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REVIEW ARTICLE

# Glioblastoma stem cells (GSCs) epigenetic plasticity and interconversion between differentiated non-GSCs and GSCs



Ahmad R. Safa<sup>a,b,\*</sup>, Mohammad Reza Saadatzadeh<sup>a,c</sup>,  
Aaron A. Cohen-Gadol<sup>c</sup>, Karen E. Pollok<sup>a,b,d</sup>,  
Khadijeh Bijangi-Vishehsaraei<sup>b,e</sup>

<sup>a</sup> Indiana University Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202, USA

<sup>b</sup> Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

<sup>c</sup> Department of Neurosurgery, IU School of Medicine and Goodman Campbell Brain and Spine, Indiana University School of Medicine, Indianapolis, IN 46202, USA

<sup>d</sup> Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202, USA

<sup>e</sup> Otolaryngology-Head and Neck Surgery, Indiana University School of Medicine, Indianapolis, IN 46202, USA

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**Abstract** Cancer stem cells (CSCs) or cancer initiating cells (CICs) maintain self-renewal and multilineage differentiation properties of various tumors, as well as the cellular heterogeneity consisting of several subpopulations within tumors. CSCs display the malignant phenotype, self-renewal ability, altered genomic stability, specific epigenetic signature, and most of the time can be phenotyped by cell surface markers (e.g., CD133, CD24, and CD44). Numerous studies support the concept that non-stem cancer cells (non-CSCs) are sensitive to cancer therapy while CSCs are relatively resistant to treatment. In glioblastoma stem cells (GSCs), there is clonal heterogeneity at the genetic level with distinct tumorigenic potential, and defined GSC marker expression resulting from clonal evolution which is likely to influence disease progression and response to treatment. Another level of complexity in glioblastoma multiforme (GBM) tumors is the dynamic equilibrium between GSCs and differentiated non-GSCs, and the potential for non-GSCs to revert (dedifferentiate) to GSCs due to epigenetic alteration which confers phenotypic plasticity to the tumor cell population. Moreover, exposure of the differentiated

\* Corresponding author. Indiana University Simon Cancer Center, Indiana University School of Medicine, 980 W. Walnut Street, R3-C524, Indianapolis, IN 46202, USA. Tel.: +1 317 278 4952 (office); fax: +1 317 274 8046.

E-mail address: [asafa@iupui.edu](mailto:asafa@iupui.edu) (A.R. Safa).

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GBM cells to therapeutic doses of temozolomide (TMZ) or ionizing radiation (IR) increases the GSC pool both *in vitro* and *in vivo*. This review describes various subtypes of GBM, discusses the evolution of CSC models and epigenetic plasticity, as well as interconversion between GSCs and differentiated non-GSCs, and offers strategies to potentially eliminate GSCs.

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## Introduction

Glioblastoma multiforme (GBM) comprises the largest group of brain tumors which respond very poorly to current therapies.<sup>1</sup> In the United States, approximately 13,000 people die annually from GBM, and it is disappointing that only about 10% of patients survive 5 years.<sup>2–4</sup> The combination of radiotherapy and adjunct temozolomide (TMZ) has increased the survival of patients with GBM, but the median survival of GBM patients is only about 14.6 months.<sup>5</sup> The highly aggressive nature of GBM is due to multiple genetic alterations which result in augmented cytoprotective and survival pathways as well as numerous defects in the apoptotic signaling machinery and epigenetic alterations (Fig. 1).

A growing body of evidence indicates that rare populations of cancer cells, termed cancer stem cells (CSCs) or cancer initiating cells (CICs), play a significant role in several cancers, including GBM.<sup>6–8</sup> GBM tumors display high degree of phenotypic, cellular, genetic, and epigenetic heterogeneity, and it is believed that a major problem in the unresponsiveness of GBM tumors to therapy is the existence of GBM stem cells (GSCs) within the tumor which are most crucial for driving invasive tumor growth and relapse.<sup>6,9</sup> Emerging results have revealed that in GBM and other malignancies, CSC enrichment may occur either from an increased symmetric self-renewal division rate of CSCs or a reprogramming of non-CSC to CSCs and conferring phenotypic plasticity to the tumor population.<sup>10</sup> The concept of interconversion of CSCs and non-CSCs has provided major complexity in understanding the role of CSCs in tumor heterogeneity, a potential mechanism for therapeutic relapse, resistance to anticancer therapies, and developing therapeutic strategies. In this review we describe various subtypes of GBM, discuss the evolution of CSC models and epigenetic plasticity as well as interconversion between GSCs and differentiated non-GSCs, and offer strategies to potentially eliminate GSCs. Understanding GBM tumor cell plasticity and its underlying molecular mechanisms will help in the design of more effective therapies against GBM and preventing tumor recurrence.

## Glioblastoma multiforme (GBM)

GBM comprises the most common and very aggressive form of primary brain tumors which respond very poorly to the current therapies.<sup>1,2</sup> This most malignant brain tumor is designated as World Health Organization (WHO) grade IV astrocytoma which expresses the astrocyte marker, glial

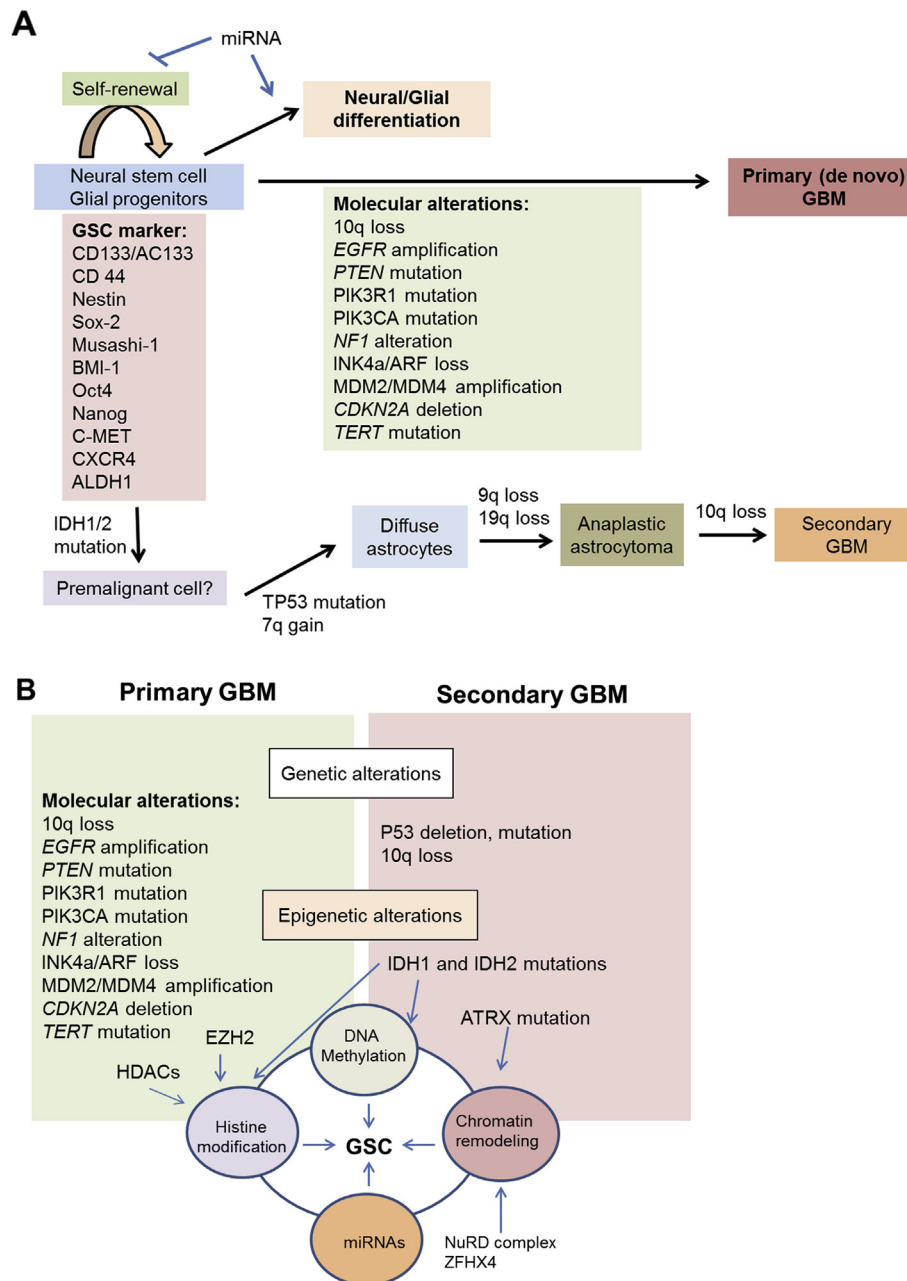
fibrillary acidic protein (GFAP).<sup>11–14</sup> Initiation and recurrence of primary GBM may be caused by a subpopulation of GSCs which may derive from mutated neural stem and precursor cells.<sup>8–14</sup> GBM tumors developed from lower-grade astrocytomas or oligodendrogliomas are termed secondary GBMs (Fig. 1). While primary and secondary GBM's are histologically similar, they are genetically different.<sup>15,16</sup> Primary GBM frequently displays molecular alterations in EGFR, PDGFRA, PTEN, p53 tumor suppressor protein, NF1, CDKN2A/B, and telomerase reverse transcriptase (TERT) promoter mutations (see Fig. 1).<sup>16,17</sup> Furthermore, as reported by Cadieux et al, global hypomethylation is frequently observed in primary human GBM.<sup>18</sup>

Primary GBM is heterogeneous in nature, and based on its patterns of gene expression and genetic changes, four different subtypes including proneural, neural, classical and mesenchymal have been identified.<sup>19,20</sup> While the biological significance and origin of these GBM subtypes are unclear, patients with specific GBM subtypes exhibit distinct survival times and different responses to therapy.<sup>12,19,20</sup> A high frequency of isocitrate dehydrogenase 1 (IDH1) mutations and O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation among young adult patients with primary GBM compared to other subtypes correlates with increased survival.<sup>21</sup> The classical subtype is associated with a high frequency of EGFR aberrations and low expression of p53 tumor suppressor protein mutations.<sup>22</sup> The mesenchymal subtype displays loss of the tumor suppressor gene NF1 with high CD44 and MERTK expression, and the neural subtype does not express any particular alterations of specific genes or pathways.<sup>12,22</sup>

The most complete information has been provided by The Cancer Genome Atlas (TCGA) Research Network which published a report by analysis of copy number, methylation patterns, expression profiling, and whole-genome sequencing of GBM samples.<sup>20</sup> Many genes including EGFR, PDGFRA, CDK4, MDM2, MDM4, MET, CDK6, N-Myc, Cyclin D2, PIK3CA, and AKT3 have been found amplified in GBM, further contributing to the complexity in developing therapies to treat GBM.<sup>20</sup> Moreover, significant abnormalities in several signaling pathways including the receptor tyrosine kinase pathway, the p53 pathway, and the RB pathway were found.<sup>12,16,20</sup>

## Cancer stem cell model

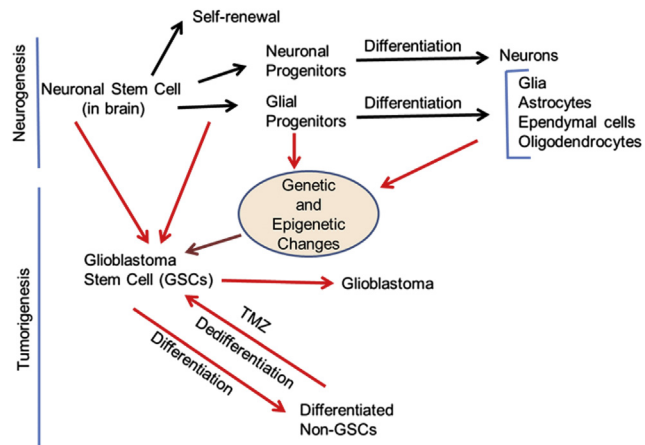
GBM tumors display a great degree of phenotypic and functional heterogeneity.<sup>7,8,12,13</sup> Heterogeneity among tumor cells arises within a single tumor as a result of genetic and epigenetic changes (Fig. 2) as well as different



**Figure 1 Genetic alterations and aberrant signaling pathways in primary and secondary GBM. A.** The continued growth and recurrence of primary GBM is due to the presence of GSCs which express various protein markers and display self-renewal and tumorigenic potential. Modified from Masui et al.<sup>15</sup> **B.** Epigenetic changes in GBM. Numerous molecular alterations shown in this figure and described in the text occur in primary GBM. Mutations in p53 tumor suppressor protein (p53) and ATRX typically occur in low-grade gliomas and secondary GBM. Mutation of the IDH1 gene is commonly found in low-grade gliomas and secondary GBM, but is rare in primary GBM. Mutation of IDH1 leads to aberrant DNA methylation and mutations in the important chromatin modifier ATRX, affecting chromatin structure. Figure was modified from Kondo et al.<sup>16</sup>

microenvironments within different regions of tumor.<sup>23,24</sup> The genetic alterations and epigenetic changes of the cells within the same tumor is not well characterized, and for future personalized medicine strategies, it is necessary to explore intratumoral heterogeneity with respect to the phenotype and genotype of the tumor as well as evaluating its epigenetic alterations to achieve effective treatment for

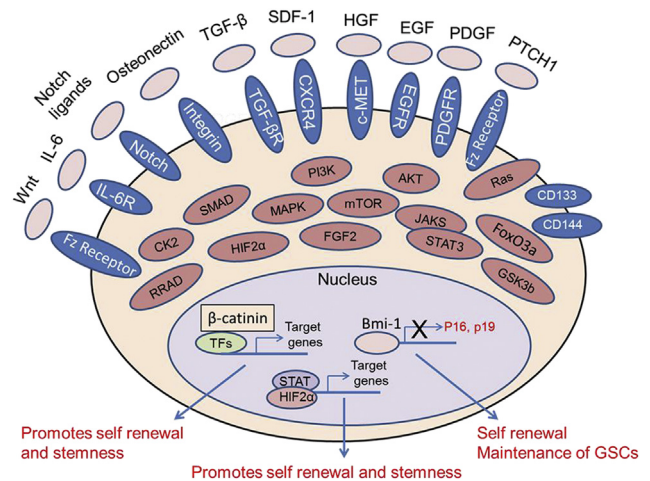
GBM.<sup>25,26</sup> To better understand intratumoral heterogeneity in a given GBM tumor, Sottoriva et al demonstrated that investigating genome-wide GBM intratumoral genomic heterogeneity can be used to reveal tumor evolution.<sup>25</sup> Furthermore, the authors showed that based on gene expression levels, tumor fragments from different anatomical regions of the same patient tumor may be classified into



**Figure 2 Relationship between neuronal stem cells, differentiation, GSCs, cancer initiation, and dedifferentiation.** NSCs are able to differentiate into neural progenitors. Neural progenitors differentiate into neurons and glial progenitors differentiate to oligodendrocytes, ependymal cells, and astrocytes. GBM is initiated from the transformation of NSCs into GSCs. Similarly, glial progenitors are able to trigger tumor development following malignant transformation of normal progenitor cells. Astrocytes, neurons, oligodendrocytes, and ependymal cells also have the potential to initiate tumorigenesis.<sup>33</sup>

different GBM subtypes.<sup>25</sup> Significantly, by using single-molecule techniques, the authors described the clonal composition of single tumor fragments and showed that a hierarchy of mitotic clones coexists within the same fragment of tumor. These impressive results unraveled the complexity of GBM tumors with respect to their heterogeneity which represents the signature of GBM clonal evolution at the single patient level.<sup>25</sup> These results demonstrate the urgent need for personalized medicine and the difficulty in developing effective therapies for each GBM patient.

The origin of tumor cell heterogeneity may occur from clonal evolution and from differentiation of CSCs.<sup>7,27–32</sup> The CSC model well explains the versatility and plasticity of heterogeneous tumor populations. This model discusses how very small subpopulations of CSCs drive cancer progression and how small subpopulations of cancer cell types with specific features are produced within a given tumor.<sup>26</sup> CSCs are characterized by their ability to generate xenografts representing the initial tumor in immunodeficient animals and to divide asymmetrically to allow self-renewal as well as differentiation into a non-CSC population (Fig. 3). However, recent experimental evidence showing CSC plasticity suggests that the tumor cell populations are dynamic, and both CSCs and non-CSCs are capable of interconversion (Figs. 3 and 4) due to environmental factors.<sup>7,8,33–39</sup> The dedifferentiation of non-CSCs to CSCs further complicates the generation of tumor heterogeneity and CSC-targeted therapy.<sup>39,40</sup> As stated by Vries et al, any tumor cell can revert to a CSC after gaining a clonal advantage over the original CSC during its development.<sup>28</sup> While much evidence supports the CSC model in several cancers, reliability on cell surface markers for identifying authentic CSCs is limited. However, clonal analysis and lineage tracing demonstrating



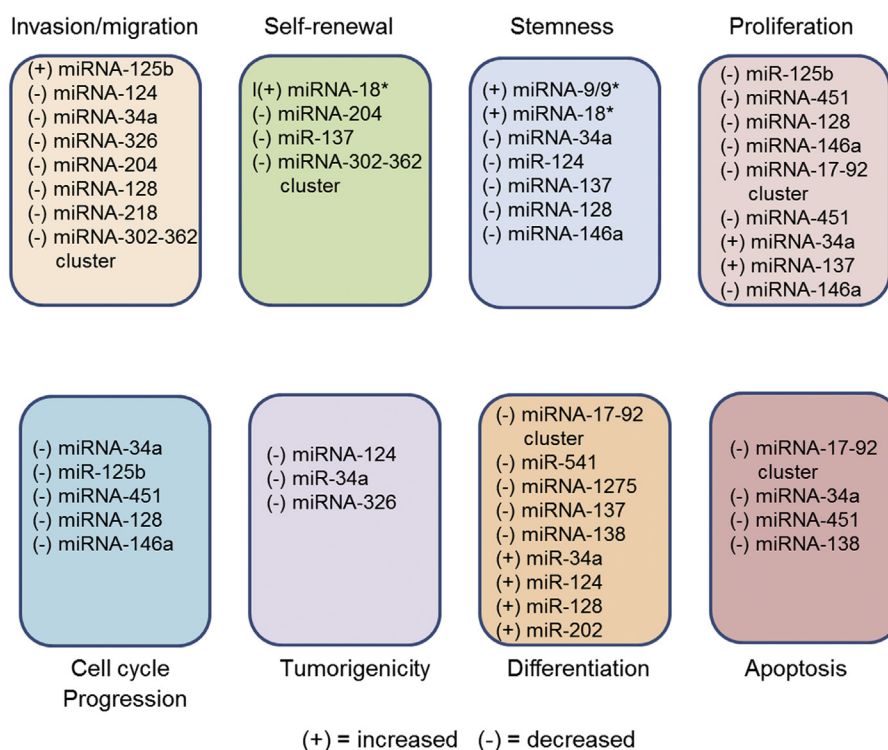
**Figure 3 Multiple signaling networks in GSCs.** A complex and integrated signaling network governs self-renewal, stemness, and maintenance of CSCs including GSCs. As shown in this figure, this network of proteins belong to many pivotal cellular pathways and include several plasma membrane receptors, cytoplasmic signaling proteins, specific transcription factors, growth factors, and ligands.

the hierarchical organization of tumors *in vivo* provide strong evidence in support of the CSC concept.<sup>41,42</sup> In support of this CSC concept, Cheng et al by *in vivo* cell lineage tracing also showed that GSCs contribute to vascular pericytes that may remodel perivascular niches.<sup>43</sup>

The relationship between neuronal stem cells (NSCs) and GSCs as well as differentiation of these stem cells are shown in Fig. 2. GSCs like other CSCs are a rare population of slow growing cells in tumors which display various “stemness” properties including (1) the ability to self-renew and differentiate into distinct lineages through different intermediate progenitors, (2) co-existence or heterogeneity of cells with different differentiation capacities providing the cellular hierarchy within the tumor, and (3) GSCs have the ability to initiate tumors in intracranial xenograft models in immunodeficient animals that recapitulate phenotypic characteristics of the initial tumor including tumor cell heterogeneity, invasiveness, migration and metastasis, tumor hypoxic response; resistance to drugs and radiation; resistance of tumors to apoptosis stimuli, and vascular characteristics.<sup>2,6–8,44,45</sup> Mounting evidence shows that the stem cell niche, i.e., the environment in which GSCs reside, is responsible for the maintenance of these cells with respect to “stemness” and therapeutic response.<sup>36,46–48</sup> The intimate network of various cell types and niche paracrine factors are responsible for controlling the necessary signaling pathways that regulate the properties of GSCs. As shown in Fig. 3, numerous signaling pathways maintain stemness and regulate the tumor propagating capacity of CSCs including GSCs.

**GSC specific markers**

The role of the cell surface protein CD133 (pronin) as a cancer stem cell marker in GBM has been extensively investigated. While the CD133 identifies GSCs that form



**Figure 4** MicroRNAs identified in GSCs. A summary of deregulated microRNAs regulating various cellular processes is listed. This is summary of the previously reported publications cited in the reference list.<sup>16,64,96,97,130</sup>

neurospheres and generate heterogeneous tumors when transplanted in immune-compromised mice, CD133-negative cells displaying similar properties have also been reported.<sup>49–54</sup> Interestingly, Brescia et al through clonal analysis reported that actually there is not a hierarchical relation between CD133-positive and CD133-negative cells, and in fact CD133 is capable of changing its subcellular localization between the cytoplasm and the plasma membrane of GSC neurospheres.<sup>49</sup> Significantly, these authors demonstrated that silencing CD133 in human GBM neurospheres using lentivirus-mediated short hairpin RNA impaired the self-renewal and tumorigenic capacity of neurosphere cells. Interestingly, hypoxia significantly increased the percentage of CD133-positive cells from 69% to 92%.<sup>55</sup> These data collectively suggest that CD133 is indispensable for GSC function and essential for maintaining the self-renewal and tumorigenic potential of GBM stem cells.<sup>55</sup> Moreover, Denysenko et al demonstrated that CD133-positive cell lines showed increased proliferation rates in neurospheres and increased differentiation potential towards neuronal lineages, while cell lines with low CD133 expression showed mesenchymal properties *in vitro*.<sup>56</sup> Moreover, other factors may collaborate with CD133 and increase the stemness of GSCs. For instance, EGFRvIII contributes to stemness through coexpression with CD133.<sup>57</sup> Moreover, while other biomarkers have been investigated in GBM including L1CAM, SOX2, CXCR4, Integrin  $\alpha$ -6, and CD36, their roles in GSCs are not well defined.<sup>57</sup>

While tumor heterogeneity is evident in all four clinically relevant subtypes of GBM as described above, molecular signaling in GSCs in individual subtypes is poorly characterized.<sup>58</sup> In light of this, Mao et al recently identified and

characterized two mutually exclusive GSC subtypes, proneural (PN) and mesenchymal (Mes) GSCs.<sup>58</sup> Mes GSCs showed more aggressive phenotypes both *in vitro* and in intracranial xenografts of GBM in mice, and were very resistant to radiation compared with PN GSCs. Interestingly, both the glycolytic pathway and ALDH1A3 activities were robustly elevated in Mes but not PN GSCs, and inhibition of ALDH1A3 attenuated the growth of Mes but not PN GSCs.

Recent results clearly show the heterogeneity of GSCs that display intrinsically distinct tumorigenic ability. By combining ploidy-based flow sorting with array-comparative genomic hybridization, Stieber et al found that primary GBMs are either mono- or polygenomic tumors (64% versus 36%, respectively) within primary GBMs.<sup>26</sup> The authors showed that monogenomic tumors are composed of a pseudodiploid tumor clone and normal stromal cells, whereas polygenomic tumors consisted of multiple tumor clones and always contain a pseudodiploid subpopulation. While multiple tumor GSC clones could generate spheroids as well as spheroid-based xenografts, genetically distinct clones had different tumorigenic potential. Interestingly, genetically distinct tumor cell populations displayed putative GSC markers including CD133, CD15 (SSEA-1), A2B5, and CD44. Therefore, the clonal heterogeneity at the genetic level, tumorigenic potential, and GSC marker expression may influence GBM progression and govern its response to treatment.<sup>26</sup>

### GBM heterogeneity and GSC plasticity

Recent research efforts have been directed toward selectively targeting CSCs for therapy.<sup>29</sup> However, therapeutic

response is influenced by the stemness of a tumor which is defined by cancer genetics, epigenetics, microenvironment, and dedifferentiation or conversion of non-CSCs to CSCs (Fig. 2).<sup>7,8,59–63</sup> These processes determine stemness and resistance to drugs and ionizing radiation in GBM tumors. Moreover, growing evidence reveals a high degree of plasticity of cancer cells with the ability to effectively and reversibly transit between differentiated and CSC phenotypes in response to microenvironmental factors like hypoxia.<sup>62–67</sup> Therefore, the capacity of tumor cells to mutually interconvert is directed by genetic, epigenetic, and microenvironmental regulation by which tumor cells alter their phenotypic and functional role which contributes to tumor growth.<sup>62–67</sup> A new model explaining the differential ability of tumor cells to interconvert explains the concept of “CSC plasticity” in which many cells within the tumor can serve as stem cells with various degrees of “stemness” regulated by microenvironmental factors.<sup>68,69</sup> Indeed, Chaffer et al demonstrated that CSC cells can arise *de novo* from more differentiated cell types and that hierarchical models of stem cell biology achieve bidirectional interconversion between stem and non-stem compartments (Fig. 2).<sup>68</sup>

It has been demonstrated that GSCs can be more resistant to conventional anticancer agents like TMZ than their differentiated GBM cells.<sup>70,71</sup> Conversely, other reports have shown that primary GSCs are sensitive to TMZ therapy, and significant expansion of different GSC subpopulations after treatment of GBM patients with TMZ has been detected.<sup>37,72,73</sup> It has been reported that the chemoresistance of GSCs correlated with elevated levels of the detoxifying protein MGMT, which confers strong intrinsic resistance to these cells, and that extrinsic factors and conversion of non-CSCs to new CSCs contributes to the resistance of CSC to TMZ.<sup>74–76</sup> To understand GBM post-therapy, Auffinger et al recently investigated the properties of GSCs after primary chemotherapy with TMZ.<sup>37</sup> These authors first showed that exposure of patient-derived as well as established GBM cell lines to therapeutic doses of TMZ increases the GSC pool over time both *in vitro* and *in vivo*. Secondly, by performing lineage-tracing analysis of the expanded GSC pool, they showed that such increase by TMZ was the result of a phenotypic shift in the non-GSC population to a GSC-like state which expressed pluripotency and stemness markers such as CD133, SOX2, Oct4, and Nestin. Moreover, these new GSCs served as a reservoir for initiating relapse of the tumors.<sup>37</sup> The phenomenon of spontaneous conversion of a non-CSC population into a CSC-like population has also been reported in breast cancer.<sup>61</sup> Therefore, collectively, these results plus published data on other tumors indicate that the tight cellular hierarchy within a tumor (i.e., the initial CSC hypothesis) does not control CSCs, and the cellular heterogeneity of the tumor plus cellular plasticity control the stemness of CSCs including GSCs.<sup>37,61,77,78</sup>

The identification of GSCs has advanced our knowledge of the molecular mechanisms involved in regulating GBM development. However, the specific intrinsic factors that govern GSCs self-renewal, stemness, differentiation, and dedifferentiation of GBM tumor cells to GSCs are not understood.<sup>7,8,37,79</sup> Moreover, emerging evidence has revealed that specific GBM microenvironments (niches) also

play a crucial role in maintaining the stemness of GSCs, and that changes in the niches may lead to these processes in GSCs.<sup>47,80,81</sup> Delineating the molecular mechanisms by which cellular plasticity is influenced by niche factors can govern the interconversion of non-CSCs to CSCs and enhance the “stemness” of the tumor. This information should provide an important direction for developing potentially effective therapies and therapeutic strategies for targeting the heterogeneous GSC subpopulations as well as the bulk of the tumor population with the aim of eradicating GBM.

## Transcription factors and GCSs

The cellular epigenetic state of an organism (or “epigenome”) incorporates a landscape of complex and flexible molecular events that create dynamic plasticity in response to environmental cues, and enables cells to function under different conditions with phenotypic and functional versatility within cell populations having identical genetic backgrounds.<sup>82–84</sup> This morphological and functional flexibility or plasticity is particularly important for CSCs which generate tumor cells that transiently expand and then undergo differentiation to form the bulk of the tumor.<sup>60,85</sup> However, the underlying molecular mechanisms operating this tumor cell plasticity is not clear. Interestingly, using combinatorial mapping of various epigenetic markers and gene expression results from GSCs, Suvà et al recently identified a core set of four neurodevelopmental transcription factors (TFs) including POU3F2, SOX2, SALL2, and OLIG2 essential for GBM propagation.<sup>86</sup> Significantly, more than 50% of the cells with all four TF (4 TF) also expressed the CSC marker CD133 compared to 4 TF-negative cells, which lack CD133. These TFs coordinately bind and activate stem-like tumor propagating cell (TPC)-specific regulatory elements. Interestingly, they are sufficient and essential to totally reprogram differentiated GBM cells and interconvert these cells to TPCs.<sup>86</sup> These exciting results revealed that these 4TFs are able to reproduce the epigenetic characteristic and phenotype of native or initial TPCs. Moreover, by reconstructing the transcriptional network controlled by these factors, Suvà et al highlighted critical interactions and a regulatory role for a chromatin-modifying complex involving RCOR2 and LSD1.<sup>86</sup> These significant findings identified the RCOR2/LSD1 histone demethylase complex as a candidate therapeutic target in human GBM stem-like TPCs.<sup>86</sup> These data establish the epigenetic basis of plasticity and evolutionary and developmental hierarchies within GBM.<sup>86</sup>

Another critical transcription factor playing an important role in the GSC phenotype is FOXM1, a master regulator of mitotic progression of cancer cells. FOXM1 forms a protein complex with the mitotic kinase maternal embryonic leucine zipper kinase (MELK) in GSCs, leading to phosphorylation and activation of FOXM1.<sup>87</sup> Activated FOXM1 results in increased mitotic regulatory genes in GSCs. TMZ treatment enriches both FOXM1- and MELK- positive GSCs, and adding Siomycin A, a CSC-targeted agent, to TMZ treatment in mice harboring GSC-derived intracranial tumors enhanced the effects of TMZ.<sup>87</sup> Identifying and developing therapeutic agents to inhibit TFs has been very

complex. Since the protein complex of FOXM1 with the mitotic kinase MELK in GSCs plays a critical role in GSC maintenance, a specific MELK inhibitor, OTSSP167, has been shown to have *in vitro* and *in vivo* effects on various human cancer xenograft models and is a promising agent for GBM therapy.<sup>88</sup> Moreover, Minata et al used the multi-kinase inhibitor C1 and showed that it induces mitotic catastrophe in GBMs, primarily through MELK kinase inhibition.<sup>89</sup>

To further understand the regulation of GSC subpopulations, Chudnovsky et al recently identified a 397-kDa transcription factor, ZFH4, which regulates differentiation, and its suppression increased GBM-free survival in intracranial xenografts.<sup>90</sup> The authors showed that ZFH4 interacts with CHD4, a core member of the NuRD (nucleosome remodeling and deacetylase) complex. Furthermore, using expression data derived from GBM patients, they found that ZFH4 is a regulatory factor that links the chromatin remodeling NuRD complex and the GBM tumor initiating cells (TIC) or GSC state.

### Epigenetic regulation of GSCs

Known mechanisms of epigenetic gene regulation include (1) chromatin remodeling and histone modification, (2) DNA methylation, (3) regulation by polycomb group proteins (PcGs), and (4) control and regulation by microRNAs (miRNAs). Chromatin remodeling and histone modification results in histone acetylation and phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation. DNA methylation results in covalent modification of cytosine nucleotides at the C5 position of particular areas of unmethylated CpG dinucleotides.<sup>91</sup> PcGs play crucial roles in regulating many cellular processes including development, pluripotency, senescence, and cancer.<sup>92</sup> PcGs are essential epigenetic factors and some members have histone methyltransferase activity.<sup>91,93</sup>

### MicroRNAs and other epigenetic factors in GBCs

miRNAs are non-coding regulatory RNAs that are dysregulated in GSCs, suggesting they play an important role in posttranscriptional gene regulation and function in a variety of cellular processes.<sup>94</sup> Recent results have revealed that miRNAs play important regulatory roles in the GSC apoptotic pathway, differentiation, proliferation, migration and invasion, drug resistance, and radiation resistance.<sup>94,95</sup> Like CSCs from other types of cancer, GSCs are controlled by specific receptor signaling and the regulation of stem cell genes by transcription factors and miRNAs. Recently, a number of new targets for these regulators for GBM treatment have been identified (Fig. 4) and demonstrated that miRNA expression patterns are correlated with the developmental lineage and differentiation state of tumor cells, as well as innovative biomarkers.<sup>94–100</sup> Several published articles have summarized a wide range of miRNAs in GSCs and the molecular mechanisms of miRNAs involved in the signaling pathways regulating these processes, as well as potential usefulness of miRNAs for eliminating GSCs (Fig. 4).<sup>96,101–103</sup> From the viewpoint of the CSC hypothesis,

several deregulated miRNAs have been strongly implicated in regulating the GSCs self-renewal capacity, maintenance of stemness and plasticity, and resistance to drugs and radiation therapy, as well as unresponsiveness to apoptotic stimuli (Fig. 4).<sup>8,103–107</sup> Therefore, miRNAs can serve as potential targets for anti-GSC therapeutics.<sup>103,108–110</sup>

Godlewski et al demonstrated a link between miR-128, which is significantly downregulated in GBM, and the loss of GSC self-renewal, which occurs by direct regulation of the neural stem cell (NSC) self-renewal factor B lymphoma MoMLV insertion region 1 homolog (BMI1).<sup>110</sup> The polycomb repressor complex (PRC) is an epigenetic regulator of transcription and its action is mediated by two protein complexes, PRC1 and PRC2. PRC functions as an oncogene in GBM where it is involved in GSC maintenance and radioresistance.<sup>111</sup> miR-128 directly targets the mRNA of SUZ12, an important component of PRC2, in addition to BMI1, a component of PRC1.<sup>111</sup> This reduction of SUZ12 expression blocks the partially redundant functions of PRC1/PRC2, thereby significantly reducing PRC activity and its associated histone modifications.

Epigenetic modifications regulate intratumoral heterogeneity, which is usually regulated by specific GSC niches, particularly, perivascular and hypoxic region microenvironments.<sup>112</sup> Moreover, GSC survival, proliferation, and maintenance is regulated by oncogenic cytoprotective signaling pathways and epigenetic modifications (Fig. 3).<sup>113</sup> Recently, Nabils et al investigated the extent to which epigenetic differences contribute to intratumoral cellular heterogeneity by developing a high-throughput method, termed MAPit-patch.<sup>113</sup> The authors found several differentially expressed and methylated promoters that are associated with altered gene expression between NSC and GBM cell populations. In addition, considering each promoter individually, substantial epigenetic heterogeneity was observed across the sequenced molecules, indicating the presence of epigenetically distinct cellular subpopulations within a GBM tumor.<sup>113</sup> Their results showed the biological relevance of epigenetically distinct subpopulations to the phenotypic heterogeneity of tumor cell populations. Moreover, Schonberg et al demonstrated that changes in chromatin accessibility without alterations in DNA methylation may comprise a novel class of epigenetic biomarkers of GBM.<sup>112</sup> A summary of the significance and targets of GSC miRNAs is shown in Fig. 4.

While the underlying mechanisms of GSC plasticity are not well established, as discussed above, it is regulated by interconversion of GBM tumor cells to GSCs. Mechanistically, Natsume et al have shown that this conversion is accompanied by the gain or loss of polycomb repressive complex 2 (PRC2), which modifies chromatin structure.<sup>114</sup> PRC2 mediates lysine-27 trimethylation on histone H3 and affects pluripotency or development-associated genes (e.g., Nanog, Wnt1, and BMP5) in GSCs as well as alterations in the subcellular localization of EZH2, a catalytic component of PRC2. Mechanistic studies revealed that epigenetic regulation by PRC2 is a key mediator of tumor cell plasticity, which is required for the adaptation of GBM cells to their microenvironment.<sup>114</sup>

Transcriptional mechanisms that control the phenotypic conversion of differentiated tumor cells into tumor-propagating stem-like cells remain to be found. Lopez-

Bertoni recently showed that the reprogramming transcription factors Oct4 and Sox2 trigger GBM cells to change into stem-like and tumor-propagating cells via a mechanism involving direct DNA methyltransferase (DNMT) promoter transactivation, leading to global DNA methylation and DNMT-dependent downregulation of multiple miRNAs.<sup>115</sup> They showed that one of the miRNAs, miRNA-148a, inhibited GBM cell stem-like properties and tumor-propagating potential. These findings identify methylation- and microRNA-based strategies for inhibiting the GSCs, their functions, and contributions to tumor growth and recurrence.<sup>115</sup>

## Epigenetic therapy

The identification and development of drugs to correct aberrant epigenetic processes in CSCs requires an in depth understanding of the extent and roles of epigenetic reprogramming in these cells. Among many alterations, amplification and rearrangements of the epidermal growth factor receptor (EGFR) gene are frequently found in GBM. The most common variant is EGFR variant III (EGFRvIII) and this variant could be a marker for GSCs showing that epigenetic mechanisms have a role in maintaining heterogeneous EGFRvIII expression.<sup>116</sup> Demethylation induced a 20%–60% increase in the percentage of EGFRvIII-positive cells, indicating that some cells could re-express EGFRvIII. Interestingly, inhibition of histone deacetylation resulted in a 50%–80% reduction in EGFRvIII expression.<sup>116</sup>

Two main features of cancer are aberrant gene function and altered patterns of gene expression, and evidence shows that epigenetic changes in collaboration with genetic alterations cause dysregulation in cancer.<sup>117,118</sup> However, the epigenetic changes in cancer are potentially reversible, and treating CSCs with demethylating agents or HDAC inhibitors may potentially reactivate silenced tumor suppressor and TF genes.<sup>118</sup> The DNA methyltransferase (DNMT) inhibitor 5-azacytidine is an effective anticancer agent and inhibitor of GSCs.<sup>119–121</sup> Another class of epigenetic inhibitors are HDAC inhibitors. HDACs are a family of 18 deacetylating enzymes that remove acetyl groups from lysine residues of histone proteins and other proteins including TFs.<sup>122</sup> HDACs regulate the conformation and activity of chromatin and mostly function as transcriptional co-repressors as part of large multi-protein complexes.<sup>122</sup> HDAC inhibitors and DNA damaging agents synergistically inhibit the growth and induce apoptosis in GSC cells possibly because they promote an open chromatin conformation and allow more effective access of DNA damaging agents to the chromatin, resulting in the increased effectiveness of these agents.<sup>12</sup>

## Clinical significance of GSC plasticity

For the future of personalized medicine for cancer patients, delineating the molecular mechanisms to predict the therapeutic response in GBM is critically important. A major challenge is to identify molecular predictors of response to new drugs. However, in the absence of such detailed molecular mechanisms, it is still possible to some degree to predict the response of GBM tumors to therapy.

For example, in GBM cells TMZ is cytotoxic to cells by triggering DNA damage, but it can be rapidly repaired by the protein MGMT. In a subset of GBM, the MGMT promoter methylation, impairs the repair mechanism and confers chemosensitivity.<sup>123</sup> While numerous GSC targeted therapies have been identified, the usefulness of these compounds from the viewpoint of pharmacokinetics and toxicity profiles and whether they cross the blood–brain barrier (BBB) remain to be found. Repurposing FDA-approved drugs which are clinically used for other diseases may identify effective agents for GBM therapy. For example, several drugs that target epigenetic alterations, including HDAC inhibitors and DNA methyltransferase (DNMT), approved for hematological malignancies, are available for solid tumor therapy.<sup>124</sup> Recently, Jiang et al used GBM cells and GSCs to identify several FDA-approved compounds that potentially could be useful in GBM treatment.<sup>125</sup> Their findings provided the basis for the rational combination of statins and topoisomerase inhibitors for GBM therapy. Moreover, using high-throughput chemical screens, Hothi et al identified an FDA-approved agent for the treatment of alcoholism, disulfiram (DSF), as an inhibitor of human GSCs.<sup>126</sup> Interestingly, DSF is a relatively non-toxic drug that can cross the BBB, and it is a direct and potent inhibitor of human MGMT in brain tumor cells.<sup>126,127</sup> These results support the repurposing of DSF for GBM therapy.<sup>127</sup> Another group of agents potentially useful for GBM therapy are epigenetic inhibitors. For example, treating GSCs with the histone deacetylase inhibitors trichostatin A (TSA) and valproic acid (VPA) significantly reduced proliferation rates, decreased the expression of stem cell markers, and induced differentiation of these cells.<sup>128</sup> Using these agents may increase the efficacy of conventional cancer treatments for eliminating GSCs. Moreover, it has been shown that GBM patients have displayed stable disease and partial responses to the redox agent perylene-quinone hypericin (HYP), a compound targeting multiple epigenetic mechanisms.<sup>129</sup>

## Future directions

While considerable progress has been made toward isolating GSCs, it is still not clear what the molecular characteristics of authentic GSCs are. Therefore, identifying the specific and reliable biomarkers of GSCs is critical. Current studies have shown the presence of distinct subpopulations of GSCs within a single GBM tumor. Therefore, it would be critically important to develop therapeutic strategies that contain agents targeting different signaling pathways and/or employing effective multi-targeting agents to eradicate these GSCs which display several phenotypic, genotypic and epigenetic characteristics. Mounting evidence supports a model of tumorigenicity with considerable plasticity between the non-GSC and GSC subpopulations within a GBM tumor, and particularly interconversion of the differentiated non-GSCs to GSCs upon chemotherapy treatment. Investigating specific niche factors which influence the interconversion between GSCs and non-GSCs will provide significant information on the role of microenvironment on GSC plasticity. Moreover, understanding the molecular mechanisms of how cellular



plasticity can govern the interconversion of non-CSCs to CSCs and enhance the “stemness” of the tumor is required for developing effective therapeutic strategies to treat GBM. Targeting the mechanisms associated with drug- and ionizing radiations (IR)-induced dedifferentiation and plasticity may potentially lead to the development of rational therapeutic strategies for treatment of GBM.

## Conflicts of interest

No author has a conflict of interest

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