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## Glioblastoma cancer stem cell biology: Potential theranostic targets

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## ABSTRACT

Glioblastoma multiforme (GBM) is among the most incurable cancers. GBMs survival rate has not markedly improved, despite new radical surgery protocols, the introduction of new anticancer drugs, new treatment protocols, and advances in radiation techniques. The low efficacy of therapy, and short interval between remission and recurrence, could be attributed to the resistance of a small fraction of tumorigenic cells to treatment. The existence and importance of cancer stem cells (CSCs) is perceived by some as controversial. Experimental evidences suggest that the presence of therapy-resistant glioblastoma stem cells (GSCs) could explain tumor recurrence and metastasis. Some scientists, including most of the authors of this review, believe that GSCs are the driving force behind GBM relapses, whereas others however, question the existence of GSCs. Evidence has accumulated indicating that non-tumorigenic cancer cells with high heterogeneity, could undergo reprogramming and become GSCs. Hence, targeting GSCs as the “root cells” initiating malignancy has been proposed to eradicate this devastating disease. Most standard treatments fail to completely eradicate GSCs, which can then cause the recurrence of the disease. To effectively target GSCs, a comprehensive understanding of the biology of GSCs as well as the mechanisms by which these cells survive during treatment and develop into new tumor, is urgently needed. Herein, we provide an overview of the molecular features of GSCs, and elaborate how to facilitate their detection and efficient targeting for therapeutic interventions. We also discuss GBM classifications based on the molecular stem cell subtypes with a focus on potential therapeutic approaches.

**Abbreviations:** BMP, bone morphogenic protein; CD, cluster of differentiation; CSC, cancer stem cell; CNS, central nervous system; CTGF, connective tissue growth factor; CSF, colony stimulating factor; Dov, dovitinib; EMT, epithelial-mesenchymal transition; FABP, fatty acid-binding protein; FGF, fibroblast growth factor; GLI, glioma-associated oncogene; GSCs, glioblastoma cancer stem cells; HIF, hypoxia-inducible factor; HO, heme oxygenase; ID, inhibitor of differentiation; IL, interleukin; MDSC, myeloid-derived suppressor cell; MMP, matrix metalloproteinase; NSC, neural stem cell; PcG, polycomb group; PDGF, platelet-derived growth factor; PLAGL, pleomorphic adenoma gene-like; PRC, polycomb repressive complex; RGC, radial ganglion cell; SDF, stromal cell-derived factor; SHH, sonic hedgehog; TAM, tumor-associated macrophage; TMZ, temozolomide; TGF, transforming growth factor; TP, thymidine phosphorylase; TRKA, tyrosine receptor kinase type A; VEGF, vascular endothelial growth factor; ZEB1, zinc finger E-box binding homeobox 1

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## Introduction

Among the many types of primary tumors, glioblastoma multiforme (GBM) is a highly aggressive and lethal cancer that is considered incurable. The current treatment is limited to gross total resection followed by radical chemo- and radiotherapy. The chemotherapeutics that have been used include alkylating agents such as nitrosoureas, which induce cytotoxicity due to the formation of DNA cross-linking, and temozolomide (TMZ), which promotes apoptosis via generation of single- and double-strand breaks in DNA (Brandes et al., 2016).

Targeted therapies such as the administration of anti-angiogenic agents, anti-epidermal growth factor receptor (EGFR), and phosphoinositide 3-kinase (PI3K) inhibitors as an adjuvant for second line treatment have also been used; however. These approaches have proven to be only marginally effective, and the poor median survival of GBM patients currently being 15 months, has not improved significantly (Schwartzentruber et al., 2012). Despite advances in the current understanding of the molecular and cellular biology of GBM, treatment strategies have not changed considerably. Moreover, the neurotoxicity of the chemotherapy can induce the formation of secondary gliomas (Dimov et al., 2011).

Other treatment complications have arisen owing to the stemness of a rare subpopulation of tumor cells called glioblastoma stem cells (GSCs) or tumor-initiating cells, which have stem cell characteristics but are not necessarily derived from normal stem cells (Pointer et al., 2014). They are able to self-renew, proliferate, and differentiate into various cell types, which underlies the cellular heterogeneity in GBM. GSCs give rise to new tumor cells after therapeutic eradication of the bulk of the tumor (Chen et al., 2012). Although the concept of cancer stem cells (CSCs) is controversial for some types of cancer, accumulating evidence supports this concept, suggesting that GSCs may be a primary contributing factor for tumor recurrence (Akbari-Birgani et al., 2016; Farahani et al., 2014; Hombach-Klonisch et al., 2017; Wasik et al., 2014). Indeed, GSCs harbor capacities for self-renewal, differentiation, and plasticity, as well as increased chemo- and radio-resistance (Ahmad and Amiji, 2017; Dey et al., 2010). The mechanisms underlying drug resistance include, drug metabolic inactivation, inhibition of pro-drug to bioactive drug conversion, increased double strand DNA repair, decreased drug influx and enhanced drug efflux. Energy-dependent drug efflux lowers intracellular drug concentration. This mechanism mostly operates via increased expression of the ATP-binding cassette super family (ABC) of transporters (Hiddings et al., 2014). ABC transporters are commonly overexpressed in GSC, in particular the ABCG2 (Wee et al., 2016). Many novel approaches have been tested to achieve effective tumor cell targeted therapy. For example, variety of drugs coupled to nano-vehicles have been employed to target specific intra-cellular compartments to enhance tumor cell drug sensitivity. For more details, see reviews (Bar-Zeev et al., 2017; Li et al., 2016; Livney and Assaraf, 2013).

High expression of aldehyde dehydrogenase-I by GSCs is another mechanism underlying GSC chemoresistance. Aldehyde dehydrogenase detoxifies alkylating agents and reduces their reactivity by converting drugs' aldehyde groups into carboxylic acid. O<sup>6</sup>-methyl-guanine DNA-methyl transferase (MGMT), a detoxifier enzyme, is also considered to contribute to chemoresistance (Safa et al., 2015; Soehngen et al., 2014). Finally, high mobility group protein A2 (HMGA2), which is a structural chromatin protein, is overexpressed in GSCs (Yi et al., 2016). These are the most typically discussed topics in the literature. Indeed, GSCs have recently been identified as potential therapeutic targets owing to their roles in tumor initiation and recurrence (McCord et al., 2009; Zhou et al., 2015b). Moreover, glioblastoma tumors exhibit proliferative and hyper-angiogenic phenotypes that can vary substantially depending on patient age and the extent of necrosis and hemorrhage (Barajas et al., 2015; Burger et al., 1985). In the present review, we discuss GBM classifications based on the molecular stem cell subtypes with a focus on potential therapeutic approaches.

## Characterization and isolation of GSCs

For characterization of CSCs, technologies such as fluorescence activated cell sorter (FACS) (Ablett et al., 2012; Witt et al., 2016), and magnetic activated cell sorting (MACS) (Torre-Healy et al., 2017) provide data for identification of single CSCs among cell populations. These technologies take advantage of cellular granularity (FACS only), size, and expression of well-defined surface markers (Hasmim et al., 2016; Prestegarden et al., 2010). CD133, CD44, and CD24 are the most common markers used as stemness identification tools, although several studies have not confirmed their reproducibility and accuracy owing to the genetic heterogeneity of CSCs (Dantas-Barbosa et al., 2015; Hiddings et al., 2014). Furthermore, microenvironmental signals in the *in vivo* niches or during *in vitro* isolation and cultivation impose epigenetic changes, and variations in CSC phenotypes are also possible. Consequently, there are many challenges that still need to be overcome.

To isolate and identify GSCs, specific and precise criteria are needed. Singh et al., described appropriate methods for GSCs characterization based on the functional properties of isolated GSCs. Additionally, neurosphere assays can be applied to assess GSC proliferation *in vitro* (Lathia and Liu, 2017; Singh et al., 2003). In this approach, researchers cultivate cells in an appropriate concentration of growth factors, and the frequencies of GSCs in tumors can be determined (Lathia and Liu, 2017; Singh et al., 2004). Another approach to identify GSCs is the use of specific surface markers to define sub-populations within the tumor that are lineage-specific. For instance, the aberrantly reactivated EGFR pathway, which causes genetic alteration, has been detected in GBM (Flavahan et al., 2016) and results in EGFR promoter alterations which facilitate EGFR overexpression (Erfani et al., 2015; Iacopino et al., 2014). Recently, Erfani et al, developed a method for the isolation of the EGFR + population of tumor cells in which fluorescence-activated cell sorting is used, to select EGFR + cells based on their affinities towards EGF ligands. This further allowed the determination that these cells displayed stemness properties by the functional characterization including a highly proliferative neurosphere, tri-lineage differentiation, as well as the expression of Sox2 and nestin (Erfani et al., 2015) The most common GSC surface markers are listed in Table 1.

## GSC tumor microenvironment –the GBM niche

Researchers have started to focus on determining the origin and identity of cells that induce the development of GBM and/or promote metastasis and relapse. Several hypotheses have been proposed, including dedifferentiation of ordinary neural cells, transformation of undifferentiated precursor cells, and proliferation of neural stem cells (NSCs) (Friedmann-Morvinski and Verma, 2014). According to these hypotheses, when genetic mutations in oncogenes accumulate in normal brain cells, dedifferentiation (a process similar to reprogramming) may occur, or accumulation of genetic mutations in NSCs may cause the NSCs to form cancer cells (Campos et al., 2016). The nature and properties of stem cell niche is an important factor in determining the fate of cancer stem cells (Farahani et al., 2014; Plaks et al., 2015).

In the adult mammalian brain, two neurogenic niches have been identified: the subventricular zone (SVZ) located in the forebrain lateral ventricle, and the sub-granular zone (SGZ) located in the hippocampus in the dentate gyrus. Stem cells in both the quiescent and active mitotic states reside in these two regions (Bayin et al., 2015). The tumor niche can be divided into three distinct areas, including the perivascular niche, which is characterized by non-malignant cells such as reactive astrocytes, fibroblasts, pericytes, neural progenitor cells, and a variety of immune cells, as well as malignant cells, including GSCs and tumor cells surrounding disorganized blood vessels (Hambardzumyan and Bergers, 2015). The interactions among these cells promote GSC survival and growth. Angiogenic factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-

**Table 1**  
Common surface markers on GSCs.

Marker	Origin	Normal function	Reference
Nestin	Mammalian CNS stem cells during development	Proliferation/migration	(Prestegarden et al., 2010)
CD133/Prominin	Normal human NSCs, several cancers and brain tumor cells	Effects on cell polarity, migration, stem cell-adjacent cell interactions, and ECM	(Rizzino, 2009; Sun et al., 2016)
Musashi-1	Its expression is correlated with the proliferation and grade of malignancy	Inhibition of mRNA translation	(Chen et al., 2017; Kaneko et al., 2000)
SOX2 and HMG box	Multipotent NSCs and ESCs	Sustain neural and embryonic stem cell pluripotency	(Rizzino, 2009)
CXCR4	Neurons and glial cells during embryogenesis and in the adult brain	Ligand for CXCL12/SDF-1, lymphocyte homing, apoptosis, and stem cell movement	(J Richardson, 2016; Zhu et al., 2002)
CD44	Stem cells and CSCs	Moderates homing of stem cells as an adhesion molecule	(Ariza et al., 1995; Pietras et al., 2014; Xu et al., 2010)
L1CAM(CD171)	Neural cells	Maintain survival and tumor growth, GSC radiosensitivity and DNA damage response regulation, neuron adhesion molecule	(Jackson et al., 2014; Samatov et al., 2016)
CD15(SSEA-1)	Developing brain stem cells and adult SVZ	Specific progenitor cell marker	(Son et al., 2009)
A2B5	Early stage of gliomagenesis and tumor propagation		(Auvergne et al., 2016; Auvergne et al., 2013; Ogden et al., 2008)

derived growth factor (PDGF), are important mediators of angiogenesis, which affect the oxygen supply to the tumor. These angiogenic factors may be produced by the tumor, especially under hypoxic conditions, and could facilitate the formation of the tumor blood vessels (Calabrese et al., 2007; Farahani et al., 2014; Plaks et al., 2015). Furthermore, VEGF causes pericytes to separate from each other and the vascular basement membrane to be disrupted. The specific role of VEGF in pericyte disintegration leads to the formation of abnormal and leaky blood vessels, which are enlarged and vulnerable to hemorrhage. This phenomenon is phenotypically similar to the process in kidney glomeruli, called glomeruloid microvascular proliferation (GMP), and is a hallmark of GBM (Dvorak, 2015). The leakiness of the GBM vessels has severe consequences on the blood brain barrier (BBB) and disrupts its firm structure (Abbott, 2013).

Following BBB disruption, tumor-derived chemokines/cytokines attract immunomodulatory cells, which can enter the brain and secrete more angiogenic factors, while suppressing immune function. The interaction among these factors, tumor cells, and GSCs results in tumor progression (Hambardzumyan and Bergers, 2015). The major populations of cells involved in this process at the tumor niche are monocytes, myeloid-derived suppressor cells (MDSCs) (Kohanbash and Okada, 2012), and neutrophils (Bergers and Song, 2005; Feng et al., 2015; Liang et al., 2014). Moreover, tumor-associated macrophages (TAMs) are common infiltrating cells in the perivascular niche adjacent to GSCs (Mantovani et al., 2002; Zhou et al., 2015a). These cells promote neovascularization by producing heme oxygenase-1 (HO-1) and thymidine phosphorylase (TP), which are strongly associated with neo-angiogenesis (Hirano et al., 2001). High expression of chemo-attractants by TAMs, including VEGF, interleukin (IL)-6, IL-1 $\beta$ , colony stimulating factor (CSF), and stromal cell-derived factor 1 $\alpha$  (SDF1 $\alpha$ ), results in recruitment of polarized macrophages and monocytes. This generates an immunosuppressive phenotype and facilitates tumor progression. TAMs can also induce matrix metalloproteinase 9 (MMP9) expression by releasing transforming growth factor- $\beta$  (TGF- $\beta$ ), which further promotes GSC proliferation (Badie and Schartner, 2001; Hambardzumyan et al., 2016). In turn, GSCs can release periostin in the aforementioned niche, which acts as another chemoattractant for TAMs (Zhou et al., 2015a).

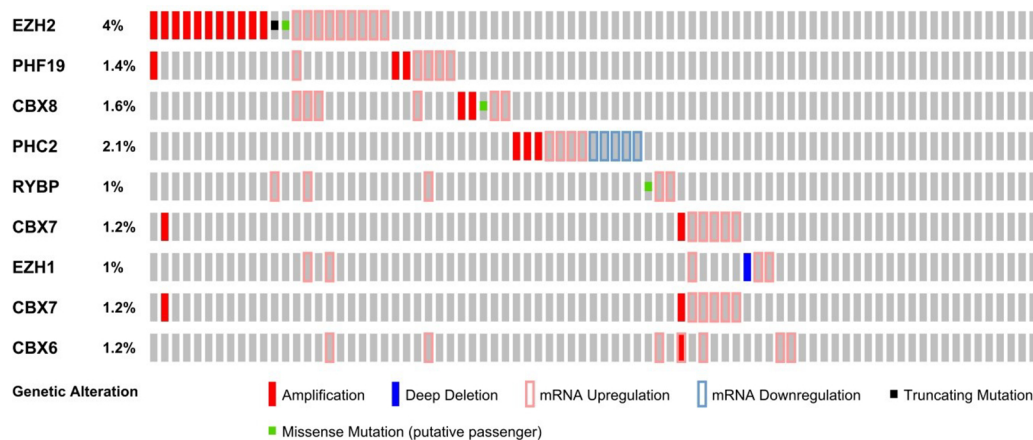
The perinecrotic or hypoxic niche, which can be identified by cells around the necrosis center (i.e., irregular architecture, blind ends, absence of smooth muscles and high permeability), is created from inefficient blood supply, which causes hypoxia, subsequently induces pseudo-palisading necrosis, and is an important regulator of tumor growth, cell maintenance, stemness induction, and immune surveillance (Ishii et al., 2016; Semenza, 2010; Soeda et al., 2009)

Hypoxia-inducible factors (HIF1 and HIF2) are the main proteins upregulated in response to low oxygen tension. They are strong inducers of VEGF and IL-8, which promote angiogenesis and invasion (Brat, 2011; Filatova et al., 2013; Schito and Semenza, 2016; Zagzag et al., 2006) and also activate genes involved in dedifferentiation and self-renewal. Some studies have found GSCs as an enriched population in the hypoxic niche (Seidel et al., 2010; Uribe et al., 2017). Indeed, hypoxia induces stemness characteristics, which can be measured by increased expression of CD133 and other GSC markers (Bar et al., 2010; Silver and Lathia, 2017; Soeda et al., 2009). Hence, GSCs and tumor cells may be maintained in the hypoxic niche, even after chemo/radiotherapy, and the death of cells in the necrotic area results in release of pro-inflammatory signals, which converts inflammatory cells to immunosuppressive cells (Casazza et al., 2013; Qian and Pollard, 2010; Rivera and Bergers, 2015). These events cause the cells to lose function and induce angiogenesis. Furthermore, hypoxia stimulates the differentiation of GSCs into endothelial cells, which could explain the growth of tumors and GSCs in a direction from the necrotic area towards the neovascular region (Soda et al., 2011).

The invasive niche for GBM has also been identified. GBM tumor cells have the unique ability to use normal blood vessels to migrate, and

**Table 2**  
Genes commonly mutated in GBM.

Gene	Function	Expression status	Reference
<i>EGFR</i>	Regulates processes involved in cell growth, division, and survival	Gain of function/amplification	(Mazzoleni et al., 2010)
<i>IDH1</i>	Produces NADPH	Gain of function/amplification	(Cohen et al., 2013)
<i>PDGFRA</i>	Regulates processes involved in cell growth, division, and survival	Gain of function/amplification	(Furnari et al., 2015; Koschmann et al., 2016; McLendon et al., 2008)
<i>HDM2</i>	Regulates processes involved in cell growth, division, and survival	Gain of function/amplification	(de Toledo et al., 2000; Lathia and Liu, 2017; Noushmehr et al., 2010)
<i>PIK3CA</i>	Regulates processes involved in cell growth, division, and survival	Gain of function/amplification	(Gallia et al., 2006; Zhao et al., 2017)
<i>TERT</i>	Induces EGFR expression/involved in cell renewal	Gain of function/amplification	(Beck et al., 2011)
<i>PIK3R1</i>	Regulates processes involved in cell growth, division, and survival	Gain of function/amplification	(McLendon et al., 2008; Zhao et al., 2017)
<i>PTEN</i>	Regulates cell signaling involved in cell proliferation and survival	Loss of function/deletion	(Benitez et al., 2017; Zheng et al., 2008)
<i>TP53</i>	Check point protein involved in apoptosis	Loss of function/deletion	(Daniele et al., 2014)
<i>CDKN2A</i>	Cell cycle regulation/retinoblastoma activation	Loss of function/deletion	(Faiq et al., 2015; Parsons et al., 2008)
<i>NF1</i>	Regulates cell signaling involved in cell proliferation and survival	Loss of function/deletion	(Verhaak et al., 2010)
<i>ATRX</i>	Regulates cell division	Loss of function /deletion	(Cottini et al., 2013; Schwartzentruber et al., 2012)
<i>RB</i>	Regulation of cell cycle	Loss of function/deletion	(Cenciarelli et al., 2016)



**Fig. 1.** TCGA analysis of PcGs in GBM patients. Due to the lack of a GSC database, the diagram shows PcG alterations with special consideration of the occurrence frequency and type of mutation.

they invade the normal brain parenchyma in this manner (Cuddapah et al., 2014). Complete deletion of MMP2 and MMP9 can enhance perivascular invasiveness and reduce angiogenesis, in contrast to the aforementioned niches (Du et al., 2008; Silver and Lathia, 2017). Astrocytes are the key cells in this niche. Exchange of ions and metabolites between blood vessels and the brain occurs via direct contact between astrocyte-end feet and pericytes and/or endothelial cells (Mathiisen et al., 2010; Silver and Lathia, 2017). The invasive niche of glioma cells can also induce astrocyte proliferation and migration by paracrine interaction. These reactive astrocytes can release connective tissue growth factor (CTGF), which binds to tropomyosin receptor kinase type A (TRKA) and integrin-1 $\beta$  that are located on the surface of GSCs. This leads to tumor cell infiltration via induction of zinc finger E-box binding homeobox-1 (ZEB1), a transcription factor involved in the epithelial-mesenchymal transition (EMT) (Abbott, 2013; Edwards et al., 2011).

Studies have shown that astrocytes, which are reactive to growth factors, cytokines, and metabolites, express sonic hedgehog (SHH); SHH is attached to the membrane protein Patch1, leading to activation of GLI and promoting stemness properties. Thus, astrocytes are important cells in the maintenance of GSCs and invasiveness of tumors (Becher et al., 2008). On the basis of these findings, larger tumors are expected to have expanded invasive niches, resulting in induction of neo-angiogenesis through hypoxia.

## Genetic events involved in GSC biology

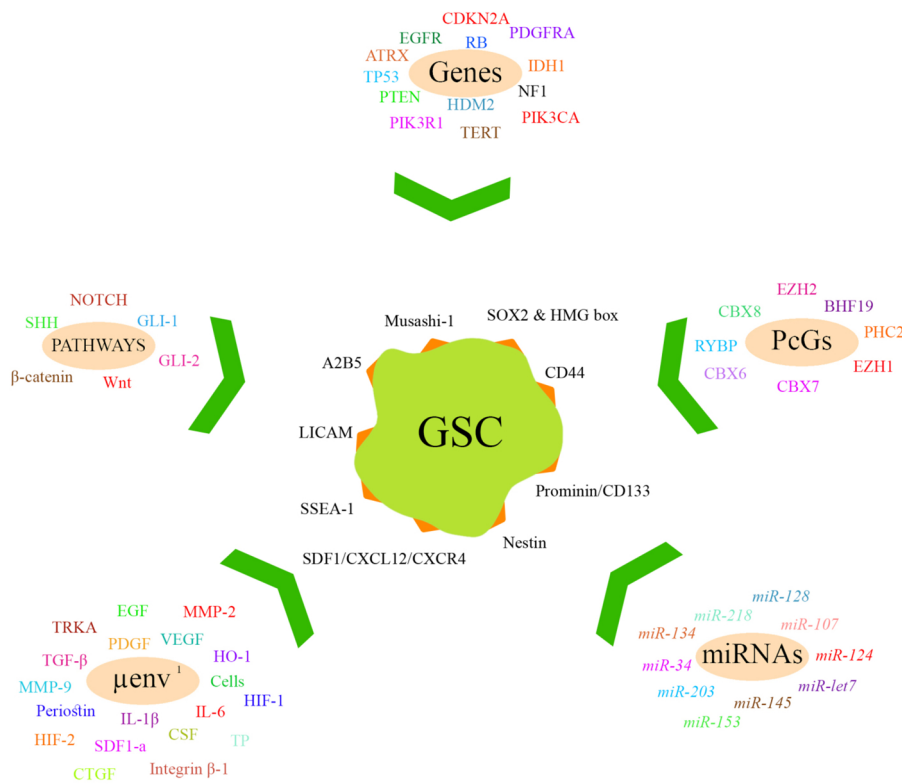
To obtain basic knowledge for targeting GSCs and efficient treatment of GBM, the genetic and epigenetic alterations in these cells must be well defined, and the molecular pathways and cellular interactions between GSCs and the tumor microenvironment as well as normal cells must be evaluated. Seventy-four genetic mutations have been detected using accessible data in The Cancer Genome Atlas (TCGA) via the c bio portal (Table 2). This database has been used to categorize mutations, and the most frequently identified signaling pathways are discussed below, and listed in Table 4.

## The most common pathways involved in maintenance of GSCs

### Notch signaling pathway

Notch signaling plays important roles in cell fate, proliferation and migration, also it is involved in maintaining cellular quiescence and regulating neural (NSC) differentiation (Saito et al., 2017). The cleavage of notch receptor by  $\gamma$ -secretase by the jagged family or delta ligands binding, leads to the translocation of Notch intracellular domain (NICD) to the nucleolus; this results in the formation a complex with RBPJ and MAML in the nucleus and activation of the hairy and enhancer of split (HES) and HEY genes, thereby promoting the





1.  $\mu$ env = Microenvironments

**Fig. 2.** Molecular interactions among GSCs and aforementioned factors. A brief graphical explanation is provided for the intracellular and extracellular factors that promote or inhibit stemness.

**Table 3**  
miRNAs involved in the regulation of GBM fate.

miRNA	Gene targeted	Changed Expression in GBM	Function	Reference
miR-218	BMI1, LEF1, IKKBK, ECOP, CDK6	downregulated	Inhibits glioma stem-like cells	(Liu et al., 2012; Mathew et al., 2014; Zhang et al., 2011)
miR-107	Notch-2, SLLA4, CDK6	downregulated	Downregulation of Nestin and CD133	(Chen et al., 2013; He et al., 2013)
miR-128	P70S6K1, SUZ12, BMI1, PDGFRA, EGFR, E2F3a, WEE1, MSII	downregulated	Suppresses PRC activity, early events in gliomagenesis	(Ciafire et al., 2005; Papagiannakopoulos et al., 2012; Rooj et al., 2016; Zhang et al., 2009)
miR-134	KRAS, STAT5B	downregulated	Stimulates differentiation	(Zhang et al., 2014)
miR-153	BCL2, MCL1, IRS1	downregulated	Impairs self-renewal and differentiation	(Xu et al., 2011; Zhao et al., 2013)
miR-203	PLD2, SNAI	downregulated	Inhibits stemness and glioma cell migration	(Deng et al., 2016; Liao et al., 2015)
miR-let-7	NRAS, KRAS, CCND1	downregulated	Possible anti-tumorigenic effects	(Guo et al., 2013; Wang et al., 2013)
miR-124	SNAI1, PIM3, NRAS, SOS	downregulated	Decreases self-renewal and migration	(Lv and Yang, 2013; Xia et al., 2012)
miR-34	MET, NOTCH1, NOTCH2,	downregulated	Induces apoptosis and inhibit invasion	(Guessous et al., 2010; Li et al., 2009)
miR-145	Sox2	downregulated	Contributes to silencing of c-Myc	(Speranza et al., 2012)

maintenance of multipotency (Bayin et al., 2017; Dantas-Barbosa et al., 2015).

CD133-positive GSCs overexpress Notch signaling pathway activators, including inhibitor of differentiation 4 (ID4) and a cellular chaperon; fatty acid-binding protein 7 (FABP7), which directly affect radial ganglion cell (RGC) migration. High expression of these genes promotes infiltrating potential of GBM tumors (Kaloshi et al., 2007); thus, the Notch signaling pathway is thought to promote migration. The activation mechanisms of oncogenic signaling in GSCs by Notch, remain unclear, however it had been shown that an extracellular matrix protein Tenascin-C, increased Notch activation in GSCs (Sarkar et al., 2017). Notch signaling activation is also involved in maintaining the stemness of GSCs, and determining glial cell fate. Thus, inactivation of this pathway may be an effective method for blocking GSCs and limiting

tumor growth.

*Sonic hedgehog (SHH)/glioma-associated oncogene (GLI) signaling pathway*

During embryonic development, SHH plays a critical role in organogenesis and especially neural progenitor regulation. However, this is not the case in quiescent adult tissues, with the exception of repair and tissue maintenance. Hence, this is an important pathway affecting the self-renewal and tumorigenicity of GSCs (Clement et al., 2007; Honorato et al., 2018). The majority of GSCs have activated the SHH/GLI-pathway and it also causes the up-regulation of drug efflux P-glycoprotein (ABCB1), ABCG2/BCRP, ABCC1/MRP1 MGMT, BMI, (Hombach-Klonisch et al., 2017; Shahi et al., 2016). Recently, studies have shown that SHH/GLI activity is crucial for Nanog regulation, and

**Table 4**  
Surface markers and other potential molecular targets in GSCs.

	Marker Identifier	Agent	Mechanism	In vivo	In vitro	Clinical trial	Reference
<b>Cell Surface Marker</b>	CD133	Carbon nanotube-conjugated anti-CD133(CD133-SWNT)	Directly targets GSCs, kills them synergistically with photothermolysis	+	+	Not yet	(Wang et al., 2011)
	CD44/Osteopontin	CD44 antagonist	Decreases GSC survival by enhancing Hippo signalling, inhibiting growth signalling from ErbB/c-Met RTKs	+	+	Not yet	(Xu et al., 2010)
<b>Signalling pathways</b>	NOTCH	NOTCH siRNA	Downregulation of NANOG, N-cadherin, CD133, and Oct-4	+	+	Not yet	(Wang et al., 2012)
	NOTCH/NOTCH ligand	OMP-59R5 (Tarextumab), BMS-906024	Induces radiosensitivity, reduces microvessels	+	+	Solid tumors other than GBM	(Dragu et al., 2015)
	TGFβRI inhibitor	LY2109761	Inhibits invasiveness and induce apoptosis	+	+	Not yet	(Mengxian et al., 2011)
	Targeting Wnt pathway	Celastrol	Inhibits GBM growth by protecting against Axin degradation and βcatenin loss.	+	+	Not yet	(Kardosh et al., 2008; McCord et al., 2009; Sareddy et al., 2013)
		SEN461	Inhibits GBM growth viaβ-catenin phosphorylation and stabilization of Axin.	+	+	Not yet	(De Robertis et al., 2013)
		XAV939	Induction of GSC differentiation	+	+	Not yet	(Huang et al., 2009; Lee et al., 2016)
<b>MicroRNAs</b>	miR-34-a		Suppression of anti-apoptotic genes, increases sensitivity to chemo/radiotherapy	+	+	Not yet	(Guessous et al., 2010; Li et al., 2009)
	miR-145		RTKs, EGFR, PDGFR-α targeting, NSC differentiation	+	+	Not yet	(Speranza et al., 2012)
	miR-128			+	+	Not yet	(Claire et al., 2005)

binding of SHH/GLI1/GLI2 to the Nanog promoter leads to activation of Nanog expression. The transcription factor Nanog is one of the ‘master regulators’ of the expression of several stemness factors. Conversely, p53 downregulates Nanog expression by decreasing GLI1 expression and activity. Furthermore, loss of p53 leads to activation of SHH signaling, upregulation of Nanog, and maintenance of stemness properties (Abou-Antoun et al., 2017; Ma et al., 2017; Zbinden et al., 2010). In contrast, bone morphogenic protein (BMP), which may also act as a growth factor and have pro-differentiation activities in stem cells, is downregulated by GLI. Piccirillo et al., showed that this pathway blocked the proliferation of GSCs (Piccirillo et al., 2006; Shahi et al., 2016). Furthermore, inhibition of SHH could potentiate the therapeutic effect of TMZ, one of the key drugs employed to treat glioblastoma. Hence, the aforementioned studies affirm the contribution of this pathway to GSCs chemoresistance and its targeting could potentially increase chemotherapy efficacy (Honorato et al., 2018).

*The Wnt/β-catenin signaling pathway*

During CNS development, the Wnt signaling pathway is a crucial player in self-renewal, differentiation, and NSC development specifically in axis patterning and the differentiation of posterior and anterior structure of CNS. (Kalani et al., 2008; Zuccarini et al., 2018). However, aberrant activation of this pathway in the CNS leads to transformation into brain tumors. The regulatory association between the Wnt pathway and maintenance of GSCs involves genetic and epigenetic mechanisms (Pulvirenti et al., 2011). With regard to genetic regulation, overexpression of pleomorphic adenoma gene-like2 (PLAGL2) results in upregulation of Wnt components such as frizzled (FZD) 2, FZD9, and Wnt6, thereby activating the canonical Wnt pathway (Zheng et al., 2010; Zuccarini et al., 2018). The other genetic event affecting Wnt is FoxM1, which directly binds to the Sox2 promoter and induces stemness programming and maintenance of GSCs (Lee et al., 2015).

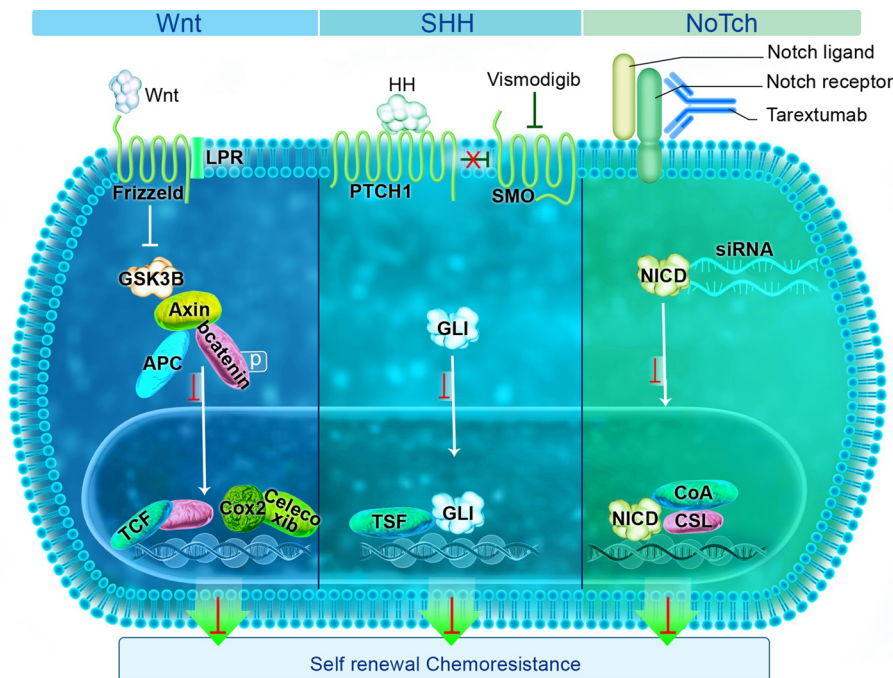
Epigenetic factors affect Wnt signaling; the transmembrane protein Evi, which is overexpressed in GBM, is regulated via epigenetic mechanisms and alters the canonical and non-canonical Wnt pathways (Ahmad and Amiji, 2017; Augustin et al., 2012; Bartscherer et al., 2006). Moreover, a Wnt family member; Wnt5A promotes endothelial differentiation of GSCs which is associated with neovascularization, hence providing environment that facilitates tumor cell growth and invasion (Binda et al., 2017; Hu et al., 2016).

These studies have shown that canonical and non-canonical Wnt signaling pathways are involved in maintenance and gliomagenesis of GSCs. Furthermore, Wnt signaling induces TMZ resistance through promotion of O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) expression, which protects the genome from anticancer therapeutics like TMZ, that act as alkylating agents (Lee et al., 2016; Wickström et al., 2015). Broad and unspecific targeting of the Wnt signaling pathway is not a viable option because of its key role in the aforementioned physiologic functions in the brain and in other tissues and organs. The blockade of such a crucial signaling system, which controls vital functions will certainly cause serious side effects in patients. Though, it would be essential to design strategies based on targeting the Wnt axis at the tumor level (Zuccarini et al., 2018).

**Epigenetic changes in GSCs**

Epigenetic heterogeneity is involved in regulation of GSCs. Epigenetic mechanisms are considered a dynamic interface between physical, social and metabolic signals of the external world, allowing changes in gene expression in response to the environment (Herman and Baylin, 2003). Indeed, GBM harbors not only genetic- but also epigenetic alterations, which in concert regulate cancer cell gene expression (Mack et al., 2016).

Epigenetic changes may involve: (i) DNA methylation especially within gene-promotor area (CpG islands), hence affecting gene



**Fig. 3.** Schematic diagram of the signaling pathways most involved in GSC maintenance and their components, which are already used as targets of most commonly used drugs. *Wnt signaling:* Binding of wnt proteins with the Frizzled and low density lipoprotein receptor-related protein/α2 macroglobulin receptor (LRP) families as their receptor results in disassembling of Axin, adenomatous polyposis coli (APC), and formation of the GSK3β complex through stabilization of β-catenin. This produces a signal to the β-catenin to migrate to the nucleus and make a connection with T cell factor (TCF) to activate their target gene, which is involved in stem cell maintenance. Celecoxib, a member of the non-steroidal anti-inflammatory drug (NSAID) family, targets the cyclooxygenase2 (COX2) gene and XAV939 and SEN461, two small molecules that have shown therapeutic promise for the treatment of Wnt-induced gliomas by stabilizing Axin, such that the amount of cytoplasmic phosphorylated β-catenin increases and then β-catenin cannot move to the nucleolus. *SHH pathway:* Binding of HH ligands to 12 transmembrane patched homologue1 (PTCH1) causes 7-transmembrane protein smoothed homologue (SMO) release. Thus, GLI is activated via a multistep process and moves to the nucleolus to induce transcription and regulate genes while stimulating cell proliferation, self-renewal, and

chemoresistance. Vismodegib binds to SMO and blocks the rest of the pathway. *Notch pathway:* The interaction between notch ligand and the notch receptor initiates Notch signaling, which proceeds by the sequential cleavage of Notch; therefore, Notch intracellular domain (NICD) is released from the membrane and moves toward the nucleolus. There, it forms a transcriptional activating complex with the nuclear transcription factor CSL and the co activator (CoA). This complex induces target genes involved in self-renewal and chemoresistance. Tarextumab is a monoclonal antibody (Ab) that targets Notch receptors and blocks the whole pathway. Blockade of this pathway appears to restrict self-renewal.

expression. Specifically, promoter hypermethylation (addition of methyl groups to cytosine residues in DNA) results in gene silencing. Hypermethylation of tumor-suppressor genes, such as *TP53*, can promote cancer development (Noushmehr et al., 2010). Conversely, hypomethylation (decreased methylation) causes activation of genes that are normally silenced or suppressed, such as oncogenes (Feinberg and Vogelstein, 1983; Noushmehr et al., 2010). (ii) Post-translational modifications of histones, i.e., methylation, acetylation, phosphorylation, sumoylation, and ubiquitylation, affect the chromatin architecture and result in epigenetic changes in the regions around enhancers, promoters, and other regulatory elements. Mutations in the histone modification pathway can promote and enhance GBM formation (Tessarz and Kouzarides, 2014). (iii) Polycomb group proteins (PcGs) play a crucial role in embryonic development and can cause gene silencing through chromatin remodeling (Fig. 1). Dysregulation of these proteins leads to tumor progression, invasion, and metastasis (Koppens and Van Lohuizen, 2016). The TCGA database, reveals a numbers of PcGs with altered expression in GBM versus the normal brain as shown in Fig. 2. Among the listed proteins, BMI1, one of the members of polycomb repressive complex1 (PRC1), prevents GSCs from differentiating into mature neurons and also inhibits apoptosis (Abdoh et al., 2009). EZH2, a component of the polycomb repressive complex2 (PRC2), is also thought to be involved in sustaining GSCs through the activation of STAT3 (Kim et al., 2013). Owing to intricate interactions among these proteins, they are thought to also be involved in inducing GBM progression and invasion. (iv) Modifications in microRNAs (miRNA), which are non-coding functional RNAs that have crucial effects on post-transcriptional regulation (Table 3). miRNA expression is greatly altered in GSCs versus the normal brain (Nagarajan and Costello, 2009). Moller et al., demonstrated altered expression of 351 miRNAs in GBM, with 256 overexpressed and 95 under-expressed (Møller et al., 2013).

### Conclusions and perspectives regarding therapeutic agents for eradication of GSCs

Despite major advances in research, treatment of GBM continues to be a major challenge. Surgical resection followed by radical chemoradiotherapy is a standard treatment protocol in patients with GBM (Tome-Garcia et al., 2017). However, the only benefit that these treatments can achieve is shrinking the tumor size by killing active cancer cells. Tumor metastasis or recurrence is common after such treatments owing to molecular heterogeneity and the unique tumor microenvironment in GMB (McCord et al., 2009). Therefore, it is crucial to design strategies that focus on targeting GSCs and their microenvironment components to eradicate GSCs in combination with traditional therapies, which may lead to more effective treatment. Other available treatment approaches include specific chemotherapeutic agents, immunotherapies, radiotherapies, gene therapies, and induction of GSC differentiation to normal cells; these treatment modalities may have applications in the targeting of GSCs with the goal of eradicating minimal residual disease and blocking tumor recurrence (Cho et al., 2013).

Currently, many studies are underway to target GSCs for more effective treatment. For example, Thanasupawat et al., tested a small molecule inhibitor, dovitinib (Dov; TK1258, CHIR258) (Thanasupawat et al., 2017). Dov is a benzimidazole-quinolinone compound that acts as a receptor tyrosine kinase inhibitor and down-regulates HMGA2. Considering the special function of HMGA2 protein in self-renewal, its down-regulation should reduce the size of GB tumors. Dov also causes reduced expression of MGMT, which is an important enzyme that plays an important role in the repair of DNA damage caused by the alkylating activity of TMZ (as shown above), and is a potential chemoresistance determinant. Dov damages DNA by its localization in the minor groove, and it targets topoisomerase I and II. Combinatorial treatment of TMZ with Dov enhances DNA damage and apoptosis in GB cells (Thanasupawat et al., 2017). The pathway blockers demonstrated in Fig. 3 are other examples of these stemness-targeted drugs. Selected,



potential targets for GMB-therapy have been listed in Table 4.

Salinomycin is another potentially promising experimental drug that preferentially kills GSCs as well as other types of CSCs (Jangamreddy et al., 2013, 2015; Xipell et al., 2016). While phase I clinical trials testing the potential use of salinomycin in glioblastoma yet await to be published, the drug and its derivatives, are currently being intensively explored as an anti-CSCs agent for several types of malignancies.

In the current review, we highlighted the molecular markers that can be utilized to isolate GSCs from normal stem cells. These promising therapeutic targets may afford a systematic, well-organized approach for cancer treatment and prevent further exposure of patients to the severe side effects of chemoradiotherapies and more invasive approaches. Further laboratory and clinical investigations are needed to identify the appropriate selection of targets for promising treatments.

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