Review Article

Targeting glioma stem cells: A novel framework for brain tumors

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The past decade has seen a dramatic increase in stem cell research that focuses on glioma stem cells (GSC) and their mechanisms of action, revealing multiple potential targets for primary malignant brain tumors. Herein, we present a novel framework for considering GSC targets based on direct and indirect strategies. Direct strategies target GSC molecular pathways to overcome their resistance to radiation and chemotherapy, block their function or induce their differentiation. Indirect strategies target the microenvironment of the GSC, namely the perivascular, hypoxic and immune niches. Progress made on GSC targets is reviewed in detail and specific pathways are identified in context of the proposed framework. The potential barriers for translation to the clinical setting are also discussed. Overall, targeting GSC provides an unprecedented opportunity for revolutionary approaches to treat high-grade gliomas that continue to have a poor patient prognosis. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.02064.x, 2011)

Gliomas are brain tumors that arise from glial cells and they account for over 30% of all primary brain and central nervous system tumors diagnosed in the US.[1] Gliomas are classified by the World Health Organization into four grades of ascending malignancy; Grades III and IV are considered high-grade gliomas (HGG) and are associated with a poor prognosis.[2] Grade IV gliomas, or glioblastoma multiforme (GBM), are the most malignant and most common, accounting for over half of all gliomas.[1] Patients with GBM have a median survival of 14.6 months and an overall survival of only 10% at 5 years, even after gold-standard treatment with surgery, ionizing radiation (IR), and temozolomide (TMZ).[3]

The concept of a cancer stem cell (CSC) was first proposed in the context of acute myeloid leukemia[4,5] and later extended to a number of solid organ malignancies. Several groups identified CSC in samples from patients with gliomas.[6–10] Consistent with the general definition of CSC, glioma stem cells (GSC) demonstrate a capacity for self-renewal, multipotency, and induction of tumorigenesis. The identification of GSC prompted the proposal of a hierarchical model of tumorigenesis (hypothesizing that only the GSC subset can induce tumorigenesis) in contrast to the stochastic model (proposing that tumor cells are heterogeneous and that virtually any of them can function as a GSC or tumor-initiating cell).[11] However, subsequent data have lent support to the stochastic model. Under certain conditions, non-GSC can become GSC and exhibit an enhanced ability to form neurospheres, thereby suggesting that the GSC state may be plastic.[12–14]

A fundamental issue regarding GSC is identification of GSC-specific markers. The GSC population was first associated with the expression of the surface marker Cluster of Differentiation (CD) 133.[6–9] The specificity of CD133 expression is under question, with groups reporting the identification of GSC that

are CD133 negative.[15] Discrepancies in the literature are affected, at least in part, by the different methods and techniques used in the detection of CD133 and factors that can influence its detection.[16] Clinical studies have shown that CD133 expression in histological samples of HGG correlates with patient survival and clinical course,[17,18] although some argue that it is not a prognostically significant factor.[19] Despite the controversy, it remains the most frequently used marker of GSC to date. Others have proposed markers such as A2B5,[20] stage-specific embryonic antigen,[21] and aldehyde dehydrogenase 1 (ALDH1),[22] or an altogether marker-independent identification of GSC.[23] An interesting concept that has evolved in the glioma literature is the concomitant use of different stem cell markers rather than focusing on a single marker. The addition of the neural stem cell marker (NSC) Nestin (an intermediate filament protein expressed during embryogenesis) to CD133 led to significantly improved clinical prognostic accuracy.[24] The embryonic stem cell marker signature (Oct4, Sox2, and Nanog) correlates with glioma aggressiveness and has been proposed as a tool in predicting GSC responses to therapy.[25–27] Similarly, there is a positive correlation between Nanog and CD133 expression in the pathological grade of clinical glioma samples, as well as in GSC formation.[28,29] Together, these results suggest that instead of focusing on one specific marker, it may be more fruitful to use multiple markers concomitantly, constituting a ''stemness signature''. Overall, controversy persists with regard to the functional significance of GSC based on their frequency, propagation rate, and correlation between tumorigenicity and differently defined stem cell markers. Nonetheless, evidence has accrued in support of a pivotal role for GSC.

A significant amount of research has been devoted to unraveling the mechanisms of action behind GSC, yielding multiple potential targets. As data on GSC accumulate, development of a framework for considering GSC targets becomes important not only for conceptualizing currently available data, but also for designing combinatory approaches. This paper presents a novel framework for GSC targets based fundamentally on the broad division of direct and indirect targeting strategies. Direct strategies target GSC activity and ⁄ or function, whereas indirect strategies target the microenvironment or GSC niches. Pathways identified in the literature thus far are reviewed in the context of this framework.

Direct GSC targeting

Direct GSC targeting strategies may involve several approaches (Table 1). Because it has been postulated that HGG resistance to standard treatment is due, at least in part, to the presence of

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Table 1. Direct glioma stem cell targeting strategies

Approach	Mechanism	Target
Overcoming resistance to standard treatment	Ionizing radiation	
	DNA repair	Chk1/Chk2
		L1CAM/NBS-1
		Bmi1
	Non-DNA repair	Notch/Akt
		Hedgehog/Gli
		SirT1
	Temozolomide	
	DNA repair	MGMT
	Non-DNA repair	ABCG2/BRCP1/ Akt/Tie2
		Notch
		Hedgehog/Gli
Blocking function	Growth factor	EGF/EGFR/Akt
	Intracellular	Akt
		EZH2
		Bmi1
		miR-128
		miR-326
		miR-21
	Developmental	L1CAM
		Notch
		Hedgehog/Gli
	Immune	STAT-3
		IL-6
		MMP-13
Inducing differentiation	Growth factor	BMP
		IFN-β
		RAR
		PPARγ
		TGF-β
	Transcriptional	Smad 2/3
		Sox11
		GSK3β/Bmi1
	Post-translational	miR-124
		miR-137
		miR-451
		miR-17-92

The approaches include overcoming resistance to standard therapy, blocking function, and inducing differentiation. In the first two approaches, targets are upregulated and/or enriched in glioma stem cells (GSC), with the exception of miR-128 and miR-326, which are downregulated. With the third approach, targeting increases signaling, with the exception of glycogen synthase kinase (GSK) 3β and miR-17-92 (which are upregulated in GSC such that targeting decreases signaling). ABCG2, ATP-binding cassette sub-family G member 2; BMP, bone morphogenetic protein; BRCP1, breast cancer resistance protein 1; EGF, epidermal growth factor; EGFR, EGF receptor; EZH2, enhancer of Zeste homolog 2; IFN-β, interferon-β; IL-6, interleukin 6; MGMT, O^6-methylguanine-DNA-methyltransferase; miR, micro RNA; MMP-13, matrix metalloproteinase 13; NBS-1, nijmegen breakage syndrome 1; L1CAM, L1 cell adhesion molecule; PPARγ, peroxisome proliferator-activated receptor γ; RAR, retinoic acid receptor; STAT-3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor-β.

GSC, the first approach involves overcoming the resistance of GSC to standard treatment. The second approach involves directly blocking GSC function and/or activity, whereas the third (and last) involves inducing GSC differentiation into less tumorigenic cells that are more susceptible to a greater repertoire of therapies.

Overcoming GSC resistance to standard treatment. Standard treatment currently involves the use of both IR and TMZ. Mechanisms of GSC resistance involve those based on DNA repair, as well as those independent of DNA repair. DNA repair is more efficient in CD133$^+$ GSC, resulting in a greater fraction of CD133$^+$ cells in the tumor bed after IR.[30,31] Mechanisms include Chk1 and Chk2 DNA checkpoint kinases,[30] the L1 cell adhesion molecule (L1CAM)-regulated nijmegen breakage syndrome 1 (NBS-1) component of the complex activating early checkpoint responses,[32] and the Bmi1 polycomb protein.[33] Checkpoint responses have a critical role in determining cellular response to IR. Experiments have demonstrated that CD133$^+$ GSC preferentially activate Chk1 and Chk2 during IR and that concomitant blockade of Chk1 and Chk2 with the inhibitor debromohymedialdisine significantly reduces GSC radioresistance.[20] Knockdown of L1CAM1 and the L1CAM1-regulated NBS-1 component (which are generally more elevated in GSC) attenuated the DNA repair capacity of GSC.[32] Polycomb group proteins typically function as epigenetic silencers and form large complexes that affect gene expression through histone modification and chromatin compaction.[33] Bmi1 is a polycomb protein, part of the polycomb repressor complex 1, that regulates histone methylation and becomes enriched in the chromatin fraction of GSC after IR.[22] Partial knockdown of Bmi1 decreases GSC radioresistance.[22] The results regarding the role of L1CAM-1, NBS-1, and Bmi1 were obtained with siRNA and knockdown technology, rather than pharmaceutical compounds,[32,33] making these targets somewhat less appealing at this time from a translational perspective.

Mechanisms for radiosensitization not based on DNA repair include the Notch, Hedgehog/Gli, and SirT1 pathways. The Notch pathway, important in the regulation of GSC self-renewal, is upregulated in GSC.[34,35] Downregulation of the Notch pathway using γ-secretase inhibitors (GSI) enhances IR-induced GSC apoptosis and decreases clonogenic survival.[35] It is reported that GSC radiosensitization occurs via downregulation of the serine/threonine protein kinase Akt rather than through DNA-based mechanisms.[35] The Hedgehog–Gli pathway, known as a key regulator of embryogenesis, has also been found to be active and essential in GSC.[36,37] Concomitant pharmacologic inhibition of the Hedgehog–Gli pathway by cyclopamine and IR significantly enhances the IR-induced reduction of the GSC growth rate compared with IR alone.[38] SirT1 is a sirtuin type of histone deacetylase (HDAC) that binds selected histones and deacetylates several transcriptions factors.[39] Regarded as a mediator for cellular longevity, SirT1 is overexpressed in CD133$^+$ GSC.[39] Knockdown of SirT1 expression significantly augments IR-mediated apoptosis of CD133$^+$ GSC and renders IR more effective *in vivo*.[39]

With respect to mechanisms of chemoresistance based on DNA repair, evidence points to differential DNA methylation in GSC. Compared with non-GSC, GSC exhibit significantly higher expression of O^6-methylguanine-DNA-methyltransferase (MGMT) and are more resistant to TMZ treatment, in accordance with the well-known association between MGMT levels and TMZ response.[40] It should be noted that epigenetic mechanisms may also impair the application of non-standard chemotherapeutic agents; for example, GSC have a hypermethylated caspase-8 promoter that renders them resistant to therapies based on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[41] Mechanisms for chemoresistance based on non-DNA repair involve ATP-binding cassette (ABC) transporter protein expression and the Notch and Hedgehog–Gli pathways. Studies have shown that GSC express high levels of breast cancer resistance protein 1 (BRCP1), also known as ATP-binding cassette sub-family G member 2 (ABCG2), an ABC transporter protein responsible for drug efflux.[40,42] This has been associated not only with increased drug efflux in GSC,[40] but also with increased Akt expression[42] and Tie2 activation.[43] Inhibiting both the Notch and Hedgehog–Gli pathways with GSI and cyclopamine, respectively, sensitizes GSC to TMZ

treatment to a greater degree than with each agent alone, which already demonstrated TMZ-sensitizing effects.[36] Figure 1 schematically depicts the multiple complex signaling pathways involved in GSC resistance to standard therapy. The Notch and Hedgehog–Gli pathways are highlighted because they are implicated in GSC resistance to both IR and TMZ and can be targeted pharmacologically, making them attractive from a translational standpoint.

Blocking GSC function. A second major therapeutic approach involves blocking GSC function. Several targets have been investigated, including growth and intracellular signaling factors, and developmental and immune pathways. Of the multiple growth factors tested, only epidermal growth factor (EGF) was capable of promoting sphere formation and enhancing the self-renewal capabilities of GSC that express the EGF receptor (EGFR).[44] Inhibition of EGFR signaling inhibited proliferation and induced apoptosis of GSC.[44,45] The EGFR tyrosine kinase inhibitors tested included not only the experimental pharmacologic agent AG1478, but also the clinically approved drugs erlotinib and gefitinib.[44,45] A caveat to the effectiveness of EGFR inhibitors may be the presence of phosphatase and tensin homolog (PTEN); some GSC lines with low PTEN may be insensitive to EGFR inhibitors.[45]

One of the main effector mechanisms downstream of EGFR inhibition in GSC is downregulation of Akt signaling.[44,45] In addition to being an effector molecule in several signaling pathways, Akt merits consideration as a stand-alone target for blocking GSC. Akt is part of the phosphatidylinositol 3-kinase (PI3K)–Akt pathway (also known as the protein kinase B pathway), which is a critical regulator of multiple cellular functions, such as proliferation, survival, angiogenesis, metabolism, and glucose uptake, and is associated with loss of PTEN.[46] The

CD133^{+} GSC are more dependent on Akt signaling than non-GSC.[47] Consequently, small-molecule inhibition of Akt signaling with a phosphatidylinositol ether lipid analog (AKTIII) reduced the number of viable GSC by suppressing neurosphere formation and inducing apoptosis, and also significantly reduced the motility and invasiveness of both GSC and non-GSC.[47] The use of the phlorotannin compound Eckol also blocked Akt signaling (as well as signaling via the Ras–Raf-1–Erk pathway) and similarly attenuated CD133^{+} GSC formation and anchorage-independent GSC growth.[48] Inhibition of Akt by a small molecule inhibitor (A-44365) decreased GSC proliferation across a panel of lines.[49] Interestingly, in a set of clinical HGG samples, increased Akt signaling correlated with poor patient prognosis.[50] Clinical trials are in progress testing the use of the Akt inhibitor perifosine, alone and in combination, in a number of malignancies, including glioma (http://www.clinicaltrials.gov). Both these signaling pathways (i.e. EGF/EGFR and Akt) can be inhibited pharmacologically and inhibitors for both are in clinical use (Fig. 2).

Other intracellular signaling pathways that have been investigated as potential targets include maternal embryonic leucine zipper (MELK), enhancer of Zeste homolog 2 (EZH2), Bmi1, and several different micro-RNAs (miR). A serine/threonine kinase, MELK is involved in cell survival and NSC self-renewal.[51] It has been reported that MELK is overexpressed in glioma cells, including both GSC and non-GSC, and that reduction of MELK expression by siRNA markedly decreases glioma cell proliferation and survival.[51] However, given that downregulation of MELK also affects the proliferative capacity of NSC,[51] its suitability as a specific GSC target is questionable. As a component of the polycomb repressive protein complex 2, EZH2 is involved in epigenetic-mediated gene silencing via
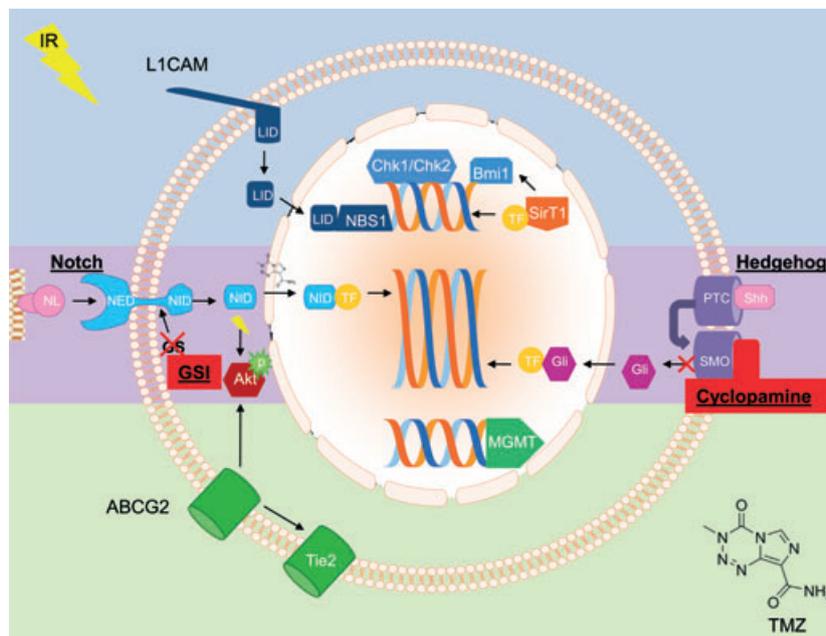
Fig. 1. Complex signaling pathways underlie glioma stem cell (GSC) resistance to standard therapy consisting of ionizing radiation (IR) and temozolomide (TMZ). Mechanisms include those based on more efficient DNA repair, as well as those independent of DNA repair. With respect to IR (blue shading), GSC have upregulated expression of checkpoint kinases Chk1/Chk2 and the L1 cell adhesion molecule (L1CAM)-regulated NBS-1 component of the complex activating early checkpoint responses, as well as Bmi1 and the histone deacetylase SirT1. With respect to TMZ (green shading), GSC have significantly higher expression of O^6-methylguanine-DNA-methyltransferase (MGMT) and upregulated expression of the ATP-binding cassette sub-family G member 2 (ABCG2) drug transporter protein responsible for drug efflux. The upregulation of the Notch and Hedgehog–Gli pathways (purple shading) has been implicated in GSC resistance to both IR and TMZ (albeit with minor downstream differences in Notch signaling). Both the Notch and Hedgehog–Gli pathways may be inhibited pharmacologically by γ-secretase inhibitors (GSI) and cyclopamine, respectively, rendering them attractive from a translational viewpoint. LID, L1CAM intracellular domain; NBS-1, nijmegen breakage syndrome-1; NED, notch extracellular domain; NID, notch intracellular domain; NL, notch ligand; p, phosphorylated; PTC, patched; Shh, sonic hedgehog; SMO, smoothened; TF, transcription factor.
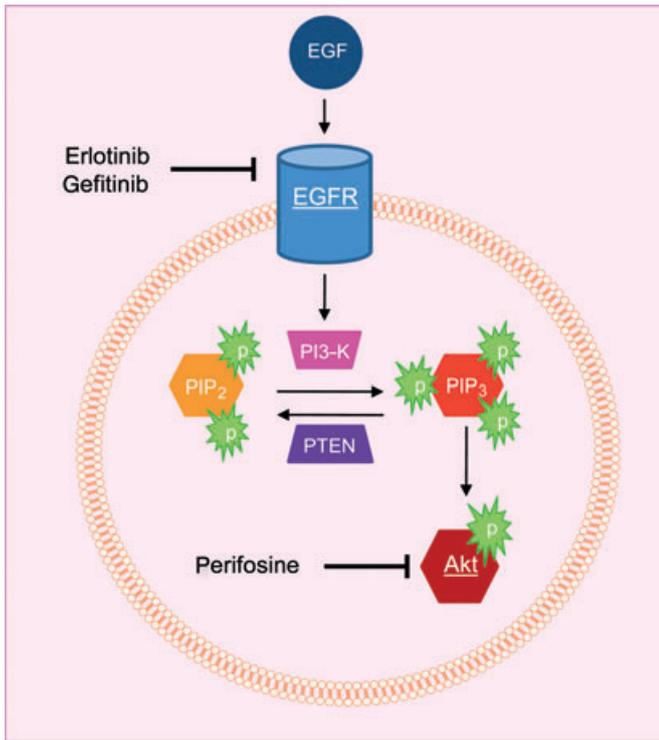
Fig. 2. The Akt pathway is one of the effector mechanism of epidermal growth factor (EGF)/EGF receptor (EGFR), but warrants consideration as a stand-alone target. Akt is a serine-threonine kinase that is part of the phosphatidylinositol 3-kinase (PI3-K) signaling pathway. Glioma stem cells (GSC) are more dependent on Akt signaling than non-GSC, rendering Akt a potential target preferentially affecting GSC. Upon EGFR activation, PI3-K stimulates the production of phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) from phosphatidylinositol 4,5-bisphosphate (PIP$_2$), which, in turn, stimulates the phosphorylation of Akt. Akt is a critical regulator of multiple cellular functions, such as proliferation, survival and metabolism. Phosphatase and tensin homolog (PTEN) can directly counteract and reverse the effect of PI3-K. Both EGFR and Akt may be inhibited by drugs used in the clinical setting. Erlotinib and gefitinib inhibit EGFR, whereas perifosine inhibits Akt. p, phosphorylated.

trimethylation and histone modification.[52] It has been shown that EZH2 is highly expressed and/or enriched in GSC.[52] The inhibition of EZH2, either pharmacologically by the *S*-adenosyl-homocysteine hydrolase inhibitor 3-deazaneplanocin or by shRNA, significantly attenuates GSC self-renewal *in vitro* and interferes with GSC tumor-initiating capacity *in vivo*, specifically via repression of *c-myc*.[52] Similarly, knockdown of Bmi1 (highly expressed in CD133$^+$ GSC) inhibits their clonogenic capacity *in vitro* and brain tumor formation *in vivo*, supporting the concept that Bmi1 plays a role in CD133$^+$ GSC function.[53] In addition, Bmi1 was found to be the downstream effector molecule for miR-128, which is downregulated in GSC.[54] Overexpression of miR-128 by transfection led to the downregulation of Bmi1 and a decrease in GSC self-renewal capabilities.[54] Overexpression of miR-326, again by transfection, in GSC (which have low baseline expression) also led to an increase in GSC apoptosis and mitigated GSC invasive properties.[55] Interestingly, the authors demonstrated a negative feedback loop between miR-326 and Notch signaling.[55] In contrast to miR-128 and miR-326, which are downregulated in GSC, miR-21, known as an anti-apoptotic factor, is upregulated in GSC.[56] Administration of interferon (IFN)-β significantly reduced tumoral miR-21 levels, which are also negatively regulated by signal transducer and activators of transcription (STAT)-3.[56]

Turning towards developmental pathways, several have emerged as regulators of GSC function. The cellular adhesion molecule L1CAM, that plays a role in axonal guidance and neuronal migration, is overexpressed in GSC and its inhibition by lentiviral-mediated shRNA not only disrupts neurosphere formation, but also induces GSC apoptosis and inhibits growth via modulation of the olig2 and p21 transcription factors.[57] Blocking Notch pathway signaling (which is upregulated in GSC) with GSI can deplete the number of CD133$^+$ GSC and inhibit the growth of xenografts.[58] Similarly, cyclopamine-mediated inhibition of the Hedgehog–Gli pathway (upregulated in GSC) also depletes GSC.[38] As a clinical correlate, increased Hedgehog signaling may be associated with a shorter survival time for patients with GBM.[59]

Finally, increasing evidence has linked immune pathways to GSC. For example, STAT-3 signaling reportedly plays a role in the maintenance of GSC: it is upregulated in glioma and has an anti-apoptotic role.[60] Inhibition of STAT-3, either pharmacologically by two distinct small-molecule inhibitors (S31-201 and STA-21) or by shRNA, may decrease proliferation and increase apoptosis.[60] Upstream of STAT-3 is interleukin (IL)-6. Data have shown that GSC preferentially express the IL-6 receptors IL-6Rα and gp130.[61] Downregulating the receptors and/or IL-6 ligand by shRNA attenuated GSC growth and survival, whereas administration of anti-IL-6 reduced the growth of GSC-derived xenografts.[61] Matrix metalloproteinase protein (MMP)-13, traditionally known as a collagenolytic enzyme, is expressed specifically in GSC and inhibition of MMP-13 by shRNA has been shown to suppress GSC migratory and invasive properties.[62]

Inducing GSC differentiation. The third major direct therapeutic approach involves inducing GSC differentiation into non-GSC with lower tumorigenic potential. Several targets have emerged from the literature. Bone morphogenetic proteins (BMP) are a group of pivotal cancer signaling molecules that bind to BMP surface receptors and can mediate the differentiation of GSC.[63] Treatment of GSC with BMP, by direct implantation of BMP-bearing beads, induced their differentiation and decreased their tumorigenic potential.[63] Enthusiasm has been somewhat tempered by a subsequent study showing that GSC may epigenetically modulate BMP receptor expression to a more resistant phenotype that can evade BMP-induced differentiation.[64] However, it remains an important target and clinical trials using BMP are being designed in Europe.

Recent evidence has also documented a role for IFN-β signaling in GSC differentiation. *In vitro* treatment of GSC with IFN-β reduced GSC proliferation via STAT-3-mediated differentiation into oligondendrocytes.[65]

Another agent under consideration for inducing GSC differentiation is all-*trans* retinoic acid (ATRA), a vitamin A derivative that binds to the nuclear retinoic acid receptor (RAR) and has made acute promyelocytic leukemia a curable disease.[66] Treatment of GSC by ATRA had marked prodifferentiation effects with functional consequences, such as deceased GSC tumorigenicity, motility and impaired cytokine secretion.[66] Similar effects were reported by a different group using 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$, an agonist of the peroxisome proliferator-activated receptor (PPAR) γ, but these were mediated via the EGF/EGFR and STAT-3 signaling pathways.[67]

Transforming growth factor (TGF)-β has also been shown to have regulatory effects on GSC differentiation, albeit with different mechanisms of action.[68,69] In one study, treatment of GSC with TGF-β induced leukemia inhibitory factor (LIF) via the Smad-2/3 transcriptional complex to prevent GSC differentiation; notably, treatment of normal neural stem/progenitor cells with TGF-β did not induce either LIF or differentiation.[68] In a different study, TGF-β maintained GSC markers and prevented differentiation via Sry-related HMG-Box factors

(Sox), specifically Sox2 and Sox4.[69] In addition, Sox11 alone may have prodifferentiation effects on GSC (which typically lack Sox11) in that its overexpression inhibits GSC tumorigenesis.[70]

Inhibition of glycogen synthase kinase (GSK) 3β, a serine/threonine kinase implicated in NSC differentiation, either directly (by siRNA or pharmacologically by SB216763 and lithium chloride) or indirectly via upstream inhibition of Bmi1, decreases GSC stemness and induces differentiation markers.[71]

Finally, recent work has identified several miRs as post-translational regulators of GSC differentiation.[72,73] Overexpression of miR-124 and miR-137, both of which are low in HGG, induced the differentiation of CD133+ GSC, but notably also had the same effect on normal NSC,[72] rendering specific delivery to GSC a greater concern. Overexpression of miR-451 inhibited neurosphere and tumor growth,[73] pointing to functional consequences of miR regulation. The miR-17–92 cluster is upregulated in GSC and repression, via transfection of an inhibitor, induced GSC apoptosis and reduced GSC proliferation.[74]

Indirect GSC targeting

A niche is not only an anatomic structural unit surrounding stem cells, but may also be a functional unit providing complex and dynamic interactions with stem cells.[75] Niches are major determinants of the microenvironment and may serve as the basis for indirect targeting of GSC. Indirect GSC targeting strategies (Table 2) involve three niches: (i) the perivascular niche (via angiogenic pathways); (ii) the hypoxic niche (via hypoxia-inducible factors); and (iii) the immune niche (via costimulatory, regulatory and suppressive pathways).

Perivascular niche. The perivascular niche is a key component of the NSC niche.[76] Neural stem cell (NSC) cluster in locations that are densely vascular and they are maintained in their undifferentiated and self-renewing state by signaling molecules secreted by endothelial cells.[76,77] Compelling data have also been put forward supporting a perivascular niche for GSC.[78] Immunofluorescent analysis of HGG specimens revealed that GSC (expressing both CD133 and Nestin) are closely associated with tumor vasculature (visualized by CD34).[78] These results were reproduced in vitro, where it was demon-strated that CD133+ GSC physically interact with endothelial cells.[78]

As with NSC, GSC are maintained in a state of undifferentiation and self-renewal by endothelial-derived factors.[78,79] Investigation of potential endothelial-mediated signaling pathways revealed mammalian target of rapamycin (mTOR) as a key player,[79] and therefore potential target. Inhibition of mTOR (either pharmacologically with rapamycin, PP242, and PI103, or with siRNA) halted endothelial-mediated GSC formation and expansion.[79]

Intracranial coimplantation of GSC with endothelial cells significantly accelerated tumor growth in a cell dose-specific manner and significantly increased the number of CD133+ GSC compared with implantation of GSC alone.[78] Treatment of experimental glioma with bevacizumab, a well-known anti-angiogenic agent that acts by inhibiting vascular endothelial growth factor (VEGF), depleted the tumor vasculature and significantly decreased the number of tumoral CD133+ GSC, which was also associated with significantly smaller tumor size.[78] That landmark study not only confirmed the presence of a perivascular niche, but also identified the perivascular niche as a therapeutic target. Initial observations noted that tumors originating from implantation of CD133+ GSC were significantly more vascular and hemorrhagic, and examination of proangiogenic factors revealed that GSC promoted angiogenesis primarily through VEGF.[80] The anti-VEGF antibody bevacizumab inhibited the effects of GSC on endothelial cells and reduced experimental tumor growth.[80] Studies have also shown the inhibition of GSC VEGF by ATRA, previously described as a GSC prodifferentiation agent that signals through the RAR.[66] Subsequent studies found that GSC cultures also expressed high levels of stromal derived factor-1 (SDF-1) and that treatment of glioma-bearing mice with a small-molecule SDF-1 antagonist (AMD3100) significantly decreased angiogenesis in vivo.[81]

Finally, interesting new data have revealed a role for targeting the IFN-β pathway as a mean to modulate the perivascular niche.[82] Treatment of experimental glioma with IFN-β decreased the number of GSC in vivo, in association with a significant increase in the number of perivascular cells and physical distance between GSC and endothelial cells of the vascular niche.[82] The decrease in GSC was notably absent in vitro, corroborating that the decrease in GSC in vivo was secondary to effects on the perivascular niche.

The combination of anti-angiogenic and cytotoxic treatments to decrease the GSC fraction has been tested in rodents[83] and a clinical trial is ongoing to test the effects of anti-VEGF-based therapy alone and in combination with cytotoxic agents.[84] Overall, the perivascular niche has garnered attention as an important therapeutic target.[85]

Hypoxic niche. Hypoxia has been reported to play a key role in the maintenance and regulation of NSC, leading to the recognition of a "hypoxic niche".[86,87] In the setting of hypoxia with oxygen and glucose deprivation, NSC upregulate baseline expression of hypoxia-inducible factor (HIF)-1α, which, in turn, upregulates the expression of VEGF as a protective mechanism to stimulate angiogenesis and renormalize the microenvironment.[86] Blockade of VEGF (using a receptor antibody) partly reverses the protection afforded by increased HIF-1α expression.[86]

Multiple reports have also established key regulatory functions of the hypoxic microenvironment on GSC, leading to the recognition of a hypoxic stem cell niche also for GSC.[13,14,88–90] Hypoxia can increase GSC stemness. Expression of several stem cell markers in GSC (e.g. CD133, A2B5, Nestin, Oct4, and Sox2) is upregulated,[13,88–90] whereas the expression of differentiation markers (e.g. glial fibrillary acidic protein) is downregulated.[13,89] The proliferation rate and self-renewal potential of GSC are also significantly increased.[13,14,89] Mechanistic analysis revealed roles for HIF-1α and HIF-2α. In the setting of

Table 2. Indirect glioma stem cell targeting strategies

Approach	Mechanism	Target
Perivascular niche	Angiogenic	VEGF
		mTOR
		SDF-1
		RAR
		IFN-β
Hypoxic niche	Hypoxia inducible	HIF-2α
		VEGF
Immune niche	Co-stimulatory	B7-H1
	Regulatory	T_{reg}
	Suppressive	TGF-β
		PGE_2
		CCL2
		Galectin-3
		STAT-3

These indirect strategies are directed at the perivascular, hypoxic and immune niches. CCL2, chemokine (C-C motif) ligand 2; HIF-2α, hypoxia-inducible factor-2α; IFN-β, interferon-β; mTOR, mammalian target of rapamycin; PGE_2, prostaglandin E_2; RAR, retinoic acid receptor; SDF-1, stromal derived factor-1; STAT-3, signal transducer and activator of transcription 3; T_{reg}, regulatory T cells; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

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severe hypoxia (i.e. 1% oxygen), the expression of HIF-1α and HIF-2α is upregulated in GSC.[88–90] The degree of hypoxia influences expression, with milder hypoxia (i.e. 7% oxygen) associated with only minimal HIF-1α expression and moderate upregulation of HIF-2α expression.[13] Upregulation of HIF-1α in hypoxia is a shared molecular response between GSC and both non-stem glioma cells and NSC. The expression of HIF-2α, however, is unique to GSC.[14,88] Expression of HIF-2α, but not HIF-1α, correlates with HGG patient survival.[88] Of note, HIF-2α is expressed by GSC under normoxic conditions.[88,89] Histopathologic evaluation of clinical HGG specimens reveals enrichment of GSC in both vascular and hypoxic, or necrotic, areas.[88,90] In one study,[88] this was determined by colocalization of CD133 and HIF-2α-positive staining, consistent with the GSC expression of HIF-2α in normoxia. These results highlight the presence of an anatomically separate hypoxic niche for GSC.[14,88] Functional studies revealed overlapping yet distinct roles for HIF-1α and HIF-2α. Knockdown of HIF-1α in GSC did not affect stem cell marker expression, but significantly reduced both neurosphere formation[90] and VEGF expression,[88] with downstream effects on endothelial cell proliferation. Knockdown of HIF-2α in GSC significantly diminished stem cell marker expression (consistent with previously described downstream targets, such as Oct4, Sox2, and c-myc), in addition to reducing neurosphere formation and decreasing VEGF expression to a greater extent that HIF-1α, with functional downstream effects on endothelial cells.[88] In non-GSC, knockdown of HIF-2α had no effect on VEGF production, consistent with its largely absent expression.[88] Interestingly, over-expression of HIF-2α in non-GSC reprograms the non-GSC into GSC,[14] highlighting the importance of the hypoxic regulatory microenvironment. In vivo, knockdown of either HIF decreased tumor formation and survival of tumor-bearing mice.[88] Together, these data demonstrate that the hypoxic microenvironment is a distinct anatomic niche enriched with GSC that can be targeted by downregulation of HIF-2α, with downstream effects on stem cell marker expression and VEGF signaling. Blockade of VEGF can downregulate one of the effector mechanisms in the hypoxic niche and so it may be considered an indirect target of the hypoxic niche. Although blockade of VEGF signaling can functionally affect both the hypoxic and perivascular niches, this does not imply that these niches are indistinguishable. The hypoxic niche is a separate entity that merits further attention as a distinct target.

Immune niche. Finally, evidence points to the active contribution by GSC to mechanisms of immune evasion, suggesting the presence of an ''immune niche''. Mechanisms of glioma immune resistance can be divided into three areas: (i) abnormal immune cell activation; (ii) deregulation of cellular immunity; and (iii) immunosupressive secretory factors.[91] As shown schematically in Figure 3, GSC have been ascribed immunomodulatory properties in all three areas, which together allow the GSC to remain in an immune-privileged environment, here termed the ''immune niche''.

Considering first the area of abnormal cell activation, GSC express major histocompatibility complex (MHC) Class I and the inhibitory costimulatory molecule B7-H1 on their surface, but not MHC Class II CD40 or CD80.[92] This cocktail of surface molecule expression induces T cell anergy and apoptosis, allowing GSC evasion of the immune system.[92] B7-H1 is notable because of its link to the PTEN/Akt pathway in HGG[93] and its ability to promote T cell apoptosis,[94] making it an attractive target based on the availability of neutralizing antibodies.[91] In addition, GSC play a role in deregulation of immune responses via their ability to expand the pool of regulatory T cells (T$_{reg}$) and suppress T cell proliferation, as well as induce T cell apoptosis.[92] The T$_{reg}$ cells are disproportionately high in HGG and their presence correlates with overall malignant

behavior.[95] Recent data acquired with high-resolution in vivo imaging of T$_{reg}$ in a hematopoietic stem cell model have highlighted T$_{reg}$ as critical mediators of immune stem cell privilege,[96] further corroborating the concept of the ''immune niche''. The generation of T$_{reg}$ in HGG is affected by the presence of soluble factors, which leads into the third major area of immunoresistance. Upon stimulation, GSC secrete several candidate cytokines, including TGF-β, PGE$_2$, chemokine (C-C motif) ligand 2 (CCL2), and galectin-3.[92] Transforming growth factor-β represents a likely mechanism for T$_{reg}$ expansion by GSC.[92] Prostaglandin E$_2$ (PGE$_2$) has the potential to downregulate MHC Class II expression and enhance T$_{reg}$ proliferation.[91] Chemokine (C-C motif) ligand 2 (CCL2) may be a principal chemoattractant for T$_{reg}$ based on increased expression of the CCL2 receptor,[97] although its potential may be limited given data that it also plays a role in the migration of NSC.[98] Galectin-3 belongs to a family of adhesion molecules with affinity for β-galactosides expressed in HGG and is implicated in the induction of T cell apoptosis, thereby assisting in evasion of the immune system.[99] A common transcriptional theme underlying several of these immune molecules is STAT-3. Glioma stem cells (GSC) induce expression of STAT-3 in immune cells.[92] Blockade of STAT-3 signaling using the small-molecule inhibitor WP1066 or siRNA restored T cell number, inhibited T cell apoptosis, and reduced the size of the glioma-associated GSC-induced T$_{reg}$ pool,[92] pointing to STAT-3 as another therapeutic target for the GSC immune niche. Together, the results demonstrate the immune niche as an indirect therapeutic GSC target.

Conclusions

In the past decade there has been an explosive growth in stem cell research. Although initially confined to the area of development, the field expanded to include neuro-oncology with the discovery of GSC. Glioma stem cells (GSC) have come under intense scrutiny as the potential culprits behind the dismal survival rate in patients with HGG. Multiple candidate GSC targets have been revealed, with varying degrees of promise, and many more will likely be identified in upcoming years. Therapeutic targets reported thus far were reviewed here using a novel framework, broadly based on the division of direct and indirect strategies. Direct strategies include several approaches, such as overcoming GSC resistance to standard therapies, blocking their function and inducing differentiation. Indirect strategies encompass approaches targeting the various GSC niches, namely the perivascular, hypoxic, and immune niches. This framework may prove useful as increasing numbers of targets continue to be identified and the potential for combination strategies arises.

Irrespective of the specific target, a major obstacle of any GSC direct targeting strategy is the need to overcome selectivity with respect to normal stem cells. Similar to the sought-after selectivity for anti-tumor agents to induce cell death in tumor cells but not normal cells, agents targeting GSC will have to demonstrate that they selectively affect GSC and not normal stem cells in order for their clinical potential to be realized. From this perspective, it is possible that some of the pathways identified in GSC are too fundamental to the development and/or maintenance of normal stem cells. This, in turn, would imply that targeting them would affect, and potentially have serious adverse effects, on normal stem cells. Therefore, the requirement of selectivity is a crucial parameter to address prior to consideration for translation to the clinical setting.

Similar reasoning applies to the targeting of GSC niches. It will be important for a therapeutic strategy to specifically target the GSC niche without interfering with normal protective niche-mediated mechanisms in the setting of normal function or biologic stressors. Given the data supporting key regulatory
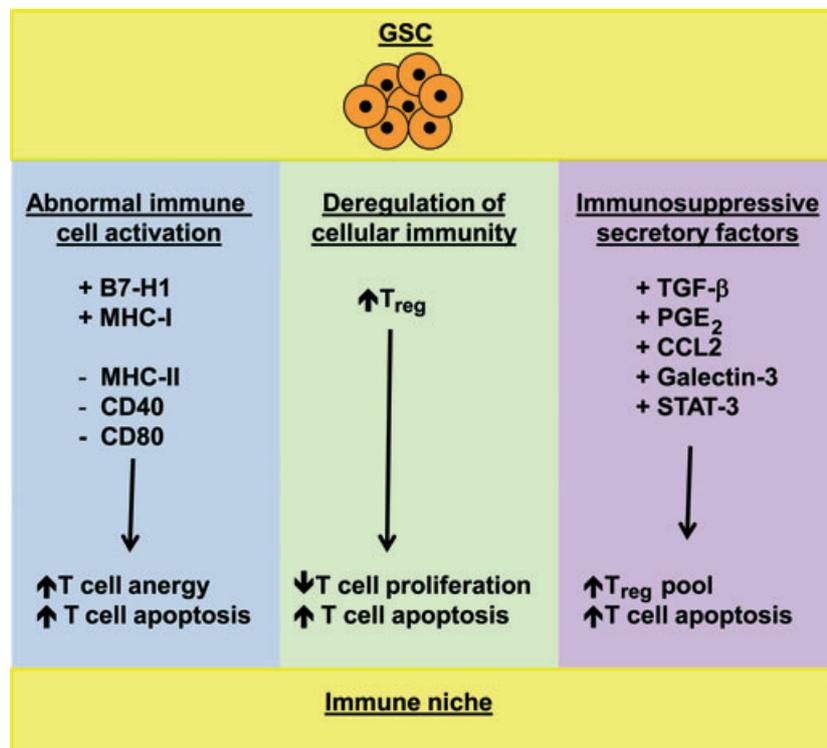
Fig. 3. Glioma stem cells (GSC) actively contribute to mechanisms of immune privilege, creating an ''immune niche''. Mechanisms of glioma immune resistance can be divided into three areas: (i) abnormal immune cell activation; (ii) deregulation of cellular immunity; and (iii) immunosuppressive factors. Glioma stem cells have been ascribed immunomodulatory properties in the three areas, leading to overall suppressed T cell proliferation, increased T cell apoptosis, and, most importantly, expansion of the regulatory T cell (T_{reg}) pool. Together, the results point to the immune niche as a potential indirect therapeutic target. CCL2, chemokine (C-C motif) ligand 2; MHC, Major histocompatibility complex; PGE_2, prostaglandin E_2; STAT-3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor-β.

functions of the microenvironment (such as the ability to transform non-GSC into GSC, thereby supporting the stochastic model of glioma initiation), combination strategies where direct GSC targeting strategies are combined with indirect strategies targeting GSC niches may be the most successful in definitive glioma treatment.

Future studies on potential candidate targets should specifically address the issue of selectivity prior to their translation to the clinical setting. Nonetheless, targeting GSC and their niches offers true potential for ameliorating the current therapeutic strategies to extend patient survival.

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