies. Due to the complicated mechanisms involved in CSC treatment resistance, it may be difficult to eradicate CSCs by a sole therapeutic strategy and thus combined and targeted strategies will be essential.

Which markers should be used to identify and isolate GSCs? Although present markers cannot be categorically and solely linked to the stem cell phenotype, they are informative towards GSC isolation. CD133 is accepted as a marker for glioma tumor-seeding cells, contributing to tumor initiation and recurrence. GBM cells are phenotypically diverse, and subpopulations grouped by expression of other markers, such as MET or SOX2, may represent distinct functional entities that contribute to the phenotypes of human GBM.

Future research needs to focus on identifying the critical pathways in CSCs not mutually essential to normal tissue. Direct targeting of GSCs will compliment conventional radiotherapy and chemotherapy. In this context, TFs expressed in GSCs should be investigated in the hope for a novel therapeutic target. More work is needed to elucidate the variability in expression as well as the significance of TFs such as SOX2 in GBM. A promising study in breast cancer showed that Zinc-finger-based artificial transcription factors used to down-regulate SOX2 expression resulted in reduction of tumor cell proliferation and colony formation (107). The inhibiting effect of engineered Zinc-finger-based artificial transcription factors on the growth of breast cancer cells *in vivo* was also verified (107). Additionally, synthetic interference peptides which can selectively target cells expressing certain TFs have also been explored in breast cancer (108). These techniques can also be translated to GBM and GSM.

The ongoing incorporation and corroboration of new data will further improve our knowledge of GBM disease origin, progression and treatment. Notwithstanding the issues outlined, the CSC theory not only informs new cancer targeting strategies through understanding developmental biology, but also provides insights into tumor maintenance, therapy resistance and recurrence. Urgent progress needs to be made in identifying molecules whose targets may eliminate GSCs, or sensitize them to chemotherapy and radiotherapy. Importantly, research is required to delineate the effects of current treatments on GSCs. This will be aided by the recognition and further investigation of the phenotypic and functional diversity of GBM among patients and the potentially different disease in GSM.

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