

Molecular Pathways of Glioblastoma and Glioblastoma Stem Cells

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1. Introduction

Glioblastoma (GBM, WHO grade IV) is a type of highly malignant brain tumor that infiltrates the brain extensively and remains virtually incurable despite being treated with gross total resection and post-operative adjuvant radiation and chemotherapy. The vast majority of patients with GBM will always develop tumor recurrence. The tumor's location, its unique feature of high motility, and its protection by the blood brain barrier make certain therapies that are effective for some other cancers ineffective against brain tumors. Overall, the 5-year survival rate is less than 10%, with a final mortality rate of close to 100 percent. The molecular mechanisms that underlie persistent tumorigenesis and treatment resistance are still poorly understood. A genome-wide expression profile analysis revealed that besides those genes associated with cell proliferation, inflammation, angiogenesis, and extracellular matrix (ECM) remodeling, a series of genes linked with neuroepithelial stem cells, mesenchymal stem cells, skeletal/cartilage development, morphogenesis, and organogenesis, were determined to be overexpressed when compared with normal brain tissue, implicating that a tissue regeneration/repair-like program is constantly activated in GBM tumors. A subset of GBM develops from lower-grade gliomas and can thus be clinically classified as "secondary," whereas some GBM occur with no prior evidence of a lower-grade tumor and can be clinically classified as "primary." Substantial genetic differences between these groups of GBM have been identified. Moreover, a molecular classification study indicated that both treatment-refractory and untreated primary GBM tumors are clustered in a group segregated from treated and untreated secondary GBM tumors, and supports the view that GBM subtypes may have derived from a distinct cell-of-origin, which is resistant to conventional therapy, therefore allowing for re-seeding tumor with molecular properties similar to untreated tumors. Thus, post-treatment tumor recurrence may mimic the scenario of post-injury tissue repair. Many adult tissues undergo renewal after injury, and hence require a new supply of cells originating from specialized tissue stem cells with the capability to undergo self-renewal and differentiation to repair damaged tissue. Recently, glioblastoma stem cells (GSC) or glioblastoma stem-like cells (GSLC), a minor subpopulation within tumor mass, were isolated and characterized as tumor-initiating cells and were hypothesized to be responsible for post-treatment recurrence because of their enhanced radio-/chemo-resistant phenotype and ability to reconstitute the original tumor tissue when grafted into mice. In contrast to the hyperproliferative,

inflammatory, and hyperangiogenic properties seen in GBM tumors, molecular analysis by gene expression profiling revealed that GSC possess neuroectodermal properties and express molecular signatures of radial glial cells (RGC) and neural crest cells (NCC), as well as portray a migratory, quiescent, and slow-growing phenotype that characterizes tumor suppressor properties. Based on the tumor stem cell model and theory, conventional cell cycle-targeted radio-chemotherapy, which aims to kill fast-growing tumor cells, would then be unable to eliminate post-operative remaining tumorigenic cells that possess quiescent stem cell properties. Thus, in order to prevent tumor recurrence, a strategy targeting essential gene pathways of GSC must be identified and incorporated into the standard treatment regimen. Identifying intrinsic and extrinsic cues, by which GSC maintain tumorigenic capacity and antiapoptotic feature to sustain tumorigenesis may highlight novel therapeutic strategies to greatly diminish the recurrence rate of GBM and provide potentially curative strategies for treating brain cancers. In this chapter, we review molecular properties of GBM tumors and GSC. We also summarize molecular signaling pathways that have been relatively well-studied in GSC and are essential for maintaining GSC stemness, tumorigenic capacity, and radio-chemoresistant phenotype.

2. Molecular properties of glioblastoma

2.1 Genetic and clinical pathways to glioblastomas

GBM remains refractory to conventional therapy. The histopathologic features that distinguish it from lower-grade astrocytic tumors are the presence of cellular atypia, mitotic figures, necrotic foci with peripheral cellular pseudopalisading, and microvascular hyperplasia (1). Two subgroups of GBM have been established based on clinical experience and have been affiliated with distinct genetic mechanisms of tumorigenesis. Secondary GBM, also known as progressive GBM, develop slowly through progression from low-grade glial tumors (WHO grade II) or anaplastic glial tumors (WHO grade III) and frequently display p53 mutation (chromosome 17) (~65%) and amplification or overexpression of platelet-derived growth factor receptor (PDGFR), but not epidermal growth factor receptor (EGFR) (2-3). Additionally, progression to secondary GBM often accompanies an allelic loss at chromosome 19q, 17p, and 10q, and a loss of expression of deleted-in-colorectal-carcinoma gene (DCC) (~50%) but rarely include PTEN mutations (5%) (4-6). The p53 mutation is usually found in the low-grade lesions, indicating p53 alteration is an early event in astrocytoma progression (7). PDGFR amplification or overexpression is also present at the early stages suggesting that it may have a role in the progression of these tumors. In contrast, loss of heterozygosity for the retinoblastoma-1 (RB1) gene was found in high-grade astrocytomas (25%) but not in low-grade astrocytomas, indicating disruption of the RB pathway is likely a significant event in the malignant transformation to GBM (8). On the other hand, primary GBM, also known as de novo GBM, seem to develop rapidly and manifest high-grade lesion from the outset and are genetically characterized by EGFR amplification/overexpression (chromosome 7) (~60%), a simultaneous loss of chromosome 10, but rarely a concurrent p53 mutation. The most common EGFR gene mutation in primary GBMs is EGFRvIII, a variant lacking exons 2-7 (corresponding to cDNA nucleotides 275-1075 encoding amino acids 6-273), which results in a truncated cell surface receptor with ligand-independent constitutive tyrosine kinase activity (9-11). This mutation presumably occurs through alternative splicing or gene rearrangements (12-13) and leads to the loss of binding activation by its normal ligand, EGF and TGF- α (14-15). Mouse double

minute 2 (MDM2) amplification that neutralizes p53 activity (16), is observed in more than 50% of primary GBM, but rarely in secondary GBM. Additionally, CDKN2A (p16INK4a) deletion, PTEN mutation, Rb protein alterations and loss of all or a portion of chromosome 10 are frequently seen in primary GBM (17-18). p16INK4a deletion is infrequent in secondary GBMs and its deletion and p53 mutation appear to be two mutually exclusive events in GBMs (19). Primary GBMs account for the vast majority of cases (60%) and typically occur in the elderly (>50 years old), whereas secondary GBMs, are less common (40%) and typically develop in younger patients (<45 y) (4). Primary and secondary GBMs are indistinguishable to the neurosurgeon as well as neuropathologist, and the clinical management of these two GBM subtypes is identical. To date, temozolomide (TMZ) administered daily with radiation therapy (RT) for six weeks, followed by adjuvant TMZ for six months, has become standard therapy for patients with newly diagnosed GBM.

2.2 Genetic characteristics of GBM link to prognosis

The overall prognosis for patients with GBM is extremely poor. However, a small proportion of patients show prolonged survival. A study indicates that different glioma-associated genomic aberrations may serve as prognostic markers in combination with histopathological findings (17). The use of comparative genomic hybridization (CGH)-based analysis of 20 primary GBMs suggests that loss of chromosome 10 and gain/amplification in chromosome 7 are most frequently observed in primary GBMs and are associated with microvascularization and poor prognosis (17). In contrast, the combination of chromosome 1p and 17p13-p14 and 19q deletions are associated with a longer survival time (5, 17, 20-21). The analysis of loss of heterozygosity (LOH) on chromosomes 19q, 1p, and 13q, using polymorphic microsatellite markers, however, has indicated that LOH on chromosome 19q was frequently found in secondary GBMs (50%) but rarely detected in primary GBMs (20), suggesting that tumor suppressor gene(s) located on chromosome 19q are frequently involved in the progression from a low-grade astrocytoma to secondary glioblastoma, but do not play a major role in the evolution of primary glioblastomas. Clinical trials indicated that patients whose tumor had a methylated promoter for the gene encoding O-6-methylguanine-DNA methyltransferase (MGMT), were more likely to benefit from the addition of TMZ to RT (22-23). A recent study further showed that pattern of, and time to, recurrence after TMZ concomitant with and adjuvant to radiotherapy are strictly correlated with MGMT methylation status (21). Recently, genomewide mutational analysis of GBM revealed somatic mutations of cytosolic isocitrate dehydrogenase 1 gene (IDH1), which catalyzes the oxidative decarboxylation of isocitrate to α -ketoglutarate, most frequently in WHO grade II and III astrocytomas and secondary GBM but rarely in primary GBM (22-23), and patient tumors with IDH1 or related mitochondrial IDH2 mutations had a improved clinical prognosis than those with wild-type IDH genes (25, 27). It is suggested that IDH mutation is a highly prognosis predictor and selective molecular signature of secondary GBM (28-29).

2.3 Molecular classification of glioblastoma subtypes

Identification of chromosomal abnormalities and cancer-associated genes in solid tumors is becoming easier as genome-wide analysis technologies improve and as the genome sequence is being completed. These technologies allow for genome-wide data acquisition in study of cancer genetics and biology, particularly in analysis of complex expression

patterns, in a rapid and efficient fashion. Moreover, since the expression of thousands of genes is analyzed simultaneously, we can expect to obtain more comprehensive information underlying the interactions of genes related to malignant transformation as well as crucial clues about alteration in the relevant genetic and biological networks (30). Since the genetic basis of human cancer is combinatorial, this approach becomes especially important when combined with computational technology (31). Likewise, genomic mutation (e.g. deletions, inversions, chromosome re-arrangement, amplification, promoter mutation) and posttranslational modifications of proteins are the key factors that induce and maintain the malignant transformation of cancer. The success of using gene expression patterns to study cancer will depend largely on how much they reflect genomic changes. If a significant portion of the effects of genomic abnormalities can be reflected at the RNA level, the gene expression patterns will be highly informative and can be analyzed to explain the molecular mechanisms underlying the pathological development and behavior of cancers (32). GBM tumor heterogeneity is likely to play a significant role in explaining the unsuccessful treatment modalities. Therefore, molecular classification with large-scale expression assays will be more prognostically and therapeutically significant (33-34) since comprehensive and unbiased information can be obtained and would allow for the development therapies specifically tailored to each subtype. Multiple studies also indicate that gene expression-based classification of malignant gliomas correlates better with survival than histological classification (35). Successful integration of molecular/genetic data into tumor status must be descriptive and partially explain known tumor behavior, pathology, and resistance to therapy, as well as provide an insight to how the deregulation of multigene networks leads to tumor development, progression and treatment resistance (32, 36-37).

2.3.1 Glioblastoma subtypes express distinct transcription profiles

To identify whether molecular profiling can distinguish GBM subtypes, we and others have performed genome-wide microarray expression profiling and identified molecular subtypes that express distinct genes associated with tumor progression and predict clinical outcome better than histological class (35, 37-38). In general, using molecular profiling, GBM can be classified into three major subtypes include proliferative, mesenchymal and neuronal phenotypes (38-39), and the poor prognosis tumor subtypes are distinguished by the molecular markers of proliferation or mesenchymal/ECM/angiogenesis, which the majority is associated with losses on chromosome 10 that span 10q23.3 and gain on chromosome 7. In particular, most cases of mesenchymal GBM had relative losses at all loci on chromosome 10 and gains of all loci on chromosome 7, whereas proliferative tumors had more heterogeneous pattern of losses chromosome 10 (38). However, upon recurrence, GBM tumors tend to shift toward mesenchymal phenotype (38, 40). In order to elucidate whether primary and secondary GBM subgroups use distinct molecular pathways as well as identify gain-of-function genes that are associated with acquisition of malignant features of GBM subtypes, we have performed a large-scale DNA microarray analysis to compare the mean level of normalized transcript levels in each of the two clinically defined GBM groups versus the grade II and III astrocytomas. We have identified shared and non-shared GBM-associated gene (GAGs) over-expressed by respective subtype (40). As anticipated, shared GAGs reflect common characteristics of hyperproliferation, hypervascularity, and apoptotic resistance in both GBM subgroups, whereas GAGs distinct to primary or secondary tumors provided information on the heterogeneous properties and apparently distinct oncogenic

mechanisms of these tumors. Secondary GBM-associated GAGs were mostly related to the mitotic cell cycle (**Figure 1A**), which corresponds to the fact that secondary GBM have high frequencies of TP53 and Rb mutations (2-3). Moreover, secondary GAGs reflect the causes and effects of such genotypic and phenotypic changes. Therefore, the molecular properties of secondary GBM support the notion that mutation or dysfunction of cell cycle regulators would be the major mechanism responsible for the development of malignant phenotype in secondary GBM. In contrast, primary GAGs highlight genes typical of a stromal/inflammatory response and are strongly associated with invasive phenotype, suggesting the importance of extracellular signaling (**Figure 1B**). The molecular properties of primary GBM thus support the view that the interplay between GBM derived bone/cartilage-associated factors and tumor-associated stromal cells play a key role in the malignant transformation of primary GBMs. To rule out the possibility that the distinct GBM progression-associated genes identified between the two subgroups are due to selection pressure (e.g. radiation or chemotherapy), we further conducted clustering-based analysis of a set of primary GBM (n=13) that are recurrent and had prior treatment and secondary GBM (n=12) samples that had been treated during grade step prior to tumor sampling, using these identified GAGs. The results indicate that both tumor subtypes, regardless of prior treatment, cluster within their clinical grouping based on gene expression of the selected GAGs (**Figure 2**). Of note, 85% of recurrent primary GBM are clustered to mesenchymal GBM and 83% of secondary GBM are clustered into cell-cycle GBM, indicating that prior treatment is not disrupting this identified gene expression signature of primary and secondary GBM nor driving the selection of the genes. These data therefore support the notion that diverse mechanisms and properties underlying distinct transformation events or perhaps distinct cells of origin of GBM subtypes.

2.3.2 Primary glioblastoma express mesenchymal stem cell properties

The most striking observation in the molecular properties of primary GBM among all is the series of genes highly expressed in mesenchymal tissues, but not in neural or glial cells, were identified. These overexpressed genes are related to osteogenesis and chondrogenesis (e.g. cartilage glycoprotein-39/YKL-40, chitinase 3-like 2, glycoprotein nmb, lysyl oxidase, lung type-I cell membrane-associated glycoprotein, collagen type V, VI, biglycan, mesenchyme homeobox 2, and fatty acid-binding protein). These molecular properties characterized a mesenchymal phenotype of these glial tumors. To access gene pathways that are potentially associated with tumor development of primary GBM, a comparative analysis of primary GBM (n = 46) relative to normal brain tissue (n = 10) was performed. As anticipated, genes that were previously identified as primary GBM progression-associated genes reappeared in this gene list, which reflected the status of inflammation, coagulation, immune/complement responses, angiogenesis, and ECM remodeling (40, Figure 1B). Strikingly, a new series of genes linked with neural stem cells (NSC), mesenchymal stem cells (MSC), skeletal/cartilage development, morphogenesis, organogenesis, and embryonic neuroepithelial stem cells was determined. It thus implicates that a tissue regeneration/repair program is constantly activated in GBMs (Figure 3). Furthermore, a subset of primary GBM tumor-derived tumor lines expresses cellular markers that are associated with MSC (CD90, CD105, CD29, and CD44) and that GBM cell cultures can be induced to differentiate into multiple mesenchymal lineage-like cell types, including adipocytes, chondrocytes, and osteocytes (41). These findings suggest

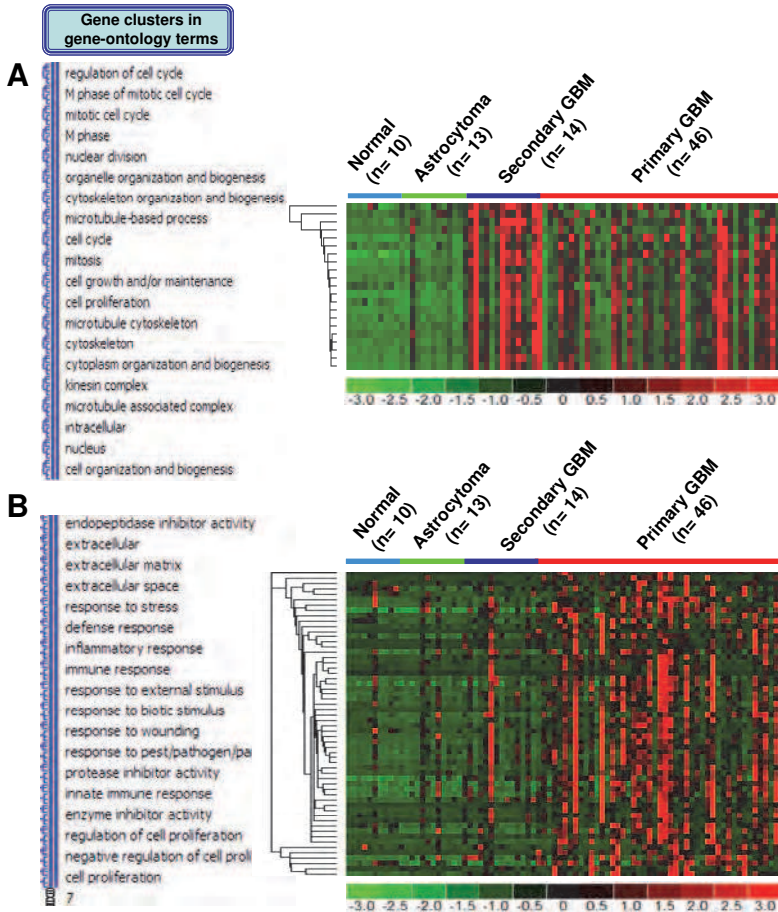


Fig. 1. Glioblastoma-associated genes (GAGs) overexpressed in GBM subtypes relative to lower grade gliomas. All plots show normalized gene expression values converted into a heat map. The log₂ of the fold difference is indicated by the heat map scale at the bottom of the Figure. Each column is an individual tissue or tumor sample organized into histologic groups defined at the top of the figure. Each row is a single probe set measurement of transcript abundance for an individual gene. All genes were filtered to select transcripts with 2.5-fold or higher expression in the respective GBM group relative lower grade astrocytomas (P value < 0.05, t -test). **A.** GAGs overexpressed uniquely in secondary glioblastomas: 21 secondary GAGs were defined as being uniquely detected with a >2.5-fold overexpression in the secondary GBM group compared with the lower-grade astrocytomas and not overexpressed within the primary glioblastoma group. **B.** GAGs overexpressed uniquely in primary GBM: 58 primary GAGs were defined as overexpressed 2.5-fold relative to lower-grade astrocytomas and not detected in the secondary glioblastomas comparison using the same criteria. Functional categories of gene clusters in gene-ontology (GO) terms were shown and were analyzed using a GO annotation-based gene function enrichment analysis (d-chip software). Gene description listed in Figure 2.

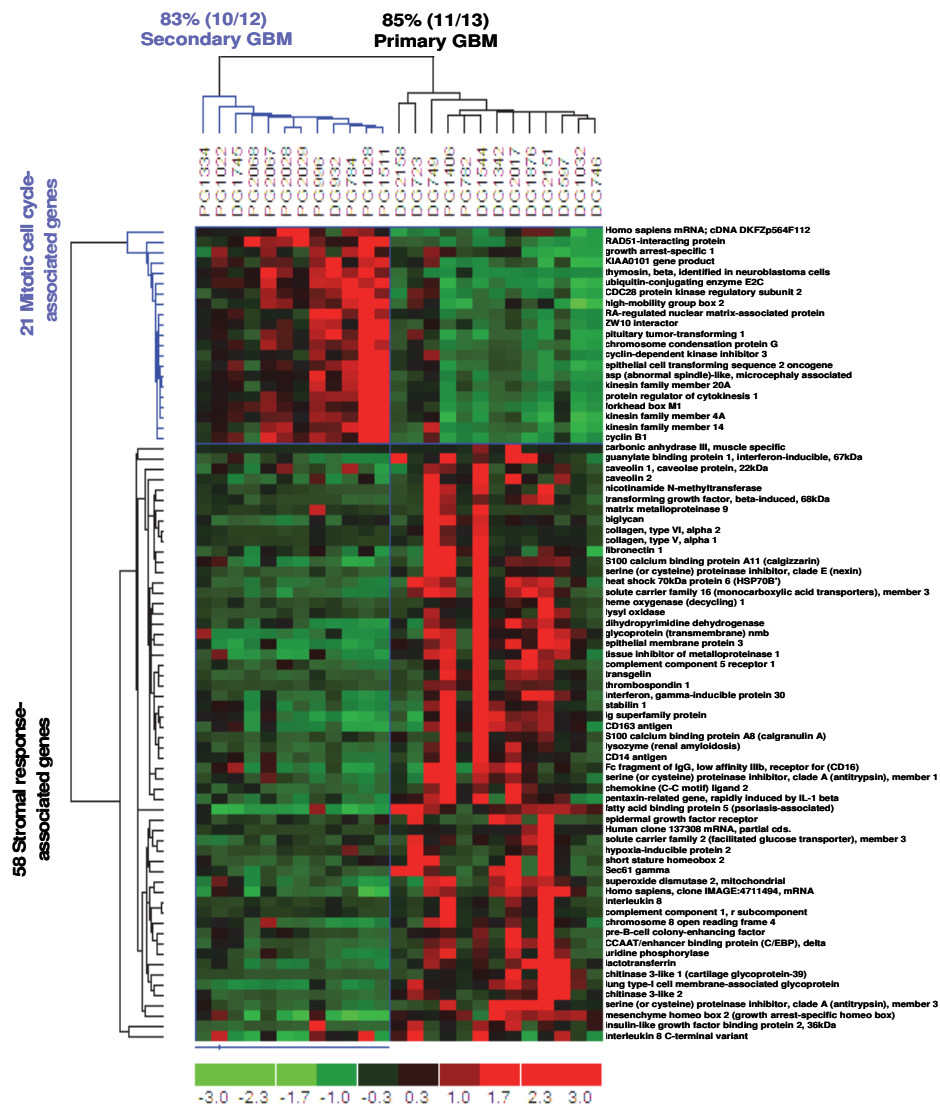


Fig. 2. Unsupervised sample clustering of primary and secondary GBM that are recurrent and had treatment using 21 secondary GAGs (Figure 1A) and 58 primary GAGs (Figure 1B). Distinct GAGs segregated GBM subtypes, suggesting they may be repopulated by GBM stem cells with distinct molecular properties. PG= progressive/secondary GBM. DG= de novo/primary GBM.

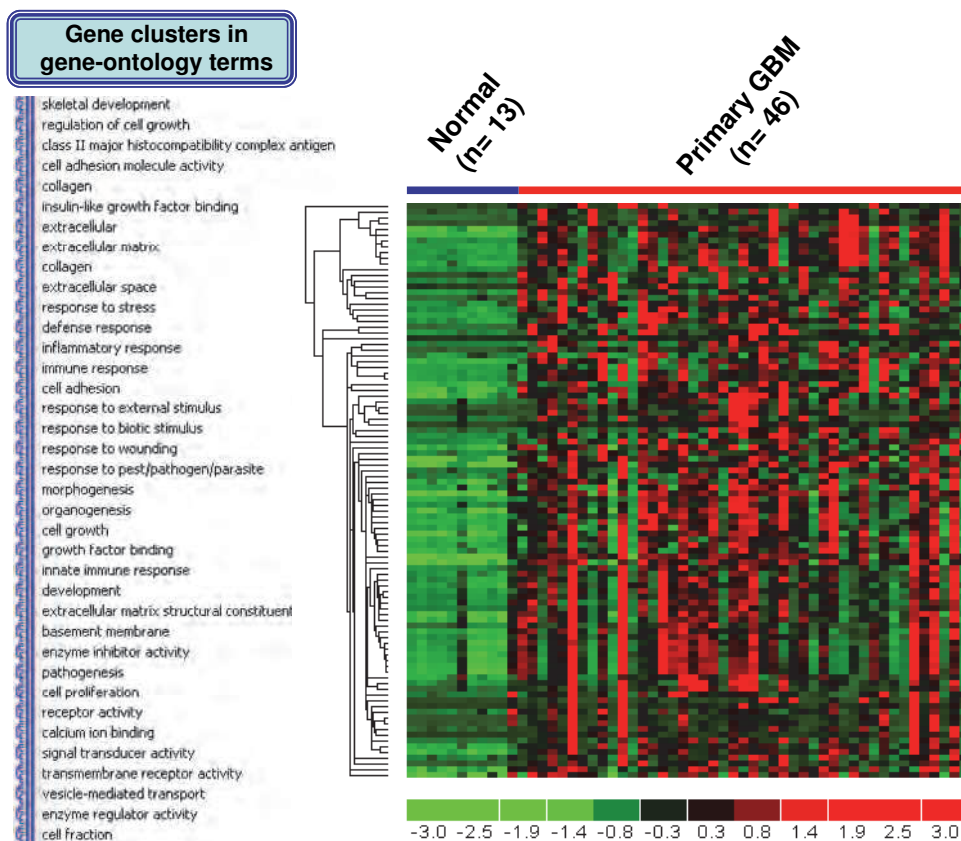


Fig. 3. A comparative analysis of gene expression profiles in primary GBM (n = 46) and normal brain tissue (n = 10). Analysis was based on a cutoff of a 2.5-fold increase in relative expression ($P < 0.05$). Top 100 primary GBM-associated genes expressed at higher levels compared with normal brain tissue were extracted. Functional categories of gene clusters in gene-ontology (GO) terms were shown.

that either a subset of primary glioblastomas derives from transformed stem cells containing MSC-like properties and retains partial phenotypic aspects of a MSC nature in tumors, or that GBMs activate a series of genes resulting in mesenchymal properties of the cancer cells to effect sustained tumor growth and malignant progression. Since primary GBM express both NSC (e.g. nestin, SOX2) and MSC makers, it is hypothesized that a subset of GBM tumors may be derived from neural crest-stem-like cells (41). The lack of MSC-like properties in secondary GBM may suggest that they originate from a different cell type of cellular origin. Further characterization of stem-like cells in GBM tumors would help to identify new targets and subsequently develop new therapeutic strategies to delay tumor progression and prevent tumor regeneration.

3. Glioblastoma stem cells

3.1 Glioblastoma contain tumorigenic stem -like cells

Tumorigenic, stem-like GBM cells, or so-called glioblastoma stem cells (GSC) have been recently isolated and characterized as GBM tumor-initiating cells by multiple groups (42-46). Although CD133/prominin, a normal NSC marker, is not an obligatory marker for GSC (46-47), CD133 was first applied as a surface marker for isolation and enrichment of GSC (42, 44-46, 48-49). Other surface markers are also reported to be used for GSC isolation and enrichment, including Musashi homolog 1 (MSI1) (50), and A2B5 (51). Through studies in both in-vitro and in-vivo GSC functional models, several essential genes and signaling pathways for maintaining tumorigenic potential have been implicated. At the functional level, GSC behave in ways similar to tissue stem cells, are capable of self-renewal and differentiation, and reconstitute the tumor tissue when grafted into mice. GSC possess a multi-lineage differentiation capacity also support for the hypothesis that cancer hierarchy is a result of developmental diversity among cancer cells in different states of differentiation (52-54). However, it is plausible that multiple genetic and/or epigenetic instabilities that take place within tumor stem cells might prevent progeny from undergoing non-proliferative terminal differentiation, leading to uncontrolled tumor growth (55-57). Tumors initiated in mouse brain by injection of patient-derived GSC often recapitulate the histopathological features of the patient tumors from which the cells were derived, indicating the ability to self-renew and reproduce the cellular heterogeneity found in human GBM tumors (44-45, 47-48). Uniquely, we found GSC isolated from treatment-refractory recurrent GBM tumors can spontaneously migrate radially outward from tumor spheres that they initiated and populated in cultures followed by spread out over the surface of the culture dish and form the secondary tumor spheres without additional factors added into the culture to influence the behavior of cells (**Figure 4**). This in-vitro observation suggests that the migratory nature of GSC is likely to be an intrinsic property that reflects inherently migratory properties of the GBM tumor of origin. Likewise, an intracranial injection of these GSC leads to the development of YKL-40+ infiltrative tumors that display hypervascularity and pseudopalisading necrosis-like features in mouse brain (**Figure 4**). Thus, it is possible that tumor recurrence in the secondary site may be due to tumor stem cells escaping from primary treatment, migrating out of core mass, infiltrating adjacent brain tissue, and continuing seeding a new tumor. Importantly, GSC were shown to resist the effects of ionizing radiation and chemotherapy (58-59) with a marked increase in activation of several checkpoint proteins in response to DNA damage, pointing to they

may be responsible for the post-treatment tumor recurrence. Thus, identification of genes and pathways confer the migratory ability, anti-apoptotic features, and tumorigenic capacity of GSC would be essential for better understanding GSC and identifying potential targets in order to eradicate and prevent them from regenerating a new tumor.

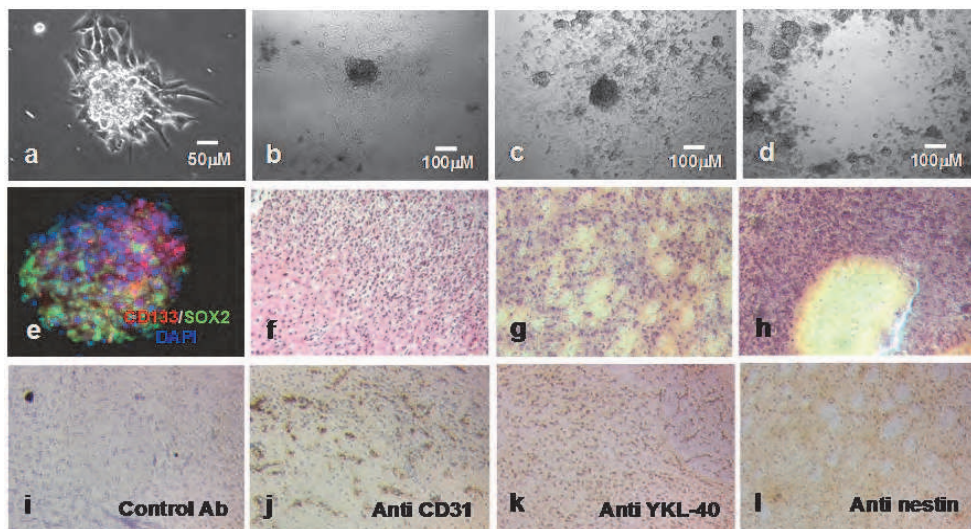


Fig. 4. Patient-derived glioblastoma stem cells (GSC) are highly motile cells and are capable of populating tumor spheres in cultures and initiating an infiltrating tumor in mouse brain. GSC isolated from treated and recurred tumor possess the ability to clonally self-renew, form primary tumor sphere (a), migrate outward (b), form secondary tumor spheres in secondary sites (c), and display pseudopalisading necrosis-like morphology (d). Immunofluorescent staining showed GSC spheres co-express CD133 and SOX2 (e) and can be propagated in cultures for indefinite passages. Brain tissues from mice injected with GSC display invasive growth of gliomas with diffuse infiltration into the surrounding tissue and exhibit hypercellular zones surrounding necrotic foci (f-h). Immunohistochemistry staining showed positivity in CD31/platelet endothelial cell adhesion molecule, YKL-40, and nestin, indicating an angiogenic progression of gliomas (i-l).

3.2 Molecular properties of tumorigenic glioblastoma stem cells

Isolation and characterization of tumorigenic GSC derived from treatment-refractory GBM tumor may have a clinical implication of identifying innovative molecular targets for the development of a more effective treatment protocol. Particularly, elucidation of essential gene pathways of GSC that confer sustained self-renewal, cell migration and cell survival will be vital important for targeting and preventing of GSC mediated-tumor recurrence. Gene expression profile analysis revealed that purified, tumorigenic CD133+ GSC derived from treatment-refractory recurrent brain tumors possess neuroectodermal properties and portray astrogligenic and chondrogenic potential. Moreover, CD133+GSC express molecular signatures for multiple adult stem cells, including RGC (e.g. fatty acid binding protein 7, secreted protein acidic and rich in cysteine-like 1), NCC (e.g. endothelial 3, Distal-less homeo

box 5/6, v-myc myelocytomatosis viral-related oncogene), NSC (e.g. SOX2, nestin), MSC (e.g. CD44, CD105), and stem cells in the small intestine and colon (e.g. Leucine-rich repeat-containing G protein-coupled receptor 5). More strikingly, in contrast to hyperproliferative and hyperangiogenic phenotype of GBM tumors, purified CD133+ GSC, not CD133+ glioblastoma spheres (containing mostly CD133- progeny), express a tumor-suppressor phenotype, which is characterized by the expression of a series of genes associated with an anti-growing, anti-inflammatory, anti-angiogenic, anti-developmental, and migrating phenotype (42). This observation implicate that these GSC may be clinically indolent/quiescent prior to undergoing proliferative cell division, which would produce proliferative and angiogenic GBM effector progeny. Thus, it is possible that some migratory, tumorigenic GBM-stem like clones may use properties of stem cell quiescence to evade first-line treatment and regrow a new tumor at a secondary site after treatment. The molecular properties of GSC also support the view that genes guarding the pools and tumorigenic potential of GSC may not be in the subgroup of genes directly controlling cell proliferation, but in the subgroup regulating cellular quiescence, development, differentiation, and survival. Analysis of the expression of the CD133 in gliomas found that both the proportion of CD133+ cells and their topological organization in clusters were significant prognostic factors for adverse progression-free survival and overall survival (60). Computational comparisons with a collection of published gene expression profiles further reveal that the CD133 gene signature transcriptionally resembles human embryonic stem cells (ES) and GSC, and this signature successfully distinguishes glioblastoma from lower-grade gliomas, and identify an aggressive glioblastoma subtype with excess mutation (61). To date, most anti-cancer therapies aim to eliminate rapidly proliferating tumor cells; thus, the discovery of treatment-resistant, quiescent GSC (42, 58-59, 62) possessing the enhanced ability to repopulate tumors provides an excellent model to explain our inability to eradicate brain tumors (**Figure 5**). The identification of genes and pathways and performing pre-clinical validation of gene function in animal experiments may facilitate the discovery and development of innovative treatment protocols for the prevention of post-treatment tumor recurrence through the targeting tumorigenic stem-like GBM cells, which is not targeted in any current anti-cancer treatment.

4. Essential gene pathways for GSC

4.1 In-vitro cultivation of tumorigenic GSC

It is plausible that the quiescent, migratory, and tumorigenic properties render GSC an excellent candidate for being responsible for post-treatment tumor recurrence. Based on the in vitro and in vivo characterization of GSC, the GSC population is being considered a dynamic fraction of cells highly sensitive to microenvironmental changes or stimulation (e.g. self-renewal and differentiation). Therefore, identifying both intrinsic and extrinsic signaling pathways by which GSC maintain the tumorigenic capacity to support continuous tumor growth will facilitate the development of novel therapeutic strategies to diminish the recurrence rate of glioblastoma tumors. Current experimental models for the study of GSC in the laboratory have been relatively standardized. In laboratory, GSC are maintained in serum-free media supplemented with epidermal growth factor (EGF) and fibroblast growth factor (FGF) and are able to propagated as a non-adherent or semi-adherent sphere cultures for indefinite passages. More importantly, GSC are capable of clonal self-renewal and proliferative differentiation, thereby allowing populate single cell-derived tumor spheres in cultures. Genome-wide expression microarray analysis of GSC have identified a series of

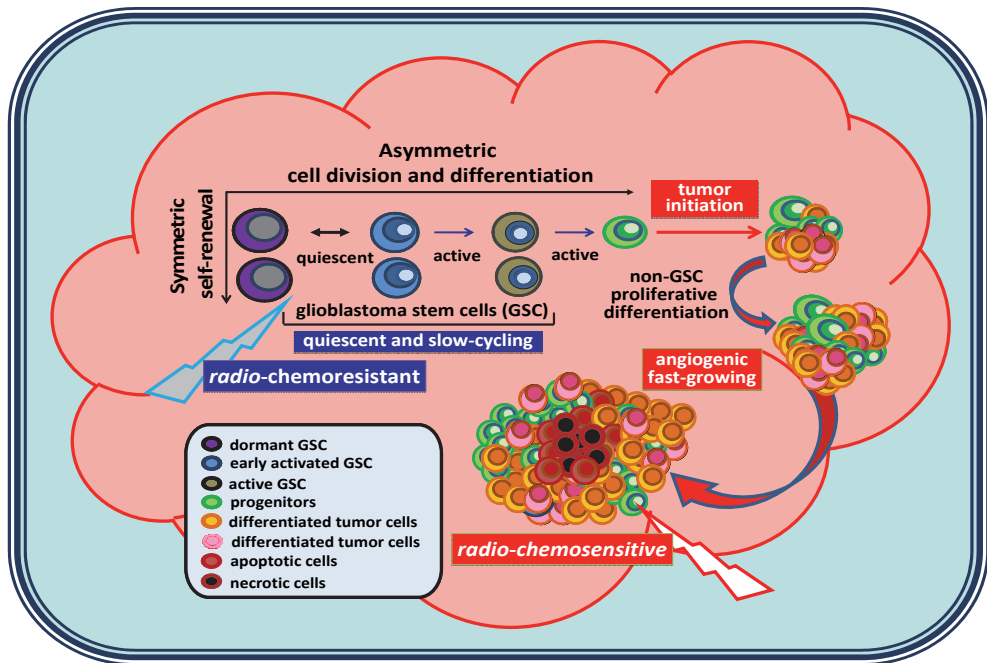


Fig. 5. A theoretical model of glioblastoma stem cells (GSC) contribute to both tumorigenesis and treatment resistance. GSC use both symmetric and asymmetric division to sustain self-renewal and proliferative differentiation to initiate and maintain a tumor. GSC contain both quiescent and active cell types; the quiescent GSC are slow-cycling, radio-chemoresistant, and capable of unlimited self-renewal, whereas the activated GSC can undergo proliferative differentiation and initiating a tumor. Progeny of GSC populate a tumor containing a heterogeneous population in different states of differentiation, and are fast-growing, angiogenic, and radio-chemosensitive. Quiescent GSC can escape from radio-chemotherapy and continually replenish tumor cells, leading to sustained tumorigenesis.

molecular markers associated with adult stem cells (42) and a recent study further showed that GSC express EC markers which can distinguish GSC from NSC (63). GSC possess tumorigenic potential, and by contrast to regular glioblastoma cell lines cultured in serum-containing media, injection of lower number of GSC into mice with severe combined immune deficiency (SCID) is able to initiate and reconstitute GBM tumors in mouse brains, which recapitulate the histopathological features of the patient tumor from which the GSC were derived (42-47). Thus, by using these well-documented in-vitro and in-vivo functional assays of GSC, which can be visualized, assayed, and quantified, investigators have discovered and established several essential gene pathways by which properties and function of GSC can be maintained; in particular, studies using small interfering RNA (siRNA)-mediated loss-of-function phenotype, a particular gene pathway that is involved in maintaining GSC can be identified and verified. Thus, by both in-vitro and in-vivo GSC functional models, several essential genes and signaling pathways for maintaining GSC stemness, tumorigenic capacity, and anti-apoptotic features have been implicated (64, Review).

4.2 Notch signaling pathway

Several Notch effector genes, including inhibitor of differentiation 4 (ID4) (65), hairy and enhancer of split 1 (HES1) (66), hairy/enhancer-of-split related with YRPW motif 1 (HEY1) (67) and fatty acid binding protein 7 (FABP7) (68), have been detected in GSC by expression microarray analysis, thereby reflecting the prolonged Notch activation (42). Notch signaling controls numerous cell fate specification events, and it has been implicated in the maintenance of cellular quiescence in many adult stem cell pools by retaining self-renewal potential, suppressing cell differentiation, and protecting them from exhaustion of their proliferative capacity (66, 69-70). The Notch signaling network is composed of a family of four Notch receptors (Notch1, Notch2, Notch3, Notch4) and five ligands from the members of the Delta-like (DLL1, DLL3, DLL4) and the Jagged (JAG1, JAG2) families. The signaling permits the gamma secretase-mediated proteolytic release of the Notch intracellular domain (NICD), which translocates into the nucleus and transactivates target genes. Notch signaling regulates NSC differentiation; induction of Notch signaling drives NSC into quiescence, whereas blocking Notch signaling stimulates NSC undergo neurogenesis (69, 71). Moreover, Notch signaling is required to convert the hypoxic stimulus into epithelial-mesenchymal transition (EMT), increased motility, and invasiveness (72). Thus, expression of Notch effector genes in GSC implies abrogation of neurogenesis, promoting a migratory phenotype and enhancing glial-fate specification. Importantly, increased expression of FABP7 was found to be associated with regions of GBM tumor infiltration (73), suggesting that Notch activation in GSC may not only maintain the stemness of GSC, but also promote a infiltrating characteristics of brain tumor. Treatment of GBM sphere cultures with gamma-secretase inhibitors (GSIs) can deplete GSC, downregulate stem cell markers (CD133, nestin, BMI1, Olig2), and inhibit self-renewal of GSC in cultures and growth of xenografts (74). The depletion of tumorigenic GSC by Notch signaling blockade was found to occur via reduced cell proliferation and increased cell apoptosis due to decreased levels of phosphorylated AKT and STAT3 (75). In addition, it has been shown that tumor endothelial cells support GSC maintenance, which is in part via Notch signaling (76) and suggested that inhibition of Notch signaling can target GSC via an endothelial cell intermediate.

4.3 Hypoxia and hypoxia-inducible factors

It has been shown that hypoxia-inducible factors (HIFs) regulate tumorigenic capacity of GSC (77-78). When glioblastoma sphere cultures are grown in 1% oxygen, hypoxic response genes, including HIF-1, HIF-2, lysyl oxidase, and vascular endothelial growth factor (VEGF), are greatly induced, in addition, both the stem-like side population and CD133+ cells were increased (79). Moreover, GSC respond to hypoxia by enhancing their self-renewal activity and anti-differentiated status (80). Loss of HIF-2 α in GSC leads to a significant decrease in both GSC proliferation and self-renewal in cultures, and attenuation of tumorigenic capacity in animals (80). Hypoxia requires Notch signaling for maintaining cells in an undifferentiated state, which occurs by recruiting HIF-1 α to the promoters of Notch-responsive target genes (81). The maintenance of GSC by a hypoxic microenvironment via enhancing the activity of other stem cell factors such as Oct4, c-Myc, and Nanog, also partially promotes and stabilizes the stem cell phenotype (77, 82). Thus, HIFs might potentially represent a promising target for depleting GSC populations.

4.4 GLI – Nanog axis

Nanog and Hedgehog (HH) are two essential regulators of stemness in ES. HH proteins act through the Patched (Ptc) and Smoothened (Smo) and ultimately activates the GLI family of transcription factors. HH-GLI signaling modulates neural progenitor proliferation and survival in the developing neural tube, and controls stem cell behavior in the postnatal and adult brain (83-85). Nanog, a pluripotency homeobox gene, is regulated by HH-GLI signaling via binding of HH effectors, Gli1 and Gli2, to the Nanog promoter, thus activating Nanog expression (86). A study shows that HH-GLI signaling regulates glioma growth, GSC self-renewal, and tumorigenic capacity, and the blockade of HH-GLI signaling by treatment with cyclopamine depletes GSC (87). Nanog was recently reported to be a novel HH-GLI mediator for expanding CD133+ GSC and promoting glioblastoma growth (88). More importantly, it was found that loss of tumor suppressor p53 activates HH-GLI signaling, thereby contributing to Nanog upregulation and leading to the promoting of GSC stemness. In contrast, the presence of p53 can negatively regulate the activity and level of GLI1, thus downregulating Nanog expression (86, 88-89). Therefore, the inversely reciprocal levels of GLI1 and p53 are consistently maintained in GSC (88). Concurrently, GLI1 upregulates Notch and downregulates bone morphogenetic protein (BMP) signaling, a pro-differentiative action on stem cells (89), implying an essential role of a functional GLI1-NANOG-p53-Notch network in maintaining stemness and tumorigenic capacity of GSC. Thus, **GLI-Nanog axis** provides a potential treatment target for the prevention of GSC-mediated tumor recurrence.

4.5 Transforming growth factor beta (TGF β) signaling

The TGF- β signaling pathway plays an essential role in the regulation of embryonic development, cell proliferation, motility and apoptosis, ECM production and modulation of immune function (90). The TGF- β superfamily comprises both growth and differentiation factors including TGF- β s, activins, inhibins, and bone morphogenetic proteins (BMPs). TGF β signaling by binding to type I and type II receptors on the cell surface. The type II receptor phosphorylates the type I receptor, which propagates the signal by phosphorylating receptor-activated SMAD (R-SMAD) proteins (91) that transduce TGF- β

family signals into a transcriptionally regulated developmental program. A recent study showed that the TGF β /activin signaling pathway is essential for the maintenance of ES cells, which is via binding of SMAD2/3 to the NANOG proximal promoter in human ES (92). Alternatively, TGF β signaling can act through Smad-independent pathways, which activate Ras/extracellular signal-regulated kinase (ERK), TGF β -activated kinase-1/p38 mitogen-activated protein kinase (MAPK)/c-Jun NH₂-terminal kinase (TAK1/P38/JNK), phosphatidylinositol 3-kinase(PI3K)/AKT, and signal transducers and activators of transcription 3 (STAT3) (93-94). A study showed that TGF β signaling promotes the self-renewal and tumorigenic capacity of GSC by Smad-dependent induction of leukemia inhibitory factor (LIF) (95). Treatment of GSC with recombinant LIF induced a rapid phosphorylation of STAT3. Thus, TGF β signaling promotes GSC self-renewal through the activation of JAK-STAT pathway by the induction of LIF secretion (95). Mice receiving GSC pre-treated with a TGF β receptor inhibitor and a JAK inhibitor significantly increased the survival rate compared to the group receiving non-treated GSC, indicating the TGF β and JAK-STAT signaling pathways play an essential role for maintaining tumorigenic potential of GSC (95).

A direct mechanistic link of STAT3 activation to GSC growth and self-renewal was further evidenced by two separated studies, demonstrating that knockdown of STAT3 signaling by a short hairpin RNA (shRNA) or inhibitors of STAT3-DNA binding, leads to loss of capacity for tumor sphere formation, induction of cell apoptosis, and a decrease in tumor-initiating capacity in animals (96-97). Therefore, these data suggest that the STAT3 signaling pathway may be a potential target for GSC-directed brain tumor therapy. Since STAT3 signaling is a downstream effector of interleukin-6 (IL-6), blockade of IL-6R alpha or IL-6 expression with shRNAs also suppresses tumor sphere formation capacity and increases survival of mice bearing intracranial glioblastoma xenografts (98). Another related study showed that autocrine TGF-beta signaling maintains stemness of GSC by induction of Sry-related HMG-box 2 (SOX2), one of the key transcription factors required in induced pluripotent stem cells, and this induction was mediated by Sox4, a direct TGF-beta target gene (99). Thus, treatment with inhibitors of TGF-beta signaling drastically deplete GSC by promoting their differentiation, and leads to less lethal potency in intracranial transplantation assay. SOX2 silencing or induction of GSC differentiation by treatment with bone morphogenetic protein 4 led to the loss of self-renewal capacity and tumorigenicity of GSC (62, 100-101), indicating the maintenance of the undifferentiated phenotype is one of the key criteria for retaining tumorigenic capacity of GSC.

4.6 Epidermal growth factor receptor (EGFR) and down-stream AKT, MEK (mitogen-activated protein kinase/ERK kinase), and ERK 1/2 signaling

EGFR is commonly amplified and/or mutated in high-grade gliomas. A study showed that EGFR signaling pathway is involved in the maintenance of GSC and is required for gliomagenesis (102). Treatment of GSC with tyrosine kinase inhibitors of EGFR signaling suppresses GSC self-renewal and induces cell apoptosis through the inhibition of phosphorylation of EGFR, AKT kinase, and ERK 1/2 (103-104). Likewise, GSC display preferential sensitivity to Akt inhibition relative to matched non-GSC cells and inhibition of Akt activity in GSC increased the survival of animal bearing human glioma xenografts (105). Similar results are also demonstrated by a targeted inactivation of MEK/ERK signaling, which led to the reduction of sphere-forming capacity of GSC accompanied by

their differentiation into neuronal and glial lineages (106). Moreover, combinational blockade of both MEK/ERK and PI3K/mTOR pathways suppressed the tumorigenic capacity of GSC more effectively than blockade of either alone (107). These results therefore indicate that the EGF/EGFR signaling and its downstream effector activation are essential for maintaining GSC, suggesting a potential molecular pathway target for depletion of GSC.

4.7 c-Myc

c-Myc belongs to a family of transcription factors containing basic, helix-loop-helix, and leucine zipper domains and it is an essential factor for normal embryonic development (108). c-Myc is an oncogenic transcription factor commonly overexpressed in a variety of human cancers. In contrast, c-Myc gene inactivation triggers telomere-independent senescence mediated by the cyclin-dependent kinase inhibitor p16INK4a, which is regulated by the polycomb group repressor Bmi-1, a direct transcriptional target of c-Myc (109). High-level of c-Myc expression was found in GSC relative to non-stem glioma cells, and knockdown of c-Myc in GSC induces cell apoptosis and leads to the loss of tumorigenic capacity (110). A recent study further showed that HIF-2 α promotes GSC self-renewal and stemness properties via enhancing the expression of c-Myc (82), and inactivation of PTEN and p53 can also lead to the increased expression of c-Myc and promotion of stemness, self-renewal and the tumorigenic capacity of GSC (111). These data suggest that the c-Myc signaling pathway is required for maintaining the self-renewal capability and tumorigenic potential of GSC, and therefore may serve as a potential signaling pathway target for a GSC-directed brain cancer therapy.

4.8 L1 cell adhesion molecule (L1CAM), Olig2, Bmi-1, integrin α 6, and A20

L1CAM is a cell adhesion molecule plays an important role in nervous system development, including neuronal migration and differentiation (112). L1CAM expression was found to be preferentially higher in GSC compared to normal neural progenitors, and knockdown of L1CAM expression via shRNA interference can lead to the loss of sphere-forming capacity, induced cell apoptosis, and suppressed tumor growth (113). The induction of GSC apoptosis by decreasing the expression of L1CAM is suggested due to the decreased expression of the basic helix-loop-helix transcription factor Olig2 and the increased expression of the p21WAF1/CIP1 tumor suppressor (113). Correspondingly, it has been shown that an Olig2-regulated lineage-restricted pathway is critical for proliferation and maintenance of tumorigenic GSC through the suppression of p21WAF1/CIP1 (114).

Bmi1 plays an essential part in the self-renewal of hematopoietic stem cells (HSC) and NSC (115-117). Bmi1 is part of the Polycomb group gene family and a member of polycomb-repressing complex 1 (PRC1), which is required to maintain the transcriptionally repressive state of many genes by chromatin remodeling and histone modification (118-119). It has been shown that Bmi-1 is highly expressed in CD133+ GSC and Bmi-1 knockdown resulted in inhibition of self-renewal capacity and induction of both cell apoptosis and cell differentiation, as well as loss of tumorigenic capacity (120). Similarly, disruption of EZH2, the main component of PRC2, robustly impairs self-renewal and tumorigenic capacity of GSC (121). This data thus suggest that PcG proteins are required for maintaining stemness, survival, and tumorigenic capacity of GSC.

Integrins are one of the major families of cell adhesion receptors that cells use to both bind to and respond to the ECM (122). Specifically, integrin $\alpha 6$ subunit is critical for the early development of the nervous system and has been shown to play a role in neuronal migration, neurite outgrowth, and axon guidance during olfactory development (123). A recent study showed that GSC highly express integrin $\alpha 6$ and their interaction with laminin on endothelial cells directly regulates the tumorigenic capacity of GSC (124). Targeting integrin $\alpha 6$ in GSCs inhibits self-renewal, proliferation, and tumor formation capacity (124), indicating integrin $\alpha 6$ is an essential factor for maintaining GSC and can be potentially used as a cellular target for depletion of GSCs.

Tumor necrosis factor, alpha-induced protein 3 (TNFAIP3) or A20, a zinc finger protein, is an NF- κ B-inducible gene. A20 can protect the cells from TNF-induced apoptosis by disrupting the recruitment of the death domain signaling molecules TRADD and RIP to the receptor signaling complex (125). A novel anti-apoptotic mechanism of A20 was recently reported and showed that A20 blocks TNF-induced apoptosis through suppression of c-jun N-terminal kinase (JNK) by targeting apoptosis signal-regulating kinase1 (ASK1) (126). A20 was overexpressed in clinical glioma tissue samples and correlates to clinical staging (127). A recent study showed that GSC overexpress A20, relative to non-stem glioma cells, and this protects GSC from cell death (128). Inhibiting A20 expression by shRNA (shRNA) decreased GSC growth and survival through mechanisms associated with decreased cell-cycle progression and decreased phosphorylation of NF-kappaB p65(RelA). By contrast, elevated levels of A20 in GSCs contributed to apoptotic resistance and were less susceptible to TNFalpha-induced cell death than matched non-stem glioma cells. A20 knockdown reduced the self-renewal ability of these cells and decreased tumorigenic potential of GSCs, thereby resulting in increased survival of mice bearing human glioma xenografts. Thus, A20 contributes to glioma maintenance likely through anti-apoptotic effects on GSC.

5. Gene pathways underlying the radio-chemoresistant phenotype of GSC

Concurrent TMZ and RT followed by adjuvant TMZ is standard for patients with newly diagnosed glioblastoma based on a large randomized phase III trial that showed survival benefit (129-130). Studies further showed that patients whose tumor had a methylated promoter for the gene encoding O6-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein, were more likely to benefit from the addition of TMZ (22, 130). Although the survival advantage of combined treatment lasts up to 5 years of follow-up, most patients successfully treated with combined therapy eventually had tumor recurrence and died (130). A significant increase in MGMT expression was found in first recurrence after treatment with RT plus TMZ, indicating either selection of MGMT-expressing cells or induction of the MGMT gene by TMZ (131). Molecular analysis of glioblastoma tumors resistance to the concomitant radio-chemotherapy with TMZ had identified a self-renewal signature dominated by homeobox (HOX) genes, which are comprised of CD133 (132). Of note, tumors with the enhanced expression of HOX genes, high EGFR expression, plus unmethylated MGMT were associated with short survival (132), implicating the association of stem-cell phenotype and radiochemoresistance. It is plausible that the quiescent stem cell nature adopted by GSC may explain the considerable resistance to chemotherapeutic agents (133-136). Moreover, quiescent cells show greater repair capacities than proliferative cells (58, 135), suggesting that slow-

cycling GSC may play a key role in the acquired or constitutive resistance to radio-chemotherapy (137).

5.1 Activation of checkpoint proteins

A study indicated a potential role of DNA damage checkpoint protein, Chk1 and Chk2 kinases in the radioresistant phenotype of CSC (58). Particularly, CD133+ GSC isolated from glioblastoma tumors preferentially activated Chk1/2 kinases, and repaired radiation-induced DNA damage more effectively than CD133- non-GSC cells (58), indicating stem-like glioblastoma cell population within tumor mass are likely responsible for the treatment resistance. This notion was further supported by the demonstrating that the radioresistant phenotype of GSC can be reversed by the treatment with a specific inhibitor of the Chk1 and Chk2 checkpoint kinases (58).

5.2 Evasion of cell-death pathway

Evidently, GSC exhibit enhanced chemoresistance to anticancer drugs (59, 138-139). The expression of ATP-binding cassette transporter ABCG2 in a tumorigenic stem-like side population (SP) distinguish them from the non-stem-like cells (138), suggesting a potential mechanism underlying chemoresistance in CSCs. Several anti-apoptotic genes (e.g. BCL-2, BCL2L1a, and MCL1) were also found to be at higher expression levels in TMZ resistant-GSC clones than those in differentiated cell lines (140). Likewise, CD133+ GSC were characterized by the enhanced expression of multidrug resistance 1 (MDR1) compared to CD133- non-stem cells (139). Moreover, the radio-resistance of GSC could be alleviated by treatment with an XIAP inhibitor (141). Thus, the radio-chemoresistance of GSC may be linked to the activation of the DNA damage checkpoint response, MGMT-mediated DNA repair, expression of both drug efflux transporters and anti-apoptotic factors, or abnormalities of cell-death pathways (59, 132,138, 141).

5.3 Constitutively active Notch and PI3K/Akt signaling

Notch signaling promotes radioresistance of GSC by upregulating PI3K/AKT pathway signaling and increasing the expression levels of myeloid cell leukemia-1(MCL1), an anti-apoptotic member of Bcl-2 family. The knockdown of Notch1 or Notch2 signaling in GSC sensitizes GSC to radiation treatment and impairs tumorigenic capacity (142), indicating a critical role of Notch/PI3K/AKT signaling in radioresistance of GSC. Moreover, addition of GSI enhances TMZ treatment of human gliomas by inhibiting neurosphere repopulation and xenograft recurrence (143), pointing out the essential role of Notch pathway in chemoprotection of GSC.

5.4 Bmi-1-mediated DNA damage response

Bmi-1 plays important roles in histone H2A ubiquitination and HOX gene silencing, and is a potent negative regulator of the Ink4a/Arf locus, which encodes the cell cycle regulators and tumor suppressor p16Ink4a and p19Arf genes (144, 145). BMI1 was enriched at the chromatin after irradiation and colocalized with ataxia-telangiectasia mutated (ATM) kinase and the histone gammaH2AX, an important DNA double strand break (DSB) repair pathway (144). A recent study showed that Bmi-1 preferentially copurified with non-homologous end joining (NHEJ) proteins in CD133+ GSC, suggesting that Bmi-1 confers

radioresistance to GSC may through the recruitment of DNA damage response machinery (145).

5.5 Insulin-like growth factor binding protein 2 (IGFBP2)

IGFBP-2 is a member of a family of six highly conserved IGFBPs that are carriers for the IGFs. The heparin-binding domain (HBD) of IGFBP2 has anabolic activity by activating IGF-I/Akt and β -catenin signaling pathways (146). IGFBP2 is known to be overexpressed in a majority of glioblastoma tumors, and its expression is inversely correlated to glioblastoma patient survival (40, 147). IGFBP2 enhances tumor invasion by upregulating matrix metalloproteinase-2 and CD24 (148, 149). Recent studies indicated that IGFBP2 is overexpressed in GSC (42, 150) and autocrine IGFBP2 is required for self-renewal and expansion of GSC (150). The knockdown of IGFBP2 expression downregulated the expression of stemness-associated gene and reduced AKT activation, and treatment with an IGFBP2 neutralizing antibody sensitized GSC to irradiation and multiple anti-neoplastic agents (150). As anticipated, recombinant IGFBP2 substantiated AKT signaling-mediated GSC viability that could be blocked by treatment with PI3K/Akt inhibitors, suggesting that IGFBP2 contributes to anti-apoptotic features of GSC.

6. Final remarks

The isolation and characterization of GSC have not only significantly changed the biological view of tumors, but has also impacted the design of effective therapies, as radio-chemoresistant, stem-like, tumorigenic glioblastoma cells may continue seeding the new tumor, despite local treatment to the tumor mass. Currently, the GSC hypothesis and model are not fully established. However, the accumulated preclinical data generated and established from both in-vitro and in-vivo GSC model systems will certainly facilitate the exploration of new concepts in tumor biology, tumor relapse and the design of potentially more effective treatment protocols that can specifically target GSC with radio-chemoresistant features. Meanwhile, since CSC share many signaling pathways with normal stem cells, exploring differences between normal and tumor stem cells may reveal novel, tumor-specific molecular targets for a safe therapy for brain cancer. Moreover, identifying the extrinsic cues and effects from their niche on GSC is also crucial as they may provide vital signaling to modulate GSC physiology and pathology (151-152). The cure for cancer requires eliminating both GSC and non-GSC populations; thus, it is important to design preclinical studies and clinical trials which evaluate the synergistic benefits of incorporating GSC-targeted therapies into conventional cancer treatments. Based on the molecular pathways of glioblastoma and GSC discussed in this chapter, I designed a therapeutic model for targeting both fast-growing, hyper-angiogenic glioblastoma tumor cells and slow-cycling, quiescent, anti-apoptotic GSC; the model theoretically and ideally, can prevent post-treatment tumor recurrence (Figure 6).

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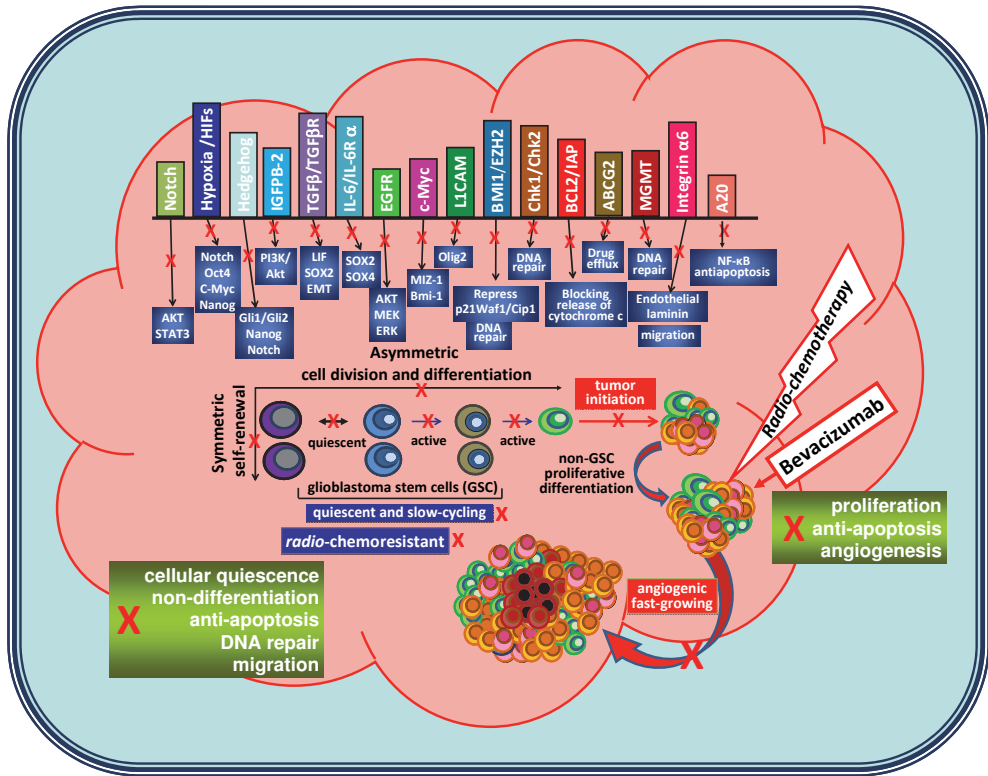


Fig. 6. A model of glioblastoma stem cell (GSC)-targeted brain cancer therapy. GSC utilize multiple stem cell associated-signaling pathways to achieve a radio-chemoresistant phenotype that sustains tumorigenesis. Essential gene pathways as indicated have been determined to be required for maintaining stemness properties, tumorigenic capacity and a radio-chemoresistant phenotype of GSC. Radiochemoresistance may be accomplished via collaboration of constitutive activation of the DNA damage checkpoint response and PI3K-Akt signaling pathway, high expression of both anti-apoptotic proteins and drug efflux transporters, and evasion of both differentiation and irreversible cell cycle arrest (cellular quiescence). In order to eradicate a tumor and prevent post-treatment tumor recurrence, a therapeutic strategy that target essential gene pathways for maintaining GSC must be developed to be fully integrated into radio-chemotherapy and anti-angiogenic therapy in order to target both quiescent GSC and fast-growing, angiogenic non-GSC populations.

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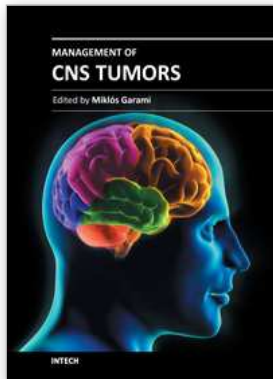
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