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REVIEW

Glioblastoma stem cells: Molecular characteristics and therapeutic implications

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Abstract

Glioblastoma Multiforme (GBM) is a grade IV astrocytoma, with a median survival of 14.6 mo. Within GBM, stem-like cells, namely glioblastoma stem cells (GSCs), have the ability to self-renew, differentiate into distinct lineages within the tumor and initiate tumor xenografts in immunocompromised animal models. More importantly, GSCs utilize cell-autonomous and tumor microenvironment-mediated mechanisms to overcome current therapeutic approaches. They are, therefore, very important therapeutic targets. Although the functional criteria defining GSCs are well defined, their molecular characteristics, the mechanisms whereby they establish the cellular hierarchy within tumors, and their contribution to tumor heterogeneity are not well understood. This review is aimed at summarizing current findings about GSCs and their therapeutic importance from a molecular and cellular point of view. A better characterization of GSCs is crucial for designing effective GSC-targeted therapies.

Key words: Glioblastoma; Glioblastoma stem cells; Self-renewal; Differentiation; Molecular markers; Therapy resistance

Core tip: Stem-like cells in glioblastoma, a malignant brain tumor, have increased tumorigenic capacity, generate tumor lineages and exhibit marked resistance to current therapies. A better understanding of these stem-like cells is necessary for designing new effective treatments. This review discusses the molecular characteristics of these cells and their therapeutic importance.

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GLIOBLASTOMA MULTIFORME

Glioblastoma Multiforme (GBM), classified by World Health Organization (WHO) as grade IV astrocytoma, is

a deadly primary brain malignancy with more than 10000 new cases in the United States annually (http://www.cbtrus.org). Despite the aggressive treatment options involving surgery and concomitant chemoradiotherapy, median survival is 14.6 mo[1]. The fact that survival has improved by only a few months over the past 50 years highlights the need for a better understanding of the disease and the design of informed therapies[2].

GBM is a highly heterogeneous tumor with distinctive histologic hallmarks including high cell density, intratumoral necrosis, vascular hyperplasia and invasion through brain parenchyma[3]. This heterogeneity is also displayed at the microscopic level, where a cellular hierarchy is dominated by the presence of stem-like cells, namely glioblastoma stem cells or GSCs[4]. In this review we will discuss the molecular and phenotypic characteristics of GSCs and their therapeutic implications.

CANCER STEM CELL HYPOTHESIS AND GLIOBLASTOMA STEM CELLS

Within multi-cellular systems, cells specialize to undertake different responsibilities, in order to maintain homeostasis. As a consequence of this specialization, every cell is not equal in its self-renewal and differentiation ability. Some cells are more stem-like, meaning that they can self-renew and give rise to different progeny through more restricted intermediate progenitors (Figure 1A)[5]. The extent of self-renewal is dictated by the developmental stage that cells are in and varies from tissue to tissue. For example, in tissues such as the gastrointestinal tract or hematopoietic system, where cellular turnover is high, adult stem cells self-renew more often, compared to more quiescent tissues such as the brain[6,7]. On the other hand, as cells differentiate, their self-renewal ability decreases and they adopt properties related to their tissue (Figure 1A)[8]. The differences in differentiation potential define a cellular hierarchy within these systems, where stem cells represent the top of this hierarchy. Lineage restriction and differentiation during physiological processes are mostly believed to be irreversible. However, pathologic conditions or experimental manipulations can cause de-differentiation[4,9]. Therefore, it is important to understand how cellular hierarchy is established and maintained in tumors in order to understand tumor biology.

Guided from research in liquid tumors, the idea of cancer cells with stem-like properties has revolutionized the field of cancer biology[10,11]. Although initially thought to be controversial, cancer stem cells (CSCs) are a proven concept for many liquid and solid tumors, including GBM.

In liquid tumors, cellular hierarchy is very well defined by the expression of surface markers. These hierarchically distinct populations were easily isolated by Fluorescence-Assisted Cell Sorting (FACS) *via* the expression of surface markers and their tumor formation ability was assessed *in vivo*[10]. These surface markers were then investigated in many solid tumors and some of them are still among the best-studied CSC markers.

Glioblastoma cells need to fulfill specific criteria to be classified as GSCs. In particular, they should be able to: (1) *self-renew* (Figure 1A); (2) differentiate into distinct lineages, a property termed *multipotency* (Figure 1A); and (3) *initiate tumors* in animal models, which recapitulate the original disease phenotype and heterogeneity (Figure 1A and B)[12,13]. Self-renewal is assessed with *in vitro* tumorsphere formation assay, a system borrowed form neural stem cell culture. In this assay, single cells are plated in suspension and their sphere formation ability is evaluated over serial passaging, which is an indicator of long-term self-renewal[14]. *In vivo* self-renewal is assayed by serial xenograft tumor formation experiments[11-13] (Figure 1B). The differentiation potential of GSCs is assessed *via* analysis of tumor-derived lineages *in vitro* and *in vivo*[15-17].

Evidence for GSCs first came from Dirks and colleagues, who isolated cells from human GBM samples based on expression of the cell surface glycoprotein CD133 (Prominin1/PROM1)[12,13]. They showed that these cells initiated orthotopic tumor xenografts in immunodeficient mice more efficiently than cells that did not express CD133.

Although the functional criteria defining GSCs are completely defined, the molecular characteristics of these cells are not understood. As expected by the heterogeneous histology of GBM, there is extensive cellular heterogeneity within GBM cells, and GSCs as well. The complex interplay of signaling pathways and lack of universal molecular markers identifying GSCs further complicate the study of these cells. More importantly, GSCs are resistant to chemoradiotherapeutic approaches and are, therefore, believed to cause tumor recurrence[18-20]. Thus, it is of major importance to understand the biology of these cells and their contribution to tumorigenesis, in order to overcome the problems current therapeutic approaches encounter. This review will focus on GSC markers, their molecular signatures and the signaling pathways important for their biology. Finally, we will discuss the therapeutic importance of these cells.

MOLECULAR MARKERS

CD133, a pentaspan transmembrane protein of unknown function, is one of the best-studied GSC markers to date. CD133 expression has been observed during embryonic development, as well as in adult neural stem cells and ependymal cells. However, CD133 knockout mice only have a mild retinal phenotype[21-23]. When isolated and injected into immunodeficient animals, CD133+ GBM cells are more tumorigenic than CD133- cells and produce xenograft tumors that phenocopy the original patient tumor[13]. Furthermore, knockdown of CD133 with shRNA impairs GSC self-renewal[24]. However, the facts that CD133- cells can also generate tumors and that some tumors do not have a CD133+ population suggest that CD133 is not a universal GSC marker[25-31].

GSCs were also expected to share common markers with neural stem cells, their normal counterparts, based

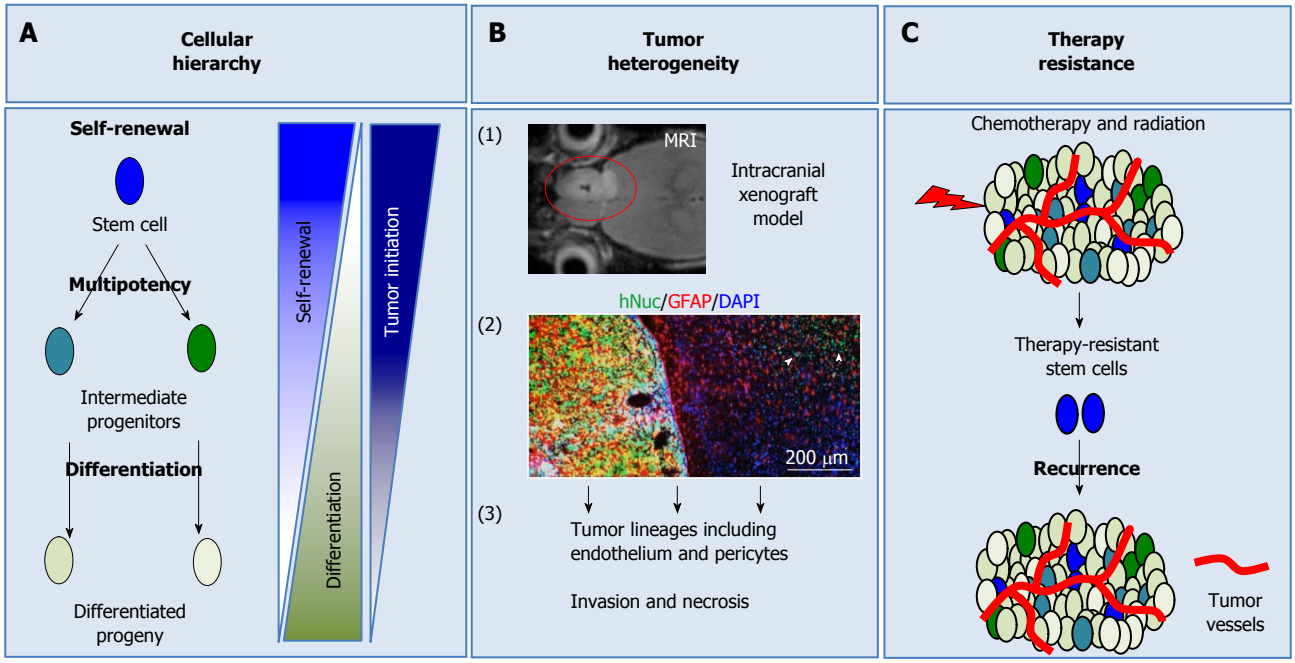

Figure 1 Biological significance of glioblastoma stem cells. A: Glioblastoma stem cells (GSCs) have the ability to self-renew and differentiate into distinct lineages through different intermediate progenitors, a property termed multipotency. Co-existence of cells with different differentiation capacities defines the cellular hierarchy within the tumor; B: GSCs have the ability to initiate tumors more efficiently than differentiated cells. Tumor initation ability can be tested *via* intracranial xenograft models in immunodeficient animals. (1) These tumors can be imaged with Magnetic Resonance Imaging (MRI); (2) Microscopic analysis shows that xenografts maintain the histologic heterogeneity of the patient tumor, including the invasion of normal surrounding brain (arrowheads) (hNuc: human nuclear antigen marking human tumor cells in mouse brain, GFAP: Glial Fibrilary Acidic Protein, DAPI: nuclear counterstain); and (3) GSCs promote tumor heterogeneity by giving rise to distinct tumor lineages including tumor endothelium and pericytes, and maintain the phenotype of the parent tumor; C: GSCs are resistant to current therapeutic approaches causing relapse of the tumor.

on the concept of stem cells sharing common signaling pathways. With this rationale, expression of neural stem cell markers was analyzed in GBM tumors. GSCs were shown to have increased expression of Nestin, an intermediate filament expressed in neural stem cells in neurogenic niches[18,32,33]. Besides Nestin, GSCs are enriched for Sox2, a transcription factor associated with multipotency and pluripotency[34,35].

Comparative gene expression analysis led to identification of more GSC markers, including Oct4, SSEA-1/CD15, Bmi-1, Musashi-1, Nanog, integrin-α6, L1CAM, A2B5 and ABC-type transporters, whose expression defines the side population (SP) on flow cytometric analysis, through the ability to extrude Hoechst dye[25,35-40]. Interestingly, some of these markers are expressed in embryonic stem cells, suggesting GSC overlap not only with NSCs but also with less differentiated stem cells as well. However, none of these markers are universal. Furthermore, the intracellular localization of some of these markers makes them less desirable candidates for selective therapeutic targeting.

SIGNALING PATHWAYS REGULATING GSC BIOLOGY

In addition to oncogenic pathways globally important to tumor biology, signaling pathways that are important for maintenance of self-renewal and regulation of differentiation receive attention in cancer stem cell biology (Table 1). In the context of GSCs, pathways known to regulate neural development are of major interest. Various signaling pathways influence GSC biology by either maintaining self-renewal or regulating differentiation. However, certain pathways can regulate either self-renewal or differentiation in the appropriate context (Table 1).

Self-renewal

Studies of pathways involved in GSC self-renewal gained momentum when Fine and colleagues started culturing tumor cells in serum-free conditions[41]. By using the mitogens epidermal growth factor (EGF) and fibroblast growth factor (FGF), they limited differentiation and promoted GSC self-renewal. These mitogens act through their receptor tyrosine kinases (RTKs) and induce activation of downstream pathways such as the Phosphoinositide 3-kinase/Akt (PI3K/Akt) and Mitogen-Activated Protein Kinase (MAPK), to induce proliferation, survival and tumorigenicity[41,42]. Furthermore, blocking the PI3K/Akt pathway has been shown to impair GSC self-renewal and tumorigenicity. Finally, knockdown of CD133 in GSCs causes downregulation of Akt phosphorylation, further highlighting the role of the PI3K/Akt pathway in GSC biology[43,44].

Originally identified in genetic screens in Drosophila as a master regulator of neurogenesis, Notch signaling plays diverse roles in nervous system development, including maintenance of self-renewal and regulation of fate decisions in neural and glial lineages[45-47]. Upon bind-

Table 1 Major signaling pathways and their roles in glioblastoma stem cell biology

Signaling pathway	Function	Ref.
Self-renewal		
Notch Signaling	Maintenance of GSCs Tumorsphere formation Tumorigenesis Asymmetric division	[50-57]
TGF-β Signaling	Regulation of self-renewal Maintenance of perivascular GSCs	[34,58]
Sonic Hedgehog Signaling	Promotion of self-renewal and migration Upregulation of stem cell associated genes Tumorigenesis	[56,61-66]
Wnt/β-catenin Signaling	Self-renewal and maintenance of GSCs Tumorigenesis Associated with bad prognosis	[15,66-71]
PI3K/Akt Signaling	Promotion of GSC self-renewal in vitro Proliferation and survival of GSCs Tumorigenesis	[41-44]
MAPK Signaling	Proliferation and survival of GSCs	[41]
Differentiation		
BMP Signaling	Inhibition of asymmetric division Differentiation and proliferation block	[72-74]
Notch Signaling	Trans-differentiation to tumor-derived endothelium	[16]
TGF-β Signaling	Trans-differentiation to vascular pericytes	[17]

GSC: Glioblastoma stem cell; TGF: Transforming growth factor.

ing to its ligands (Delta-like and Jagged), heterodimeric Notch receptors (Notch1-4) get cleaved by γ-secretase in the cytoplasm, releasing the Notch intracellular domain (NICD). NICD translocates into the nucleus where it acts as co-activator for transcription of the *Hes* and *Hey* families of genes[48]. These genes are transcriptional repressors of neurogenic genes, thereby causing maintenance of stemness in activated cells[49]. In GBM, Notch signaling is involved in several distinct processes in tumorigenesis, by regulating both self-renewal and differentiation of GSCs[16,50,53]. Blockage of Notch signaling with γ-secretase inhibitors inhibits self-renewal, as assayed by tumorsphere forming ability, and causes depletion of the CD133+ GSC population[54-56]. Furthermore, Numb, which prevents NICD from travelling to the nucleus and thus inhibits downstream signaling upon Notch activation, was shown to be asymmetrically distributed within GSCs and to promote asymmetric division. Asymmetric division of GSCs gives rise to two distinct daughter cells: a stem cell (GSC); and a more restricted and differentiated cell[57]. These findings support a role for Notch signaling in the maintenance of GBM's stem cell compartment.

Inhibitors of Notch pathway components represent promising therapeutic candidates in GBM. However, the overlapping roles with normal neural and other adult stem cell maintenance raises the question of toxicity. Of note, there are ongoing phase Ⅱ trials with Notch inhibitors in GBM patients (www.clinicaltrials.gov).

Transforming growth factor-β (TGF-β) signaling promotes GSC self-renewal through regulation of distinct mechanisms. First, it was shown to act through SRY-Related HMG-Box transcription factors Sox2 and Sox4, factors important for GSC biology, to induce self-renewal[34]. Second, blockage of TGF-β signaling decreases perivascular $CD44^{high}/Id1^{high}$ GSCs, *via* repression of inhibitors of DNA-binding proteins Id1 and Id3[58].

Sonic Hedgehog (Shh-Gli) signaling, which is highly important for brain and spinal cord patterning during embryonic development, also plays crucial functions in GSC maintenance[59,60]. It has been shown to promote GSC self-renewal and expression of stem cell genes, whereas its blockage leads to apoptosis, delay in tumorigenesis and inhibition of GSC self-renewal and migration[56,61-66].

The Wnt/β-catenin pathway induces proliferation of progenitor cells within gliomas[15,67]. Some reports suggest that Wnt signaling is important for GSC self-renewal. Overexpression of Wnt ligands, Wnt3a and Wnt1, is observed in GSCs[67]. Other Wnt pathway components were shown to promote GSC self-renewal and tumorigenicity. Some of pathway's downstream effectors such as β-catenin, Lgr5, Dishevelled 2 and Frizzled 4 are associated with negative prognosis[66,68-70]. FoxM1, which promotes nuclear localization of β-catenin, was also shown to be critical for GSC maintenance and tumorigenesis[71].

Differentiation

Bone morphogenic protein (BMP), a member of TGF-β superfamily, functions as a differentiation signal within GBM, as opposed to the previously discussed roles of other members of the TGF-β family in maintenance of self-renewal[34,72]. The difference between BMP and TGF-β's effects on GSC biology can be ascribed to distinct signaling cascades, even though they belong to the same superfamily of ligands. Also important for astrocytic differentiation in development, BMP4 treatment inhibits asymmetric division of GSCs, thereby blocking their self-renewal and depleting the stem cell compartment of the tumor[73,74]. Treatment with BMP4 leads to differentiation and proliferation block. However, a subset of GSCs manages to escape this differentiation cue *via* epigenetic silencing of BMP receptor 1B (BMPR1B)[74].

Although highly important for self-renewal, reports also suggest that Notch signaling is important for trans-differentiation of GSCs into tumor-derived endothelium[16]. Similarly, TGF-β was shown to induce GSC differentiation into vascular pericytes, supporting vessel formation and leading to further tumor growth[17].

MicroRNAs

An additional level of complexity in GSC biology is exhibited by regulatory non-coding RNAs, which are fine tuners of gene expression. Among them, microRNAs (miRNAs) have the ability to modify gene expression levels by specifically binding mostly to the 3'-UTRs of genes and causing their degradation through the RNAi machinery[75]. Besides being highly important for regulation of pluripotency and reprogramming, miRNAs play important roles in GBM tumorigenesis and GSC biology. Similar to other molecular markers enriched in GSCs, miRNAs regulating neural stem cell biology are also of main interest in GSC biology. miRNAs upregulated

in GBM and particularly in GSCs have anti-apoptotic, anti-differentiation, pro-proliferative and pro-invasion properties[40,76,77]. On the other hand, miRNAs promoting differentiation were shown to be downregulated in GBM, including miR-124, which is important for neural differentiation[78-81].

STEM CELL NICHE AND TUMOR MICROENVIRONMENT

To better understand the interplay of different signaling pathways mentioned above and how they regulate GSC biology, we need to study the niches in which GSCs reside. Besides providing crucial signals for GSC maintenance, stem cell niches and the tumor microenvironment are critical factors in the response to therapy.

Vascular niche

Endothelial cells provide signals required for self-renewal of neural stem cells and many other adult stem cell populations[82]. Similar to their normal counterparts, GSCs reside in a perivascular niche, where they maintain close contact with CD34+ endothelial cells[83-85]. This close contact facilitates presentation of Notch ligands on the surface of endothelial cells. These ligands activate Notch signaling in GSCs, thereby promoting self-renewal[85].

The perivascular niche is also subject to bidirectional cues coming from GSCs. CD133+ GSCs express higher levels of vascular endothelial growth factor (VEGF), leading to angiogenesis and increased vascularity of the tumor, when compared to their CD133- counterparts[86].

New evidence for trans-differentiation of GSCs into endothelial cells and pericytes further suggests that GSCs play a central role in maintaining the tumor microenvironment and their own niches, when presented with appropriate signaling cues[16,17].

Necrotic niche

As mentioned earlier, GBM is characterized not only by extensive vascular hyperplasia but also pronounced intratumoral necrosis. One of the main histologic hallmarks of GBM is a phenomenon called pseudopalisading necrosis (PPN), where densely packed tumor cells surround a necrotic area[87]. Although the etiology and biological significance of these areas are not well understood, they are believed to be regions of active tumor growth and neo-vascularization. Considering the importance of hypoxia in promoting self-renewal in embryonic stem cells and NSCs, pseudopalisades represent plausible niches for GSCs[88,89]. This hypothesis is further supported by studies showing immunoreactivity for CD133 in pseudopalisades[90]. Furthermore, hypoxia leads to activation of angiogenesis and neo-vascularization through the upregulation of VEGF in GSCs[91,92]. Some evidence also suggests that hypoxia reprograms CD133- GSCs to become CD133+ and induces Notch signaling, whose importance for GSC biology was mentioned above[88,89].

Keeping these findings in mind, the possibility of a necrotic niche for GSCs is biologically intriguing and represents a therapeutic challenge for systemic drug delivery methods, since these areas are devoid of blood vessels.

Invasion

The most malignant feature of GBM is its invasion of brain parenchyma. GBM cells infiltrate normal brain tissue and can be found centimeters away from the tumor core[93]. The vast majority of recurrence after surgery and chemoradiotherapy occurs within 2 cm of the resection cavity suggesting that these invading cells also have tumorigenic capacity[94-96].

Expression of C-X-C chemokine receptor type 4 (CXCR4) and its ligand, stromal derived factor 1α (SDF-1α), which are important regulators of invasion of GBM cells, is enriched in GSCs[91]. This signaling pathway also mediates recruitment of GSCs towards endothelium, causing further invasion, differentiation and endothelial cell proliferation *via* VEGF expression[92].

GSCs AS THERAPEUTIC TARGETS

Standard care for GBM is surgical resection, followed by concomitant temozolomide, an alkylating agent, and radiotherapy. GSCs represent important therapeutic targets because they have intrinsic machinery that overcomes current chemoradiotherapeutic approaches (Figure 1C). Some of the molecular mechanisms underlying GSC resistance to chemoradiotherapy are discussed below.

Chemotherapy resistance

GSCs are believed to resist chemotherapy *via* several distinct mechanisms. One such mechanism involves the active transport of chemotherapeutic agents to the extracellular space *via* ABC-type transporters on the cell surface. This mechanism also defines the side population (SP) of GBM cells on flow cytometry, through the exclusion of Hoechst dye[97]. Enrichment of stem cell markers such as CD133, CD117, CD90, CD71 and CD45 is observed in cells resistant to lethal doses of chemotherapeutic drugs[98]. Furthermore, CD133 expression is increased in recurrent tumors. Transcriptional analysis of CD133+ GSCs showed that these cells have increased expression of anti-apoptotic genes, suggesting that GSCs have intrinsic mechanisms of chemoresistance[36].

In line with these observations, more compelling evidence came from Parada and colleagues, who showed that a restricted Nestin+ GSC population was able to regenerate tumors after temozolomide treatment. Selective ablation of this population led to tumor growth arrest, consistent with the notion that GSCs resist conventional chemotherapy and cause relapse[18].

Another mechanism for chemoresistance lies in the cell cycle profiles of GSCs. Most chemotherapeutic agents target actively cycling cells. However, GSCs are mostly dormant or slow-cycling cells, thereby resisting such therapies[99].

Radioresistance

In addition to their chemoresistance, GSCs evade radiation, with radiation-resistant clones showing increased expression of GSC markers. More importantly, the Notch and TGF-β signaling pathways, which were mentioned earlier as critical for GSC self-renewal, promote radioresistance as well[51,100]. GSCs have increased DNA repair capacity. CD133+ GSCs selectively activate Chk1 and Chk2 kinases upon radiation, making them less susceptible to radiation-induced apoptosis[19].

CONCLUSION

In this review, we have summarized recent advances in understanding the biology of GSCs. We have focused on molecular markers commonly used to identify GSCs and signaling pathways that regulate important GSC characteristics, such as self-renewal, differentiation and therapy resistance. Due to their high tumorigenic potential and resistance to current therapies, GSCs represent critical drug targets. However, the lack of universal markers identifying GSCs, the complexity of signaling cascades regulating GSC biology and the large overlap between tumorigenic pathways active in both GSCs and normal stem cells complicate the development of GSC-targeted therapeutics. A better understanding of GSC biology and their contribution to cellular hierarchy and tumor heterogeneity is crucial for designing effective new therapies against gliomas and other brain malignancies.

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