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Review

Potential therapeutic implications of cancer stem cells in glioblastoma

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ABSTRACT

Glioblastoma is the most common and lethal type of primary brain tumor. Despite recent therapeutic advances in other cancers, the treatment of glioblastomas remains ineffective and essentially palliative. The treatment failure is a result of a number of causes, but we and others have demonstrated that a highly tumorigenic subpopulation of cancer cells called glioblastoma stem cells (GSCs) display relative resistance to radiation and chemotherapy. GSCs also contribute to tumor growth through the stimulation of angiogenesis, which has been shown to be a useful therapeutic target in the treatment of recurrent or progressive malignant gliomas. Cancer stem cells also have been hypothesized as a contributor to systemic metastases. While glioblastomas rarely metastasize beyond the central nervous system, glioblastomas invade into brain structures to prevent surgical cure and GSCs have an extremely invasive phenotype. Collectively, these studies and others suggest that GSCs may be important therapeutic targets not only to achieve cure but even reduce tumor relapse and improve overall survival. Many recent studies suggest that GSCs share core regulatory pathways with normal embryonic and somatic stem cells, but display important distinctions that provide clues into useful treatment targets. The cancer stem cell hypothesis may also modify our approaches in tumor imaging and biomarker development, but clinical validation waits. In this review, we summarize the current understanding of GSC biology with a focus on potential anti-GSC therapies.

Contents

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1. Introduction

Glioblastomas (GBMs, World Health Organization grade IV gliomas) are the most common type of primary brain tumors in adults. GBMs are among the most lethal and least successfully treated solid tumors [1]. Median survival of GBM patients treated with aggressive multimodal therapy, including maximal surgical resection, combined radiation and chemotherapy, and adjuvant chemotherapy is only 12–15 months [2]. Compared to the advances in the treatment of other types of tumors, the poor prognosis for GBM patients has improved minimally over decades, underscoring the challenges and difficulties in effectively detecting and treating these fatal cancers. Metastatic spread is responsible for deaths in many cancer patients, but GBMs rarely metastasize out of the central nervous system. In contrast, GBMs are highly infiltrative into the brain and spinal cord preventing surgical cure even with heroic resections. Invading tumor cells appear to be particularly resistant to cytotoxic therapy and are often protected by an intact neurovascular unit [1]. Additionally the majority of patients suffer primary treatment failure within 2–3 cm of the original resection cavity suggesting that therapeutic resistance is a common feature of the entire tumor. Collectively, these difficulties have propelled the refinement of therapy to achieve maximal efficacy with minimized toxicities. Improved intra-operative imaging and advanced surgical techniques have increased the ability to remove tumors safely as extent of surgical resection may be associated with increased patient survival [3]. After resection, patients undergo focused radiation at the primary tumor site instead of whole brain radiotherapy. Novel radiation technologies, such as intensity-modulated radiation therapy (IMRT) and proton therapy, permit delivery of higher radiation doses to tumor-bearing brain while relatively sparing normal brain. While chemotherapy has been used for decades in neuro-oncology, the oral methylator temozolomide (TMZ) has shown benefit when used concurrently with radiation and then as adjuvant chemotherapy such that it is now standard practice [2]. Many molecularly targeted therapies have been investigated in trials of malignant gliomas (glioblastomas and anaplastic gliomas) but to date only bevacizumab (Avastin) has been approved by the FDA, unfortunately lacking a definitive impact on survival [4–6]. Immunotherapies and toxin–ligand conjugates have shown promise in early trials but so far lack definitive efficacy in larger (phase III) trials. Transforming glioblastoma into a treatable entity will require new paradigms in cancer biology and the mechanisms underlying the GBM invasion, resistance and recurrence.

Most solid tumors consist of heterogeneous cancer cells, as well as recruited vasculature, inflammatory cells and stromal elements [7]. As the name indicates, glioblastoma multiforme displays striking intratumoral heterogeneity not only morphologically but also in differentiation status. Tumor heterogeneity may be derived from both genetic and non-genetic/epigenetic causes. Growing evidence from hematopoietic malignancies and solid tumors (including breast, brain, head and neck and colorectal cancers) has supported the hypothesis that a subpopulation of cancer cells in each malignancy has greater potential of tumor initiation and repopulation [8–19]. The nomenclature of these cells has been highly controversial due to the lack of definitive criteria (it is worth noting that we lack absolute definitions for somatic stem cells as well). The term tumor-initiating cell is commonly used but the evidence that these are the cells at tumor initiation is unclear. Tumor propagating cell has been proposed [20] as the defining assays involve tumor propagation into secondary hosts. Cancer stem cell or stem cell-like cancer cell terminology is imperfect due to distinct differences from stem cells and frequent confusion over a stem cell cell-of-origin but does capture the shared characteristics with normal stem cells especially somatic stem cells, including the capacities for self-renewal, differentiation, and maintained proliferation. Based on these concerns, we employ the term cancer stem cell with a firm recognition of the challenges of the name.

Glioma stem cells (GSCs) have been described by several groups [10,11,17,18]. These cells are functionally defined with self-renewal measured by serial neurosphere assay and tumor propagation by *in vivo* intracranial limiting dilution assays. Although cancer stem cells need not recapitulate normal differentiation cascades and commonly display aberrant differentiation signatures with multiple lineage markers, GSCs have been shown to differentiate into astrocytes, oligodendrocytes and neurons [10,11,17,18,21] (Fig. 1). Studies from a number of groups including ours have demonstrated that GSCs display much greater tumorigenic potential than matched non-stem tumor cells when xenotransplanted into brains of immunocompromised rodents

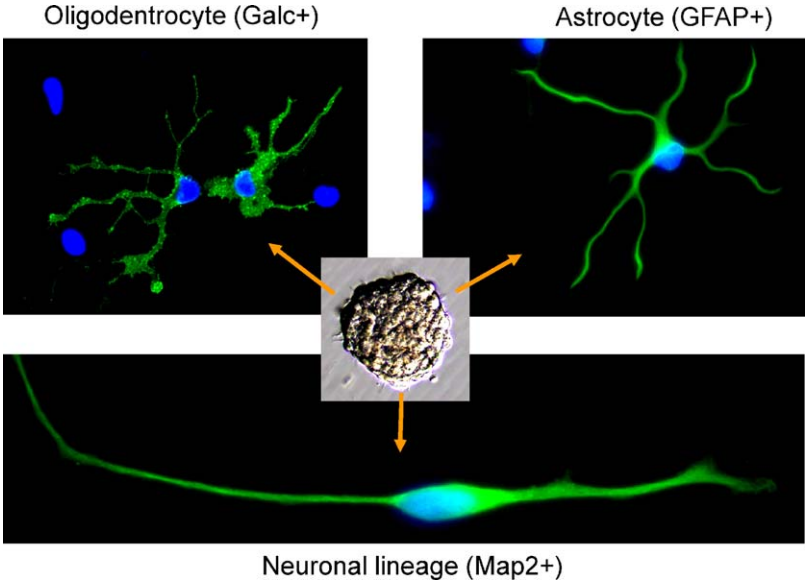

Fig. 1. Glioma stem cells display multiple-lineage differentiation potential. Immunofluorescent staining demonstrated that glioblastoma stem cells derived from a tumor specimen (T3359) formed neurospheres and differentiated into cells expressing markers for astrocytes (GFAP+), oligodentrocytes (Galc+) and neuron (Map2+) lineages upon induction of differentiation.

[10,11,17,18,21]. Although the origin of GSCs is not defined, GSCs share developmental programs with normal neural stem cells (NSCs) that endow these cells with key traits in carcinogenesis. Finally, we posit that the definition of a cancer stem cell will likely encompass a broader spectrum of behaviors in parallel to those of somatic stem cells, including the ability to modulate immune responses, interact and support the vasculature, and disperse into new locations.

GSCs have been implicated in several malignant behaviors. We found that GSCs express elevated levels of vascular endothelial growth factor (VEGF) to promote tumor angiogenesis [22]. This finding has been confirmed by others with an additional determination that stromal-derived factor-1 (SDF-1, CXCL12) is another pro-angiogenic ligand expressed by GSCs [23,24]. This is of particular significance as bevacizumab (Avastin), a humanized neutralizing anti-VEGF antibody, has demonstrated activity against GBMs and was recently approved by the United States Food and Drug Administration for the treatment of recurrent or progressive GBMs [4–6,25]. We also demonstrated that GSCs are relatively resistant to radiation due to preferential activation of the DNA damage checkpoint and lesion repair [21], while other groups have described relative resistance of GSCs to chemotherapies [26–28]. These studies may explain in part how even patients with a promising radiographic response universally suffer recurrence and/or progression of their cancers. Therefore, direct targeting of GSCs may improve the efficacy of conventional cytotoxic therapies as well as anti-angiogenic therapies. In this review, we summarize the roles of GSCs in the malignant behavior of these cancers with attention to signaling pathways involved in GSC maintenance and growth, and discuss molecular strategies for development of novel therapeutics targeting GSCs.

2. Targeting vascular niche of glioma stem cells

Florid angiogenesis is one of hallmarks of malignant gliomas [1]. Tumor growth is limited by constraints of the supportive vasculature to feed the tumor and remove waste products. Initial tumor growth occurs through vessel cooption but eventually neoangiogenesis is required although the vessels formed are abnormal and often inefficient. The degree of vascularization is significantly correlated with the glioma malignancy, tumor aggressiveness, and clinical prognosis [29]. Based on this background, a number of laboratories have aggressively investigated the relationship between the tumor vasculature and GSCs. Characterizing *in vitro* and *in vivo* growth of GSCs in relation to matched non-stem glioma cells, we determined that GSCs formed tumors with greater vascularity than the non-stem tumor cells [22]. The mechanism of this hypervascularity appeared in part due to a secreted factor from GSCs induced microvascular endothelial cell migration and vessel formation. We examined the conditioned media for expression of a series of angiogenesis regulators and noted that markedly higher levels of VEGF were the most consistent findings. Targeting VEGF effects through bevacizumab specifically blocked GSC pro-angiogenic effects both *in vitro* and *in vivo*. Using the C6 cell line, Folkins et al. showed that GSCs promote both tumor angiogenesis and vasculogenesis via VEGF and SDF-1 [24]. To understand the upstream mechanisms that drive VEGF expression in GSCs, we interrogated the role of hypoxia and the hypoxia inducible factors (HIFs). As expected, hypoxia treatment induced VEGF expression in both GSCs and non-stem glioma cells but the levels were consistently higher in GSCs [22,30]. Interestingly, HIF1α and HIF2α specifically controlled VEGF expression in GSCs in a non-redundant manner. Hypoxia can also expand the GSC fraction and regulate stem cell marker expression [31–33]. Therefore, hypoxia may function in tumor progression and therapeutic resistance through its promotion of a cancer stem cell phenotype.

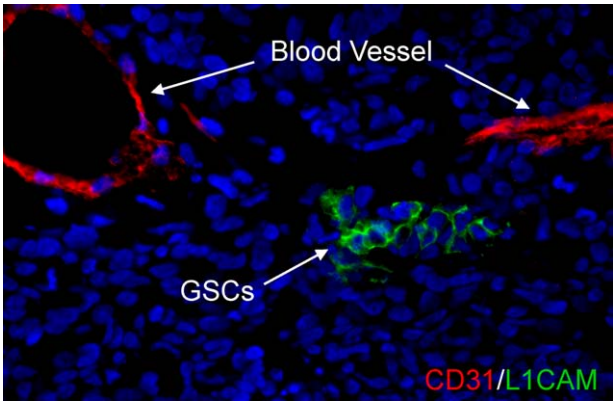

Fig. 2. Glioma stem cells are localized in vascular niches. The frozen section of GBM tumor was immunostained with anti-L1CAM antibody for glioblastoma stem cells (shown in green) and anti-CD31 antibody for endothelial cells (shown in red), and counterstained with DAPI for DNA (blue). L1CAM-positive cells (green) are located near the blood vessels (CD31+, red).

The relationship between GSCs and the vasculature is complex and bi-directional. Normal neural stem cells (NSCs) reside in perivascular locations that provide essential pro-survival and maintenance cues [34–36]. In a seminal study, Gilbertson and co-workers demonstrated that brain tumor cells that express stem cell markers reside in a perivascular niche [23]. They further showed that endothelial cells increase brain tumor stem cell survival and targeting the tumor vasculature with bevacizumab reduces the number of cancer stem cells in treated tumors. They also found that co-transplantation of GSCs with endothelial cells accelerates tumor initiation and progression [23]. Another group showed that a metronomic chemotherapy regimen decreased the growth of sphere forming C6 brain tumor cells [37]. We have observed that glioma cells bearing cancer stem cell markers, CD133, HIF2α or L1CAM (CD171), are localized near blood vessels [21,22,30] (Fig. 2). Thus, the symbiotic relationship between GSCs and vasculatures may explain the promising efficacy of anti-angiogenesis therapy with bevacizumab [4–6,25] or cediranib (AZD2171, a VEGFR inhibitor) for GBM patients in the clinical trials [38]. Thus, anti-angiogenic therapy may function as an anti-GSCs therapy. A further nuance has come from early studies that suggest that glioblastoma cells can form parts of the tumor vasculature [39]. It is likely that anti-angiogenic drugs might not only inhibit tumor vascularization to suppress GBM growth, but also directly disrupt the niches for the maintenance of GSCs, therefore weakening the "tumor roots".

3. Targeting therapeutic resistance of glioma stem cells

Glioblastoma remains to be one of the most fatal cancers despite optimal therapies. These diffusely infiltrative tumors are highly resistant to conventional radiotherapy and chemotherapy, and often recur in a local fashion despite maximal surgical resection [1]. Studies from our group and others have demonstrated that GSCs promote the therapeutic resistance and likely are responsible for the relapse of GBM [21,27,28]. The presence of a subpopulation of cancer cells within GBM possibly responsible for generating the entire mass of cancer cells has important implications for the understanding of the efficacy of current therapies and the resistance issue. It should be noted that the hierarchical relationship between the stem-like population and bulk tumor remains controversial [40], but it is possible that cancer stem cells may contribute to tumor repopulation after therapy. Radiation is the most effective non-surgical therapy for GBMs but is palliative suggesting that tumors contain resistant populations.

Indeed, we found that GSCs are more resistant to radiation than the matched non-stem glioma cells [21]. In response to radiation-induced DNA damage, GSCs preferentially activate several critical checkpoint proteins (ATM, Rad17, Chk2 and Chk1). As a result of the preferential DNA damage checkpoint activation, GSCs are more efficient in repairing the damaged DNA and more rapidly recover from the DNA damage than the matched non-stem tumor cells. Thus, GSCs are more resistant to radiation-induced apoptosis than the non-stem tumor cells *in vitro* and *in vivo*. Further, a low molecular weight inhibitor (debromohymenialdisine, DBH) of Chk2 and Chk1 checkpoint kinases abolished the radioresistance of GSCs, suggesting that targeting the DNA damage checkpoint response may sensitize GSCs to radiotherapy and thus overcome the radioresistance of GBM during treatment. Although inhibitors of checkpoint activation may be used as radiosensitizers of GSCs, consideration of the effects of the therapy on normal stem cells must also be considered, as inhibition of checkpoint activation in normal cells may lead to oncogenesis. Recently, we also showed that inhibition of the Notch pathway by the γ-secretase inhibitor or Notch shRNA renders GSCs more sensitive to radiation [41], suggesting that Notch pathway may serve as another potential therapeutic target for reducing GBM radioresistance. Additional recent studies suggest that targeting SirT1 expression or HSP90 activity can also attenuate GSC radioresistance [42,43]. It is likely that multiple mechanisms regulate cancer stem cell radioresistance, perhaps with intertumoral variation. In breast cancer stem cells, decreased radiosensitivity may be due to lower levels reactive oxygen species (ROS) [44] or activity of Wnt/β-catenin signaling [45]. To date these mechanisms have not been described in GSCs, but they give hope that several druggable targets may be targeted in GSCs to overcome radioresistance.

Besides radiotherapy, the current standard of care for newly diagnosed GBM includes adjuvant chemotherapy with temozolomide (TMZ), an oral methylating chemotherapeutic agent [2]. TMZ induces DNA alterations at several locations but achieves significant cytotoxic effect by methylating the O^6 position of guanine in DNA. This DNA adduct can be removed by the repair enzyme O^6-methylguanine-DNA-methyltransferase (MGMT) that is expressed in graded levels in GBM. MGMT expression is likely regulated at several levels but recent attention has focused on its promoter methylation that has been linked to reduced MGMT levels and greater sensitivity to TMZ treatment [46]. The addition of TMZ to GBM therapy has been potentially most effective by a radiosensitization effect [2], but TMZ is commonly used as adjuvant therapy as well. Although therapy with TMZ may slow GBM tumor growth and increases the proportion of patients surviving for two years, long-term survivors are still rare due to drug resistance and GBM recurrence. Invariable tumor recurrence after TMZ therapy indicates the presence of TMZ-resistant cancer cells in GBM. Recently, it was shown that GSCs also contribute to the chemoresistance to TMZ [26–28]. In a genetically engineered glioma mouse model, TMZ treatment increased the side population (SP), a potential measure of cancer stem cells [47] and TMZ does not inhibit GSC self-renewal [48], although it has been shown that TMZ can eliminate MGMT-negative GSCs [49]. Several other potential mechanisms of GSC drug resistance have also been reported. Increased expression of drug transporters that pump out chemotherapeutic agents may be one of critical mechanisms, including the ABC (ATP-binding cassette) drug transporters. Some studies suggest that ABC expression may enrich for cancer stem cells [16]. A side population (SP) of cancer cells isolated from tumors may represent a class of cancer stem cells with high drug efflux capacity and thus show inherently high resistance to chemotherapeutic agents [50]. SP cells express high levels of the ABC drug transporters such as ABCG2 and ABCA3 in GBM cell lines [50], suggesting that targeting these drug transporters may reduce drug resistance of GSCs. In addition, several studies have shown that the poly(ADP-ribose) polymerase-1 (PARP-1) inhibitors such as CEP-8983 and E7016 can increase sensitivity of chemoresistant GBM tumor cells to TMZ and radiation [51–53]. PARP-1 plays a critical role in DNA repair, particularly in the base excision repair of DNA strand breaks caused by ionizing radiation or DNA lesions induced by methylating agents such as TMZ [54–57]. *In vivo* studies have demonstrated PARP-1 inhibitors enhance the anti-tumor effects of radiation or chemotherapy and sensitize glioma cells to TMZ treatment [23,53]. As GSCs display preferential DNA repair capacity, it is worth of testing whether PARP-1 inhibitors can be used to overcome the radio- or chemo-resistance of GSCs.

4. Targeting glioma stem cells through specific cell surface molecules

Cell surface molecules differentially expressed in GSCs and functionally associated with the maintenance of GSC may be ideal targets. Several molecules, including CD133 [17,18], CD15 [58–60], L1CAM [61], A2B5 [62,63], have been identified on cell surface of brain tumor stem cells. Although CD133 (prominin-1) has been widely used as a marker for enrichment of GSC population from GBM tumors or xenografts, many normal cells express CD133 potentially limiting its utility as a target and the reliability of CD133 to discriminate GSCs is not absolute [64]. CD15 (stage-specific embryonic antigen-1, SSEA-1; Lewis X antigen) originally identified as a marker of mouse embryonic stem cells [65,66] has recently been used as an alternative marker to enrich GSCs from some GBM tumors (and medulloblastoma stem cells as well) in which CD133 marker is not a informative maker for GSC population [58–60]. But whether CD15 can be used as a target for GSCs is not clear because CD15 is a carbohydrate antigen rather than a distinct protein target, expressed in the normal brain including normal neural and progenitor cells [67], and the function of CD15 in normal stem cells and cancer stem cells remains poorly understood. Other surface markers such as A2B5 have been used for the enrichment of GSC population [62,63], but whether these surface markers can be used for targeting GSCs need further investigations.

In the search for specific functional surface targets for GSCs, we have identified L1CAM as a cell surface molecule that is differentially expressed in GSCs and plays critical roles in the maintenance, survival and functions of GSCs [61]. L1CAM was originally identified as a neural cell adhesion molecule in the nervous system and plays important roles in the development of nervous system [68]. This glycoprotein contains a cytoplasmic tail, a transmembrane domain and an extracellular domain that can interact with another L1CAM molecule through homophilic interaction, or binds to EGFR and FGFR, α5β1 and αvβ3 integrins, Neuropilin-1, and a number of extracellular matrix proteins through heterophilic interaction (reviewed in [69]). L1CAM mediated intra- and inter-cellular signaling plays important roles in regulating cell adhesion, migration, survival, growth and cancer cell invasion. We have found that L1CAM is highly expressed in GSCs relative to non-stem GBM tumor cells and normal neural progenitor cells [61]. Knockdown of L1CAM using specific shRNA specifically disrupts neurosphere formation and growth of GSCs *in vitro*. Targeting L1CAM in GSCs remarkably suppressed the tumor growth and increased the survival of mice bearing intracranial GBM xenografts [61]. We have determined that the molecular mechanism by which L1CAM promotes GSC maintenance and tumor growth is through up-regulation of Olig2 to suppress expression of $p21^{WAF1/CIP1}$. Our data indicate that L1CAM as a functional surface molecule may represent a novel target for development of anti-GSC specific therapeutics.

5. Targeting glioma stem cells through blocking specific signaling pathways

Although the therapeutic targeting of cancer stem cells has generated excitement [70], the understanding on the molecular signaling of cancer stem cells is in early development. While cancer stem cells share some properties with normal somatic stem or progenitor cells, they are distinct from the normal stem cells at genetic and molecular signaling levels. The identification of specific signaling pathways involved in the maintenance and functions of GSCs may be useful to develop novel strategies to improve GBM treatment. A number of singling pathways associated with cancer stem cell maintenance have been reported [70]. Here we discuss a few of the critical signaling transduction pathways mediated from external signals to nucleus in GSCs.

5.1. RTK-Akt signaling

Receptor tyrosine kinases (RTKs) transduce signaling of multiple oncogenic growth factors, including the epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) that are used in culturing GSCs [71]. Among these RTK pathways, the EGFR-mediated growth signaling through phosphoinositide 3-kinase (PI3K)/Akt is one of the most important and best characterized pathways in gliomas. Malignant gliomas, particularly GBMs, frequently display EGFR amplification and/or expression of the constitutively active variant EGFRvIII that leads to increased EGFR-Akt signaling in cancer cells [72,73]. Overexpression of EGFRvIII in genetically engineered models induces glioma-like tumors [74,75]. It is not then surprising that EGFR activity is required for maintenance of GSCs as EGFR kinase inhibitors attenuates *in vitro* GSC proliferation and neurosphere formation [76,77]. The intracellular pathways that are activated in turn upon EGFR activation are numerous but prominently the PI3K-Akt axis has been strongly linked to glioma stem cell biology [78]. Our group recently demonstrated that GSCs are more dependent on Akt signaling than non-stem cancer cells [79]. Inhibition of Akt with the pharmacologic inhibitors (SH-6 or LY294002) disrupts GSC neurosphere formation, induces apoptosis, reduces migration and invasion *in vitro*, and significantly delays intracranial tumor formation of GSCs. These results have been validated by other groups, including in a genetically engineered mouse model [47,80]. Although targeting EGFR-PI3K-Akt signaling pathway may have specific effects on GSC population to reduce tumorigenic potential, the results to date in clinical trials of EGFR inhibitors have been disappointing suggesting that alone this is an insufficient therapeutic paradigm and prompting greater focus on PI3K inhibitors.

5.2. Notch signaling

Notch proteins including four members (Notch 1–4) are transmembrane receptors that mediate short-range cellular communication through interaction with ligands (Jagged-1, -2, and Delta-like-1, -3, and -4). The Notch signaling pathway is essential for the maintenance and fate determination in somatic stem and progenitor cells by promoting self-renewal and repressing differentiation. The critical roles of Notch signaling in regulating self-renewal and determining cell fate have been well established in neural stem cells (reviewed in [81]). The activation of Notch requires sequential proteolytic cleavages by the γ-secretase complex to release its intracellular domain from membrane to nucleus. The nuclear translocation of the cleaved Notch leads to Notch-dependent transcription. Notch signaling promotes the proliferation of normal neural stem cells and is required for the maintenance of neural progenitors both *in vitro* and *in vivo* [82]. Aberrant Notch signaling has been found in several types of tumors including gliomas [83,84]. The role of Notch signaling in brain tumor stem cells was initially identified in medulloblastomas. Inhibition of Notch signaling by a γ-secretase inhibitor (GSI-18) induces differentiation and apoptosis of CD133+ stem-like cells derived from medulloblastoma and impairs the tumorigenic potential of these cells [85]. Recently, the function of Notch signaling has been linked to GSCs, as blockade of Notch signaling in GSCs attenuates the formation of neurosphere-like colonies [86]. In addition, Notch overexpression in a K-ras-induced glioblastoma mouse model increased expression of NSC marker Nestin and induced glioma formation in the NSC-rich subependymal zone [87]. As mentioned above, Notch signaling has been linked to radioresistance of GSCs by our group [88], suggesting that inhibition of Notch signaling may not only disrupt the maintenance of GSCs but also reduce the radioresistance of GSCs. Other regulators of Notch signaling, including Delta/Notch-like epidermal growth factor-related receptor (DNER) and the Notch ligand Delta-like 4 (DLL4), can also regulate glioblastoma growth [89,90]. Further, other signaling pathways – inhibitor of differentiation 4 (ID4) and CXCR4 – functionally interact with Notch signaling in brain tumors as well [91,92]. Anti-DLL4 therapies have demonstrated anti-cancer stem cell activity [93] but concern has been raised as chronic DLL4 targeting can induce neoplasia as well [94]. Although blocking Notch signaling may be a good strategy targeting GSCs, γ-secretase inhibitors are still in early clinical development for brain cancers.

5.3. Bone morphogenetic proteins (BMPs)/transforming growth factor-β (TGF-β)

The BMPs and TGFβ superfamily regulates a large number of cellular processes during development and injury responses. The BMPs instruct cell fate during neural development. Based on this background, the Vescovi group performed a seminal study that demonstrated the ability of BMP ligands to activate their canonical receptors on GSCs to induce differentiation and inhibit tumor growth [95]. This study demonstrated that direct implantation of BMP-bearing beads into glioblastomas slowed tumor growth laying the foundation for a potential therapy. The role of BMPs in GSCs became more nuanced after the Fine group showed that some GSCs epigenetically regulate BMP receptors to shift towards a fetal phenotype to escape the pro-differentiation effects of BMPs [96]. In contradistinction, TGF-β serves as a largely oncogenic stimulus in glioblastoma growth through induction of angiogenesis, immune evasion, and invasion [97]. Recent studies have added a new dimension in TGF-β oncogenesis as autocrine and paracrine loops function to maintain GSCs through induction of leukemia inhibitory factor (LIF) [98] and the SOX family members [99]. TGF-β inhibitors have already entered into clinical trial and BMPs are being considered.

5.4. Hedgehog-Gli signaling

The Sonic Hedgehog-Gli signaling is one of the key regulatory pathway during embryogenesis and is critical for the maintenance of several types of adult stem cells, including neural stem cells [100]. The binding of Hedgehog ligands to the PTCH receptor activates Gli signal transducers that then translocate into the nucleus to activate or repress transcription of downstream genes. Aberrant Hedgehog signaling has been associated with medulloblastomas, the common childhood tumors [100,101]. Hedgehog signaling is mediated not only through specific signaling molecules but also in association with a physical organelle, primary cilia, that function in medulloblastoma development [102]. Active Hedgehog-Gli signaling is also found in gliomas [103], in fact the key

intracellular mediator Gli was originally discovered in gliomas [104]. Gli activity correlates with tumor grade in a genetically engineered mouse model [105]. Several groups have studied the role of Hedgehog-Gli signaling in GSCs and found that this signaling pathway regulates self-renewal and tumorigenic potential of GSCs [48,106–108]. Treatment of GSCs with the hedgehog inhibitor cyclopamine or Gli RNA interference suppresses self-renewal and proliferation while increases apoptotic cell death. Importantly, inhibition of Hedgehog-Gli signaling enhances the efficacy of TMZ to inhibit GSC proliferation and induce cell death [48]. *In vivo* studies showed that the inhibition of the Hedgehog signaling pathway blocks GSC tumor growth, and the viable neoplastic cells after the cyclopamine treatment failed to propagate tumors *in vivo* [48]. Furthermore, cyclopamine treatment has been shown to improve the effect of radiation on GSC cell survival. Collectively, these studies indicated that Hedgehog-Gli signaling pathway is critical for GSC maintenance and targeting this pathway with pharmacologic inhibitors may suppress GSC growth and improve the efficacy of conventional therapies against malignant gliomas. Although the side effect of these inhibitors on normal stem cells needs to be carefully evaluated, recent clinical studies with the Hedgehog inhibitor GDC-0449 have shown promising responses with acceptable toxicity [109,110].

5.5. *Wnt-β-catenin signaling*

The canonical Wnt cascade is one of critical regulators in embryonic stem cells and adult stem cells. Wnt-β-catenin signaling has clearly defined roles in both normal stem cells and cancer stem cells (reviewed in [111]). In brain, the Wnt signaling pathway regulates brain development as well as proliferation and self-renewal of NSCs or neural progenitor cells in the fetal ventricular zone, the postnatal subventricular zone and hippocampus [112–116] and alterations have been linked to medulloblastoma [117,118]. Wnt signaling is activated predominantly in medulloblastoma of the classic subtype [119]. Recent studies indicate that Wnt-β-catenin signaling may contribute to radioresistance in cancer stem cells [45]. Whether Wnt-β-catenin signaling is associated with GSC maintenance and radioresistance requires further investigation, but it is possible that Wnt blockade can effectively target GSCs.

5.6. *STAT3 signaling*

The signal transducer and activator of transcription 3 (STAT3) is a critical transcriptional regulator involved in a wide range of cellular activities in the immune response, central nervous system development, stem cell maintenance and tumorigenesis. The link between the activation of STAT3 and glioblastoma biology has become increasingly evident (reviewed in [120]). Abnormal STAT3 activation has been detected in many types of cancers including solid tumors and hematopoietic malignancies. The canonical oncogenic function of STAT3 depends on its phosphorylation on Tyr^{705} that can be attributed to aberrant activity of various upstream kinases. STAT3 in conjunction with C/EBP-beta correlates with mesenchymal transformation of glioblastomas and inversely related to patient outcome [121]. Based on this background, several groups have interrogated STAT3 in GSCs. Genetic knockdown of STAT3 or inhibition of STAT3 with specific inhibitors disrupts proliferation and maintenance of GSCs [122,123]. Moreover, the phosphorylated active form of STAT3 on tyrosine-705 and serine-727 is present in GSC population and the active form of STAT3 decreases to undetectable level after differentiation induction of GSCs [123]. Several pathways upstream of STAT3 are active in GSCs – interleukin-6 (IL6), erythropoietin, and Notch – and targeting these pathways inhibits STAT3 activation and GSC growth and self-renewal [85,122,124]. STAT3 also contributes to the immune regulation by GSCs [125]. Since STAT3 is involved in many cellular activities in a wide range of cancer types, STAT3 inhibitors are undergoing clinical development. However, as STAT3 is also important for the maintenance of normal stem cells and required for critical immune responses and other normal cellular activities, targeting STAT3 may display significant side effects and is unlikely to be specific for GSCs.

5.7. *GSK3-β signaling*

GSK3-β is one of isoforms of glycogen synthase kinase 3 (GSK3) and involved in different signaling pathways regulating cell cycle, proliferation, differentiation and apoptosis. GSK3-β has been implicated in the regulation of neural stem cell differentiation and proliferation [126–129]. Recently, it has been shown that GSK3-β is involved in maintaining GSCs, as reduced GSK3-β activity either by shRNA or the specific inhibitor SB216763 or lithium chloride (LiCl) induces GSC differentiation and reduces expression of stem cell marker Sox2 and Myc [130]. In addition, down regulation of the stem cell factor Bmi1 (a member of polycomb group of proteins) reduces GSK3-β expression in GSCs [130]. Since GSK3-β signaling is very complex, the role of GSK3-β in GSCs needs further investigation, but the number of specific GSK3-β inhibitors available provides an additional therapeutic strategy for treating malignant gliomas.

6. Targeting hypoxic responses of glioma stem cells

Hypoxic conditions are commonly present in solid tumors including malignant gliomas. Hypoxia was thought to have a negative impact for tumor growth, but hypoxia actually promotes tumor angiogenesis, cancer dispersal and therapeutic resistance such as radioresistance in GBM [131]. Furthermore, recent work from our group and others has suggested that hypoxic niches play critical roles in the maintenance of cancer stem cells in tumor tissue [30,31,132,133]. Similarly, hypoxic niches are also involved in the maintenance of normal stem cells. For example, hematopoietic stem cells are maintained in hypoxic niches in bone marrow [134]. Hypoxia also prevents the differentiation of neural stem cells and promotes the self-renewal of embryonic stem (ES) cells [135–138]. Restricted oxygen concentrations also enhance the production of induced pluripotent stem cells (iPSC) [139]. GBMs frequently display areas of necrosis – necrosis serves as a grading criterium for GBM – that occur in avascular and low oxygen regions. While brain tumor stem cells have been linked to a perivascular niche, we have found an additional enrichment of GSCs around necrotic regions [30]. Other groups have found that restricted oxygen promotes a GSC phenotype [32,33]. We also found that restricted oxygen conditions increase expression of GSC markers and indicator of self-renewal and tumor growth, suggesting that the GSC state may be plastic and that microenvironmental conditions can promote the acquisition of a stem cell-like state [31,140]. These studies suggest that disrupting the microenvironment of GSCs, like the hypoxic niches, may provide a new approach targeting GSCs in malignant gliomas.

In response to hypoxia, cells undergo significant transcription modification that leads to alterations of cellular function. The cellular responses to hypoxia are mainly mediated through the hypoxia inducible factors (HIFs). We recently demonstrated that hypoxia responses in GSCs differ from non-stem cancer cells. Hypoxia differentially induces HIF2α in GSCs, while HIF1α is induced in both GSCs and non-stem GBM tumor cells by hypoxia [30]. HIF2α was essential only in GSCs and was not expressed by normal neural progenitors, suggesting that HIF2α may represent a specific target for GSCs or other cancer stem cells. Under hypoxic

conditions, GSCs display a specific gene expression profile relative to non-stem cancer cells. In addition to the increased VEGF expression, GSCs specifically up-regulate HIF2α and several HIF2α transcriptional targets such as Oct4, Glut1 and Serpin B9 under hypoxia [30]. GSCs display high levels of HIF2α under oxygen concentration as high as 5% that is within the physiologic range of oxygen in the brain and most tumor tissues [30], whereas HIF1α is induced in both GSCs and the non-stem tumor cells only at severely hypoxic conditions (<1% oxygen). Functional studies through RNA interference demonstrated that both HIF1α and HIF2α are required for GSC growth *in vitro* and GSC tumor formation *in vivo*, but only HIF1α is required for non-stem GBM tumor cell growth, suggesting that GSCs use both HIF1α and HIF2α for the hypoxia response and may be able to survive better under stress conditions. Other groups have found that HIF1α functions in the hypoxia driven expansion of GSCs [33]. Moreover, the silico analysis of HIFs expression in the REMBRANDT National Cancer Institute patient database showed that HIF2α but not HIF1α levels informed negative survival of patients. In addition, overexpression of HIF2α promotes cancer stem state in GBM [31]. These data indicate that HIF2α is a potential target specific for GSCs since HIF2α is not expressed in normal neural progenitors. However, the role HIF2α in other normal stem cells needs to be elucidated, in order to understand whether targeting HIF2α have negative impact on other normal stem cells.

7. Targeting glioma stem cell maintenance through specific transcription factors

Since GSCs share some critical characteristics with normal neural stem cells and embryonic stem cells, some important stem cell transcription factors (SCTFs) are also involved in the maintenance and functions of GSCs. These SCTFs such as Sox2, Oct4, Nanog, c-Myc, Olig2 and Bmi1 are critical for the self-renewal, proliferation, survival, and maintenance of multi-lineage differentiation potential of GSCs.

Sox2, *Oct4 and Nanog* are core components in maintaining embryonic stem cells and somatic stem cells [141–144]. They are also critical factors for cell reprogram and generation of inducible pluripotent stem cells (iPS) [145,146]. These SCTFs are highly expressed in GSCs and may be important for GSC maintenance [21,99]. Although targeting these SCTFs induces differentiation of GSCs, they present challenging targets for GSCs because these SCTFs are also highly expressed in normal somatic stem cells and are critical for maintaining normal stem cells, such as neural stem cells and hematopoietic stem cells.

c-Myc is a well known oncoprotein that has been extensively studied for its crucial role in the proliferation of both normal stem cells and cancer cells. c-Myc may be a critical link to study the relationship between "stemness" and tumorigenicity. c-Myc expression levels correlate with tumor grade in gliomas [139]. Recently, our group and others demonstrated that c-Myc expression is elevated in GSCs and it is required for maintaining GSCs *in vitro* and their tumorigenic potential *in vivo* [147]. Conditional overexpression of c-Myc in mouse astroglia leads to brain tumors resembling human malignant gliomas [148]. In addition, c-Myc prevents cell differentiation and promotes self-renewal of tumor cells derived from the pten/p53 double null mouse model [149]. These studies support an important role of c-Myc in GSC maintenance. However, the widespread effects of c-Myc in normal physiology must be considered. Although c-Myc plays crucial roles in tumorigenesis and tumor progression, it is unlikely a specific target.

Olig2 is a unique transcription factor that is almost exclusively expressed in the stem cells or progenitors in the CNS (central nervous system). Olig2 is expressed in neural progenitors fated to give rise to oligodendrocytes and subtypes of neurons [150]. Several studies have revealed that Olig2 is widely expressed in astrocytomas and is required for tumor initiation and growth [151,152], suggesting a link between Olig2 expression and GSCs. Indeed, Olig2 differential expression was found in GSC populations isolated from most cases of GBM tumor specimens obtained in our group, suggesting that Olig2 is a common marker for GSCs. This transcription factor is required for maintaining the multi-lineage differentiation potential of neural progenitors and GSCs. Olig2 mediates GSC proliferation in part through suppression of $p21^{WAF1/CIP1}$, a key cell cycle regulator [61]. Since Olig2 expression is limited in GSCs and CNS stem cells/progenitors, Olig2 could be a potential target for GSCs even it is not an ideal target.

REST (repressor element 1-silencing transcription factor) or NRSF (neuron-restricted silencing factor) is a master neuronal repressor that plays a critical role in maintaining neural stem cells by suppressing neuronal differentiation [153]. The zinc-finger domain of REST recognizes a conserved RE-1 element (21–23 base pairs) within regulatory regions of target genes to repress the transcription of the differentiation-associated genes. REST promotes oncogenesis in medulloblastomas and neuroblastomas that usually arise from neural progenitors [154,155]. This transcription repressor is also highly expressed in human glioblastomas and neuroblastomas [154]. REST is targeted for proteasomal degradation by the ubiquitin E3 ligase SCFβ-TRCP to promote neural differentiation [156]. We have observed that REST is differentially expressed in GSCs isolated from some cases of GBM tumor samples (unpublished data), suggesting that targeting REST may induce GSC differentiation.

Bmi1 is one of polycomb group genes that normally function as epigenetic silencers. Bmi1 is a positive regulator of neural stem cells and has been implicated in stem cell fate determination in several tissues [157]. Bmi1 is required for the malignant transformation of both neural stem cells and differentiated astrocytes [158]. Transformed Bmi1 wild-type neural stem cells give rise to high grade gliomas *in vivo*, but Bmi1-deficient neural stem cells only initiate less malignant type of gliomas with fewer cells expressing stem cell markers. Bmi1 is frequently overexpressed in several types of cancer including gliomas. Interestingly, Bmi1 is also highly expressed in GSCs and required for GSC self-renewal [159]. Similar finding for an essential role of Bmi1 in maintaining cancer stem cells has been shown in hepatocellular carcinomas [160].

8. Targeting glioma stem cells through induction of differentiation

One of important properties that GSCs share with normal stem cells is their multi-lineage differentiation potential, although differentiation potential is not one of essential characteristics to define a cancer stem cell. GSCs isolated from primary GBM tumors or xenografts have the capacity to differentiate into cells with the morphologies and marker profiles of astrocytes, oligodendrocytes and neurons ([21] and Fig. 1). These differentiated cells lose long-term repopulation potential *in vitro* and fail to propagate tumors *in vivo*, suggesting that inducing GSC differentiation may be a practical strategy to deplete the GSC population. Several signaling pathways involved in differentiation induction of stem cells have been identified. As noted above, the BMPs inhibit GSC proliferation and deplete GSC population by inducing the differentiation of GSCs into astroglial and neuron-like cells [95]. Targeting GSCs with BMP4 *in vivo* significantly inhibits GBM tumor growth and reduces tumor invasion [95]. Recent study by Lee et al. has confirmed that BMPs promote glial differentiation of GSCs [96], but they also found that GSCs derived from one GBM sample showed enhanced cell proliferation rather than differentiation in response to BMP

treatment. This is because GSCs from this sample lost BMPR1B expression due to epigenetic silencing by an EZH2-dependent mechanism [96]. Restoration of BMPR1B expression rescued the BMP4-induced differentiation in these GSCs. These studies suggested that individual epigenetic characteristics may determine GSC response to the differentiation-inducing agents, and BMPs in combination with epigenetic modulators may be critical to induce GSC differentiation.

In addition, there are other factors that have been implicated to promote differentiation of GSCs. Recent study showed that inactivation of PTEN (a well-known tumor suppressor) promotes undifferentiated state of glioma stem cells [161], suggesting that PTEN may promote GSC differentiation. PTEN is a phosphatase with dual-specificity for both protein and lipid. PTEN deletion or functional loss has been linked to initiation and/or progression of malignant gliomas. PTEN inactivation leads to increased expression of Myc that is critical for maintaining GSC proliferation and self-renewal, suggesting promoting PTEN function may suppress the "stemness" of GSCs. In another study, Sox11 was shown to promote GSC differentiation [162]. Overexpression of Sox11 inhibits tumorigenic potential of GSCs by promoting neuronal differentiation. Furthermore, inactivated Sox11 expression was detected in GSCs derived from some cases of GBM when a gene expression profile was analyzed between tumorigenic and non-tumorigenic clones of glioma cells. Although these factors have been shown to promote GSC differentiation, whether these factors can be used for targeting GSC in clinical applications needs further studies.

9. Regulation of glioma stem cells by micro-RNAs (miRNA)

The roles of miRNAs in regulating embryonic stem cells, somatic stem cells or cancer stem cells have received much attention in recent years. miRNAs are a group of small non-coding RNAs that potently silence gene expression through post-transcriptional modification on target mRNAs. Since a single miRNA may regulate several or many distinct mRNAs, miRNAs are powerful regulators of gene expression. miRNAs are emerging as crucial regulators of cellular proliferation and differentiation. They can function as either oncogenes or tumor suppressors in various tissues or tumors. miRNA has been shown to be critical in the regulation of glioma cell functions. For example, miRNA-21 is overexpressed in GBM tumors and blocking its function induces apoptotic cell death [163]. The roles of miRNA in GSCs have been demonstrated in two recent reports [164,165]. Levels of miR-124, miR-137 and miR-451 are significantly reduced in malignant gliomas (both grade III and grade IV) relative to normal brain and in GSCs relative to non-stem tumor cells. Overexpression of these miRNAs in GSCs inhibits proliferation and induces differentiation of GSCs, suggesting that these miRNAs have an important role in maintaining GSCs. Furthermore, external expression of miR-451 disrupts neurosphere formation and suppresses tumor growth of GSCs, indicating a tumor suppressor role of miR-451 in gliomas. These studies suggest that some critical miRNAs can be potentially used as therapeutic agents for targeting GSCs. However, how we deliver these miRNAs into GSCs or non-stem tumor cells and how we make these miRNAs to be stable targeting agents may face a great challenge in the future.

10. Conclusions

The identification of cancer stem cells and their roles in GBM progression and therapeutic resistance has altered our understanding of glioma tumor biology and causing a reevaluation of current therapies for malignant gliomas. Cancer cure requires elimination of all tumor cells, even small fractions like GSCs. Novel

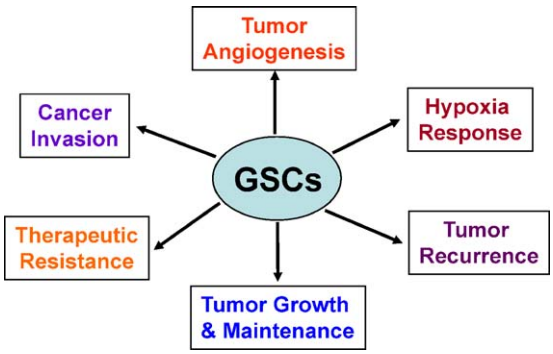

Fig. 3. A summary of roles of glioma stem cells (GSCs) in glioblastoma tumor progression and therapeutic resistance.

therapies directed against GSCs may help improve the currently dreadful record of clinical activity with conventional therapy. Although controversy still exists as to the methods for isolating and characterizing GSCs and defining the role of non-stem cancer population, GSCs represent a subpopulation of cancer cells with extraordinary capacities to promote tumor repopulation, angiogenesis, invasion and therapeutic resistance (summarized in Fig. 3), making them a critical cell population that should be targeted for anti-glioma therapies. A recent report showed that a lentival vector-mediated suicide gene therapy can eliminate invasive GSCs in xenograft models [166]. Since non-stem tumor cells (non-GSCs) may be able to reprogram into GSCs under certain conditions [31,140], we believe that eliminating both GSC and non-GSC cancer cell populations is essential to achieve therapeutic success (Fig. 4). Recent research advances in this exciting area have allowed us to gain remarkable insights into the signaling pathways that are differentially present or regulated in GSCs or non-stem cancer cells. As GSCs share critical signaling pathways with normal neural stem/progenitor cells but also significantly distinct from normal stem cells in many aspects, identification of the unique signaling pathways or molecular regulators that differentially control the phenotypes and tumorigenic potential of GSCs might offer new avenues for developing novel therapeutics against GSCs to significantly improve GBM treatment. We have discussed a number of signaling pathways or molecular targets that are potentially useful for the future development of anti-GSC therapeutics, but most of them are still far away from the clinical application except the anti-vascular niche treatment that has shown promising results in clinical trials leading to preliminary FDA approval for bevacizumab for the treatment of recurrent or progressive GBM. Some molecular regulators such as L1CAM and HIF2α that are preferentially expressed in GSCs are likely to be specific targets for GSCs, as targeting other critical pathways such as Notch, Hedgehog-gli, Wnt or STAT3 signaling pathways that are shared by normal stem cells may display significant side effect on normal cells. Additional research is certainly needed to confirm the clinical relevance of these laboratory findings and better apply these concepts to clinical practice. For example, biomarker development and the application of personalized medical therapy may be accelerated with analysis of tumor heterogeneity. However, great challenges lay ahead as GSC populations themselves are heterogeneous [167] and the GSCs may evolve over time within a patient. As the genetics of glioblastomas are becoming increasingly defined with clear subgroups of tumors evolving, our understanding of GSC diversity will certainly become more nuanced. GSCs are also a product of their environment and almost certainly not only interact with vascular niche but also with non-stem tumor cells, stromal elements and immune cells. The emerging concepts and roles of cancer stem cells are still rapidly

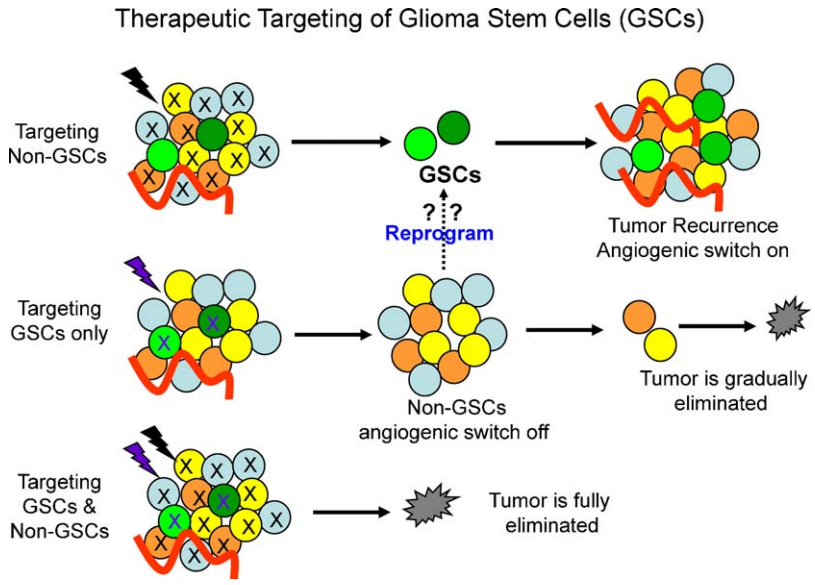

Fig. 4. Therapeutic targeting of glioma stem cells (GSCs) and non-stem cancer cells (non-GSCs). Targeting both populations of glioblastoma cancer cells is important to eliminate the tumor, since non-stem cancer cells may be able to reprogram into GSCs under certain conditions.

evolving. The road forward will likely be bumpy but these paradigms are exciting as they may bring new opportunities to a group of patients sadly lacking in effective treatment options. Since the origin of cancer stem cells in GBM from different patient may vary and they may display different genetic changes in complex tumor tissues, future treatment for GBM may rely on a unique combination of several targeted therapies based on the cellular, genetic and molecular information of the tumor in the individual patient.

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