Glioblastoma ranks among the most lethal of all human cancers. Glioblastomas display striking cellular heterogeneity, with stem-like glioblastoma stem cells (GSCs) at the apex. Although the original identification of GSCs dates back more than a decade, the purification and characterization of GSCs remains challenging. Despite these challenges, the evidence that GSCs play important roles in tumor growth and response to therapy has grown. Like normal stem cells, GSCs are functionally defined and distinguished from their differentiated tumor progeny at core transcriptional, epigenetic, and metabolic regulatory levels, suggesting that no single therapeutic modality will be universally effective against a heterogeneous GSC population. Glioblastomas induce a systemic immunosuppression with mixed responses to oncoimmunologic modalities, suggesting the potential for augmentation of response with a deeper consideration of GSCs. Unfortunately, the GSC literature has been complicated by frequent use of inferior cell lines and a lack of proper functional analyses. Collectively, glioblastoma offers a reliable cancer to study cancer stem cells to better model the human disease and inform improved biologic understanding and design of novel therapeutics.

Tumors are not homogeneous masses of neoplastic cells; rather, they contain ecosystems with diverse neoplastic populations and recruited supportive stroma. Numerous efforts to model the complexity of neoplastic populations have included not only integration of the tumor microenvironment but also efforts to explain heterogeneous tumor cells based on genetic and epigenetic diversity. The cancer stem cell hypothesis represents one element of core tumor genetics and epigenetics. The cancer stem cell hypothesis holds that tumors mimic normal tissues with hierarchically arranged and dynamically regulated populations of cells, with stem-like cells at the apex that display regenerative potential and the capacity to recapitulate the entire functional diversity present within the original tumor.

The underpinnings of cancer stem cell modeling date back to early functional studies of cancer, which showed that the injection of a single leukemic cell into mice could produce a lethal leukemia in as little as 2 wk [Furth et al. 1937]. In the modern era, the cancer stem cell model was revitalized by Dick and coworkers (Lapidot et al. 1994), who identified a subset of patient-derived leukemia cells able to traffic to the bone marrow of immunodeficient mice, with sustained proliferation and maintenance of the original leukemic cell phenotypes. Leukemia-initiating cells differentiate in vivo and possess self-renewal properties [Bonnet and Dick 1997]. These landmark studies kicked off the hunt for cancer stem cells in additional tumor types, including breast cancer [Al-Hajj et al. 2003], brain cancer [Hemmati et al. 2003, Singh et al. 2003, 2004], prostate cancer [Collins et al. 2005], colorectal cancer [O’Brien et al. 2007, Ricci-Vitiani et al. 2007], and pancreatic cancer [Li et al. 2007]. Amid the rapid pace of identification of novel cancer stem cells in a variety of cancer types, challenges to the cancer stem cell model have been raised: (1) the relevance of the cancer stem cell model to inform our understanding of the disease state and guide therapeutic development, (2) the cell of origin of cancer stem cells in many tumor types, and (3) the liberal use of the term “cancer stem cell” without strict adherence to the required functional definition.

Clinical relevance of glioblastoma stem cells (GSCs)

Glioblastoma is the most common of all primary malignant central nervous system (CNS) tumors, with a dismal 5-yr survival rate of only 5% and a median survival of <15 mo [Stupp et al. 2009, Ostrom et al. 2015].
Glioblastomas are classified by the presence of characteristic mutations, which often provide prognostic information, and also into three main tumor-intrinsic transcriptional subtypes: proneural, classical, and mesenchymal, although significant intratumoral heterogeneity exists (Wang et al. 2017a). Current standard of care includes gross total surgical resection followed by concurrent treatment with radiotherapy and temozolomide, a cytotoxic chemotherapy (Stupp et al. 2005, 2009). Tumor-treating fields (TTFs) represent a new therapeutic modality that extends overall patient survival from 16 to 20.9 mo when used in combination with radiotherapy and chemotherapy (Stupp et al. 2017).

Major contributors to the poor prognosis of glioblastoma patients include a high degree of intratumoral cellular heterogeneity and plasticity, the infiltrative and migratory nature of glioblastoma cells, and a high rate of recurrence. Recurrent tumors are frequently evolutionarily divergent from the original tumor, with distinct drivers and sensitivities, limiting the informative capacity of initial biopsies when treating recurrent disease (Kim et al. 2015b). Many of these features can be modeled through the lens of the cancer stem cell hypothesis. Functionally defined GSCs have been identified in human brain tumors (Singh et al. 2003, 2004) and play important roles in mediating therapeutic resistance through supporting radiore sistance [Bao et al. 2006a], chemoresistance [Liu et al. 2006; Chen et al. 2012], angiogenesis [Bao et al. 2006b; Cheng et al. 2013], invasion [Wakimoto et al. 2009], and recurrence [Chen et al. 2012]. Both in vitro and in vivo observations support the existence of subpopulations of human tumor cells that express stemness-related markers, are capable of initiating tumors, and recapitulate tumor heterogeneity when injected orthotopically into mice (Singh et al. 2004; Lee et al. 2006). Thus, gaining a deeper understanding of the underlying molecular processes that drive cancer stem cell maintenance, plasticity, and resilience will enhance our ability to selectively target and ablate these tumor-initiating and -propagating populations.

In glioblastoma, cancer stem cell controversies largely stem from a vague and disputed definition of GSCs. Here, we suggest a definition based on a series of practical functional criteria that verify the cellular capacity to self-renew, initiate tumors upon serial transplantation, and recapitulate tumor cell heterogeneity (Fig. 1A). This definition is inherently retrospective, as cancer stem cells can...
be conclusively identified only after performing an experiment that alters the state of the original cell, precluding a prospective identification of GSCs through the use of cell surface or other cellular markers. While it is important to agree on a functional definition for GSCs to determine their relevance in disease and mechanisms to selectively target these cells, it is equally important to understand the limitations of this model. First, the term “glioblastoma stem cell” does not make any claims about tumor cell of origin. While the presence of GSCs may imply the malignant transformation of a normal tissue stem cell, GSCs may also arise from more differentiated neoplastic cells that gain access to stem-like developmental and survival programs through genetic perturbations (Fig. 1B). Second, GSCs are not defined by the presence or absence of molecular markers. Although certain cellular features (such as CD133, CD44, and CD15) may enrich the frequency of GSCs within cellular populations, these markers are not completely sensitive or specific for GSC populations (Beier et al. 2007). Third, the GSC definition does not suggest that the stem state is static. A striking plasticity exists between different cellular states of the tumor, which allows for interconversion between GSC and non-GSC states depending on a number of factors. Microenvironmental exposures, including nutrient deprivation, hypoxia, radiation, and others, shift the dynamics of regulation of these interconversions, bringing about changes in the GSC and non-GSC pools, along with phenotypes such as proliferation or quiescence (Fig. 1C,D). Fourth, while the GSC model highlights the importance of stem-like cell populations in mediating cellular heterogeneity and disease recurrence, the GSC model does not make claims about the frequency of this cell type within tumors or diminish the role of more differentiated progeny cells that play critical roles in the maintenance of complex tumor tissue systems. Here, we provide a review of the most recent evidence regarding the existence of GSCs in glioblastoma tumors and progress made in defining their key molecular regulators, with an eye toward harnessing these breakthroughs to inform the development of novel therapeutic strategies.

**Emerging evidence of cancer stem cells in glioma and glioblastoma**

Recent technological advances, including single-cell RNA sequencing (scRNA-seq) approaches and lineage-tracing barcoding methods, have provided further evidence for the existence of a population of GSCs in human glioblastoma tumors. These studies allow for less biased identification of stem-like populations in their native environments without relying on culture, although these analyses are limited by reliance on cellular markers without validation of the required functional characteristics. scRNA-seq of primary tumor specimens captured the great extent of intratumoral heterogeneity and identified a slow-dividing quiescent population of stem-like cells in glioblastoma tumors (Patel et al. 2014). Tumor cells exist in a continuous spectrum along the stemness–differentiation axis and not as discrete populations, as are often studied in vitro (Patel et al. 2014). In oligodendrogliomas, scRNA-seq revealed the presence of undifferentiated stem-like cells with enrichment for cell cycle and proliferative transcriptional programs, suggesting that these stem-like cells support tumor growth (Tirosh et al. 2016). Single-cell sequencing of pediatric gliomas bearing histone Histone 3 Lys27 [H3K27M] mutations revealed four broad expression programs in their constituent cells, including an oligodendrocyte precursor cell (OPC)-like program, with up-regulation of cell cycle genes (Filbin et al. 2018). Undifferentiated stem-like precursor cells were also identified that may serve as a common initiating cell for both isocitrate dehydrogenase 1 [IDH1] mutant astrocytomas and oligodendrogliomas. In this study, stem cell and cell cycle genes were positively correlated at the single-cell level (Venteicher et al. 2017). While normal neural stem cells are commonly quiescent, other normal tissues (e.g., the skin and gastrointestinal systems) contain discrete self-renewing populations that can be highly proliferative or quiescent. Thus, divergent proliferative potentials for GSCs may capture this diversity, although there may also be differences between high- and low-grade gliomas or cancer stem cells that reside at different stages of the stemness gradient with distinct proliferative capacities. Lineage-tracing approaches and mathematical modeling of successive xenograft outgrowth assays using human glioblastoma cells suggest that slow-cycling stem cells undergo limited asymmetric cell division to generate a transit-amplifying progenitor-like population that generates short-lived differentiated progeny (Lan et al. 2017). Together, these granular approaches have advanced our understanding of GSCs in their native environment.

**Glioblastoma cell of origin**

Although glioblastomas represent the first and one of the most highly characterized cancers by The Cancer Genome Atlas [TCGA] at the genomic level (Brennan et al. 2013; Ceccarelli et al. 2016), controversy remains with respect to its precise cell of origin. While some contend that glioblastomas arise from a subpopulation of neural stem cells, others argue that transformation of more differentiated astrocytes may give rise to glioblastomas (Fig. 1B). In mouse models, overexpression of active Ras and Akt or inactivation of the p53 and NF1 tumor suppressors in neural progenitor cells, but not in more differentiated astrocytes, was sufficient to induce formation of glioblastoma-like lesions (Holland et al. 2000; Zhu et al. 2005; Alcantara Llaguno et al. 2009). Others show that genetic alterations in either neural stem cells or differentiated astrocytes can give rise to glioblastomas in mouse models (Bachoo et al. 2002).

Reports in the last several years favor neural stem cells, typically localized at the subventricular zone (SVZ) or subgranular zone, as the glioblastoma cell of origin. Immature outer SVZ radial glial cells share a striking transcriptional similarity to glioblastoma cells, suggesting that these cells may be early glioblastoma precursors (Pollen et al. 2015). Overexpression of mutant IDH1, a key driver of low-grade gliomas, in adult SVZ neural stem cells
induces progenitor cell hyperplasia and tumor-like nodules consistent with early gliomagenesis in a mouse model [Bardella et al. 2016]. Deletion of the tumor suppressors NF1, TP53, and PTEN in either neural or oligodendrogial precursor cells induces glioblastoma-like lesions in a mouse model. Disruption of identical tumor suppressor genes in two distinct progenitor cell populations gave rise to distinct diseases, with neural precursor-derived tumors demonstrating more aggressive phenotypes than their oligodendrocyte precursor-derived counterparts (Alcantara Llaguno et al. 2015). PTEN mutations in neural stem cells are also able to induce neoplastic transformation, while the same mutations in mesenchymal stem cells do not [Duan et al. 2015]. These data suggest that the unique underlying molecular features of different neural progenitor cell populations poise particular populations for tumorigenesis and endow the resulting tumor with distinct functional properties, highlighting the importance of understanding the cell of origin.

Compelling evidence from a series of high-throughput sequencing and mouse modeling approaches has suggested that astrocyte-like stem cells from the astrocytic ribbon layer of the SVZ are the glioblastoma cell of origin, further substantiating prior reports [Lee et al. 2018]. Whole-exome and single-cell sequencing analyses from matched human glioblastoma tissue and normal SVZ tissue revealed that (1) approximately half of patients contained shared mutations between tumor and SVZ tissue, (2) most somatic mutations and copy number alterations were tumor-private, and (3) single-cell clones from SVZ tissue with mutations shared with the tumor tissue lacked tumor-private mutations. Mouse modeling data showed that induction of mutations within the SVZ promoted tumor formation and migration from the SVZ, while the same mutations did not lead to tumor initiation when induced in cortical astrocytes (Lee et al. 2018). These recent reports highlight the importance of cellular background in the establishment of glioblastomas and suggest that neural precursor populations are uniquely situated for malignant transformation following the appropriate genetic insults. Taken together, these studies support a cancer stem cell model in gliomas whereby a small population of GSCs derived from transformed SVZ-derived neural stem cells acts to maintain tumor heterogeneity, although controversy remains regarding the applicability of mouse modeling data to the human disease. In the following sections, we review the field’s progress in understanding the defining molecular characteristics and potential targetable vulnerabilities of this stem cell population.

**Modeling glioblastoma: isolation, enrichment, and propagation of GSCs**

Strategies to isolate and enrich cancer stem cells are based on the methods used to isolate their normal counterparts: tissue stem cells. These methods involve using specific cellular markers and growing cells in defined suspension cultures to distinguish between GSCs and other nonstem tumor cells. Strategies to identify GSCs using cellular markers rely on the sensitivity and specificity of cell surface factors to enrich for stem-like populations. However, the spectrum of GSC markers has an extensive overlap with those used for identification of neural stem cells. These include intracellular proteins (SOX2, OLIG2, MYC, and NESTIN) and the cell surface markers (CD133, LiCAM, CD44, and A2B5) [Brescia et al. 2012]. As we understand the biology of GSCs in greater depth, we should re-evaluate these current methods. The neurosphere formation assay depends on self-renewal properties of GSCs, which allows for growth within defined nonadherent medium conditions. Caveats with this method include the inability to reflect the precise number of cells with in vivo tumor formation capacity and the inability to detect quiescent stem cells. An alternative to neurosphere cultures is two-dimensional adherent culture of GSCs on poly-L-lysine/laminin-coated plates, which reduces cellular differentiation [Lee et al. 2006]. However, both of these methods fail to properly model the interaction of GSCs with the various other cell types that exist in vivo.

Recently, three-dimensional GSC organoid culture systems have been developed, which more faithfully recapitulate in vivo tumor growth, cellular heterogeneity, and hypoxic gradients [Hubert et al. 2016]. CRISPR–Cas9 genomic editing can also be used to generate glioblastoma organoid models. Introduction of the HRasG12V allele into the TP53 locus in cells of cerebral organoids drove invasive phenotypes both in the organoid model and when orthotopically xenografted in immunodeficient mice with transcriptional similarities to mesenchymal tumors [Ogawa et al. 2018]. Somatic in vivo CRISPR/Cas9-mediated genomic editing approaches have also been applied to study glioblastomas in mouse models [Oldrini et al. 2018] and have allowed for the performance of large-scale screens in vivo (Chow et al. 2017). These organoid and animal systems may better model in vivo environments to improve our understanding of GSCs in their appropriate physiological context. Despite these advances, new methods for the prospective isolation and propagation of GSCs are required. The ideal in vitro assay should accurately assess the tumor formation ability of GSCs, be largely independent of growth mediated by high amounts of exogenous growth factors, incorporate various cellular interactions, and recapitulate the tumor microenvironment. Furthermore, efforts should be made to develop models that reflect the diverse mutational spectrum of glioblastoma to more completely understand the role of genetic background in cellular dependencies and therapeutic response [Table 1].

**Glioma stem cell epigenetics: interface between environment and cellular response**

While nearly every cell in the human body contains an identical genomic blueprint, epigenomic regulation of this genomic code allows for the generation of cell and developmental stage-dependent transcriptional programs through opening relevant genomic regions while sequencing others [Fig. 2A]. Appropriate epigenetic regulation is critical for the maintenance of GSCs and serves to
integrate information from numerous cellular inputs. The presumed initiating event in low-grade gliomas (along with other cancers) is mutation of IDH1 or IDH2, which generates widespread epigenomic dysregulation through generation of the glioma-CpG island methylator phenotype (G-CIMP) hypermethylator phenotype (Turcan et al. 2012). The epigenetic alterations induced by IDH mutation in vitro are partially reversible, contribute to transcriptional remodeling and deposition of histone modifications at specific genomic loci, promote the emergence of a CD24+ stem-like population, and contribute to increased genomic instability (Turcan et al. 2018). In addition to the more

| Table 1. Advantages and limitations of GSC and glioblastoma models |
|---------------------------------|----------------|----------------|----------------|
| **GSC modeling methods** | **Advantages** | **Limitations** | **References** |
| Sorting using cellular markers (CD133, CD15, and others) | Prospective identification of putative stem populations is possible | Individual stem markers are frequently disputed; no functional criteria are used; may deplete GSC heterogeneity; integrity of surface markers may be affected during single-cell dissociation | Galli et al. 2004, Singh et al. 2004, Bidlingmaier et al. 2008, Gilbert and Ross 2009, Son et al. 2009, Wan et al. 2010 |
| In vitro neurosphere culture | Uses a functional assay for self-renewal capacity; high throughput | Inability to determine tumor formation capacity, identify quiescent stem populations, and model diverse cellular interactions; reliance on artificial and unphysiological medium conditions with loss of tumor heterogeneity; contain populations of stem and differentiated cells; not ideal for assays in which homogeneity is required; percentage of enriched GSCs will vary with every sphere depending on size, passage, and technique used in propagation and culture medium | Lee et al. 2006, Wan et al. 2010, Pastrana et al. 2011 |
| Two-dimensional adherent culture on poly-L-lysine/laminin plates | May reduce differentiation compared with neurosphere culture; ideal for assays in which homogeneity is required; high throughput | Inability to determine tumor formation capacity, identify quiescent stem populations, and model diverse cellular interactions; reliance on artificial and unphysiological medium conditions with loss of tumor heterogeneity | Pollard et al. 2009 |
| Three-dimensional GSC organoid culture systems and biomaterial scaffolds | Recapitulate in vivo environment with higher fidelity than in vitro systems; model cellular and stromal interactions; model hypoxic and other biologic gradients; model multicellular and microenvironmental interactions | Decreased throughput compared with two-dimensional methods; complex procedure for initiation and maintenance; lack certain cellular interactions, including with vasculature, microglia, and others | Hubert et al. 2016, Bian et al. 2018, Ogawa et al. 2018, Langer et al. 2019 |
| Genetically engineered and syngeneic mouse modeling approaches | Allow for studies of tumor initiation and progression with ability to model cellular and immune interactions in a native in vivo environment | Systems to generate murine tumors do not fully recapitulate tumorigenesis in humans; mouse tumors may be fundamentally different from the human disease; expensive and labor-intensive | Holland et al. 2000, Zhu et al. 2005, Bardella et al. 2016, Miyai et al. 2017, Oldrini et al. 2018, Hambardzumyan et al. 2009 |
| Patient-derived xenograft methods | Allow for studies of human cancers with an ability to model cellular interactions in a more physiologic in vivo environment | Require immediate generation following tumor surgical resection; inability to assess mechanisms of tumor initiation or model adaptive immune interactions; serial passaging may deplete heterogeneity and induce genomic alterations, expensive and labor-intensive | Singh et al. 2004, Hidalgo et al. 2014, Ben-David et al. 2017, Jung et al. 2018 |
at subgroup-specific superenhancers classifies tumors and 
mothers maintaining histones 2A on Lys119. (K27-ac) acetylation of histone 3 on the sixth position of adenine bases (5mC) 5-methylcytosine; (K27-ac) acetylation of histone 3 on Lys27; (K27-me) methylation of histone 3 on Lys27; (K119-ub) ubiquitination of histone 2A on Lys119. [B] Transcriptional regulators and posttranscriptional processes modify gene expression to support GSCs. [m^6A] N^6-methyladenosine.

well-known inhibition of histone and DNA demethylases, the mutant IDH oncometabolite 2-hydroxylutarate [2-HG] (R)-2-hydroxyglutarate; (N^6-mA) N^6-methyladenine; (SmC) 5-methylcytosine; (K27-ac) acetylation of histone 3 on Lys27; (K27-me) methylation of histone 3 on Lys27; (K119-ub) ubiquitination of histone 2A on Lys119. [B] Transcriptional regulators and posttranscriptional processes modify gene expression to support GSCs. [m^6A] N^6-methyladenosine.

Figure 2. Epigenetic and posttranscriptional regulation of GSCs. [A] Critical epigenetic regulators drive GSC maintenance and response to external cues by regulating gene expression programs. (2-HG) (R)-2-hydroxylutarate; (N^6-mA) N^6-methyladenine; (SmC) 5-methylcytosine; (K27-ac) acetylation of histone 3 on Lys27; (K27-me) methylation of histone 3 on Lys27; (K119-ub) ubiquitination of histone 2A on Lys119. [B] Transcriptional regulators and posttranscriptional processes modify gene expression to support GSCs. [m^6A] N^6-methyladenosine.

Individual epigenetic and chromatin remodeling factors drive glioblastoma biology in a context-dependent manner, highlighting the microenvironment-dependent essentiality for epigenetic regulators. Under more physiologic conditions in vivo, transcription pause–release and elongation factors are required for GSC maintenance and survival, while, in cell culture conditions, these factors are dispensable [Miller et al. 2017]. Regulators of transcription elongation machinery, including RBPJ, are essential for GSC maintenance and tumor formation capacity [Xie et al. 2016]. Even in vivo, drastic intratumoral heterogeneity exists, requiring tumor cells to adapt to their unique environments. In vascular tumor regions defined by a relative abundance of oxygen and nutrients, the PRCC family epigenetic regulator EZH2 predominates and drives a proneural-like transcriptional profile, while, in nutrient-poor necrotic regions, BMI1 signaling is active to drive a mesenchymal-like transcriptional profile and promote survival in a hostile microenvironment [Jin et al. 2017]. In addition to intrinsic microenvironmental insults faced by cancer cells, GSCs also adapt to cytotoxic and targeted therapeutic agents to mediate therapy resistance. In response to inhibition of receptor tyrosine kinase signaling, KDM6A/B-mediated epigenetic remodeling promotes treatment resistance by driving a transition from a rapid proliferative state to a slowed, Notch-dependent quiescent state [Liu et al. 2017]. KDM2B also acts as an essential epigenetic remodeler in GSCs, inhibition of which sensitizes cells to lomustine therapy [Staberg et al. 2018].

Epigenetic regulation can act as a gatekeeper to cellular access to stem and differentiation programs. The pioneer transcription factor ASCL1, which serves as a master regulator of neuronal lineage differentiation, can also act to preserve differentiation capacity in GSCs, specifically when cells are treated with Notch inhibitors [Park et al. 2017]. Integrative epigenomic analyses revealed that GSCs fail to appropriately remodel their DNA methylation profile in response to differentiation cues, suggesting that epigenetic remodeling factors can lock GSCs into a more primitive state [Zhou et al. 2018].

Canonical drivers of glioblastoma, whether they act as oncogenes or tumor suppressors, can function to shape the GSC epigenome. Constitutive activation of EGFR through the exon 2–7 truncation mutation [EGFRvIII] promotes remodeling of the epigenome through overexpression of the SOX9 and FOXG1 transcription factors, which drive oncogenic and proliferative programs [Liu et al. 2015]. FOXG1 and another SOX family member, SOX2, were further implicated in repressing differentiation and supporting stem-like proliferative phenotypes in part through activation of stem regulators and repression of FOXO3 [Bulstrode et al. 2017]. A panel of transcription
factors, including SOX2, OLIG2, and ZEB1, transforms astrocytes into tumor-initiating cells even in the absence of oncogenic driver mutations, suggesting that altered epigenetic landscapes can drive tumor formation (Singh et al. 2017). Another significantly mutated gene in glioblastoma, PTEN, shapes the glioblastoma epigenome through nonenzymatic interactions with the chromatin regulator DAXX, which regulates histone H3.3 genomic localization (Benitez et al. 2017). Histone H3.3 levels can also be controlled by the chromatin regulatory element MLL5, which is critical for the maintenance of genomic architecture required for persistence in a stem cell state (Gal- lo et al. 2015).

Posttranscriptional regulation: RNAs as drivers of GSC biology

In the previous section, we discussed how GSCs fine-tune their genetic and epigenetic programs to regulate gene expression. Another complex node of gene regulation exists at the posttranscriptional level, including regulation of RNA maturation, cellular localization, stability, and alternative splicing of transcripts. These mechanisms contribute to the effective translation of transcripts into functional proteins, which ultimately carry out most cellular functions (Fig. 2B). Many of these processes are regulated by RNA-binding proteins (RBPs), a class of deeply conserved and highly abundant proteins, which form ribonucleoprotein complexes with transcripts to facilitate their functions (Hentze et al. 2018). In glioblastoma, many RBPs are expressed at high levels in patient tumors and portend poor prognosis, including SNRBP, a splicing factor that regulates RNA processing and DNA repair pathways (Correa et al. 2016). RNAi screens of patient-derived GSCs identified the splicing factor PHF5a as a selective dependency in GSCs but not in nontransformed controls, such as fibroblasts, astrocytes, and neural stem cells (Hubert et al. 2013). MYC overexpression in GSCs is associated with increased sensitivity to splicing inhibition (Hubert et al. 2013). Splicing factors can also be provided in the form of vesicular secretions from apoptotic cells that promote proliferation and therapy resistance in neighboring cells within a tumor (Pavlyukov et al. 2018). Serum microRNA levels are proposed to serve as novel microRNA-based strategies have the potential to be used in combination with conventional therapies as sensitizing agents (Anthiya et al. 2018). Further deeper screening of novel microRNAs is required for the identification of appropriate microRNA targets for glioblastoma.
**GSC metabolism: fueling tumor growth**

The metabolic dysregulation of cancer cells has been well documented for centuries and has served as an integral component of our understanding of cancer initiation, growth, and adaptation [Hanahan and Weinberg 2011; Pavlova and Thompson 2016]. Similar to other types of cancer cells, GSCs have high metabolic demands, some of which support rapid proliferation, and others that drive the maintenance of stemness (Fig. 3). Multiple reports have investigated metabolic networks underlying the bioenergetic capacity of GSCs, which up-regulate high-affinity nutrient transporters, including GLUT3, in part through aberrant integrin signaling networks to obtain sufficient glucose to support rapid metabolism and downstream pathways [Flavahan et al. 2013; Cosset et al. 2017]. Glucose obtained in this manner supplies substrates for nucleotide biosynthesis to support GSC proliferation [Wang et al. 2017c]. In addition to glucose, GSCs acquire nutrients from other sources, including glutamine and acetate, which provide bioenergetic and proliferative substrates. Glutamine is not used as an anaplerotic substrate to replenish tricarboxylic acid (TCA) cycle intermediates but is instead synthesized de novo in GSCs or taken up from surrounding tumor cells and astocytes to support purine biosynthesis [Tardito et al. 2015]. Both glucose and acetate, but not glutamine, are robustly oxidized in the TCA cycle to support energetic needs in glioblastomas and other brain tumors [Mashimo et al. 2014]. Glioblastomas rely on acetyl-CoA synthetase enzymes [namely, ACSS2] to convert acetate to acetyl-CoA and to allow for oxidation of acetate in the TCA cycle [Mashimo et al. 2014]. ACSS2 also has a nuclear role in epigenetic remodeling through repurposing acetate derived from protein deacetylation reactions to support histone acetylation at lysosomal and autophagic genes during instances of nutrient deprivation [Li et al. 2017a]. In other cancer models beyond glioma, the intersecting epigenetic and metabolic roles for ACSS2 have been well documented. ACSS2 promotes lipid biosynthesis through mediating histone acetylation under hypoxia or nutrient deprivation conditions [Gao et al. 2016]. Other metabolic enzymes, including the nicotinamide regulator NNMT [nicotinamide N-methyltransferase], also play epigenetic roles through regulating cellular methyl donor pools and act to control both histone and DNA methylation as well as protein methylation capacity [Ulanovskaya et al. 2013; Jung et al. 2017; Palanichamy et al. 2017].

GSCs must undergo frequent metabolic adaptations to survive in hostile conditions within rapidly proliferating tumors, which are characterized by low oxygen and nutrients and an abundance of wastes and necrotic tissue. Glioblastoma cells within poorly vascularized necrotic tumor regions up-regulate expression of SHMT2, a serine metabolism enzyme that can limit oxygen consumption by shifting cellular metabolism away from the TCA cycle through inhibition of pyruvate kinase [Kim et al. 2015a]. GSCs must scavenge iron from a nutrient-poor microenvironment and selectively up-regulate iron transporters to obtain this critical cofactor [Schonberg et al. 2015]. AMP-kinase [AMPK] is important for responding to oncogene-induced and metabolic stressors by regulating glycolysis and mitochondrial metabolism to support energetic requirements [Chhipa et al. 2018]. Oxidative metabolism is critical for GSC survival and is regulated through IGF2BP2, which enhances assembly of mitochondrial respiratory chain components and delivery of nuclear-encoded transcripts to the mitochondrial translation machinery [Janiszewska et al. 2012]. The FGFR3-TACC3 genetic fusion event in glioblastoma and other tumors drives mitochondrial oxidative phosphorylation to promote tumor growth [Frattini et al. 2018]. Dependency on oxidative or nonoxidative metabolism is heterogeneous throughout the tumor, with fast-cycling cells more dependent on anaerobic glycolysis, while slow-cycling cells rely on oxidative phosphorylation and lipid oxidation [Hoang-Minh et al. 2018].

Similarly to oxidative metabolism, the importance of lipid metabolism has often been overlooked in GSC biology. Fatty acid synthesis enzymes as well as fatty acid-binding proteins have essential roles in maintaining GSCs [De Rosa et al. 2012; Yasumoto et al. 2016]. Due to their location behind the neurovascular unit (also called the blood–brain or blood–tumor barrier), GSCs are sequestered from peripheral nutrient pools and thus are dependent on cholesterol metabolism and uptake for survival. These pathways can be selectively targeted using LXR agonists, which disrupt cholesterol uptake, or statins, which impair mevalonate and cholesterol synthesis [Villa et al. 2016; Wang et al. 2017b]. EGFR-driven glioblastomas are particularly dependent on lipid metabolism and fatty acid synthesis for survival [Guo et al. 2009] and up-regulate these pathways through activating SREBP-1, a master regulator of lipogenesis, through glycosylation of SCAP [Cheng et al. 2015].

![Figure 3](image-url) **Figure 3.** Multiple metabolic pathways power GSCs. GSCs depend on key enzymes to support their bioenergetic needs, requirements for proliferative and epigenetic substrates, and capacity to adapt to harsh microenvironments. (Ox. Phos) Oxidative phosphorylation; (TCA) tricarboxylic acid cycle.
findings discussed here, the roles of key metabolic enzymes and the utilization of their respective substrates are certain to be context-dependent. Caution should be used when interpreting results of metabolic profiling studies that are conducted in artificial cell culture systems replete with serum and under high-oxygen conditions.

GSC microenvironment: essential niche factors
In the previous sections, we described cell-intrinsic programs that GSCs use to adapt to widely variable microenvironental pressures. Here, we focus on key stromal signaling nodes, including those with vasculature, neurons, different tumor components, and the immune system, and explore how these interactions support GSCs (Fig. 4). At the earliest phases of tumor initiation, cancer cells coopt local vasculature dependent on vessel-derived angiopoietins and tumor-derived vascular endothelial growth factor [VEGF] (Holash et al. 1999; Bao et al. 2006b). While VEGF supports angiogenesis, its inhibition can drive acquisition of invasive mesenchymal phenotypes in certain glioblastoma patients, highlighting its complex role in the tumor ecosystem (Lu et al. 2012). To promote access to nutrients and a path for cell migration, glioma cells home to and invade along blood vessels using an Olig2–Wnt7 signaling axis (Griveau et al. 2018). In mouse models, aberrant oncogene-driven expression of tumor-localized ephrin-B2 ligands subverted normal repulsive interactions with ephrin-B2 on vascular endothelial cells to drive cancer cell invasion and proliferation (Krusche et al. 2016). Osteopontin derived from the perivascular niche serves an oncogenic role by promoting glioma cell survival and aggressiveness through a CD44–HIF2α axis-dependent activation of hypoxia response genes and maintenance of stem-like properties (Pietras et al. 2014). GSCs also respond to hypoxic environments by up-regulating vonin, which physically interacts with Notch and promotes its downstream signaling to support GSC maintenance (Man et al. 2018). Hypoxia signaling pathways are aberrantly activated in a GSC-intrinsic manner through the prevention of HIF2α degradation by high expression of ID2 (Lee et al. 2016).

In addition to receiving growth and survival signals from the vasculature, GSCs remodel vessels by differentiating into vascular pericytes or endothelial-like cells. GSC-

Figure 4. GSCs in context. Microenvironmental interactions with other tumor cells, neurons, macrophages, T cells, and the vasculature are key for supporting GSCs.
derived pericytes depend primarily on TGF-β and BMX-tyrosine kinase activity to shape their microenvironment and maintain the integrity of the blood–tumor barrier—interactions that can be targeted for therapeutic benefit (Cheng et al. 2013; Zhou et al. 2017; Shi et al. 2018). GSC-derived endothelial-like cells rely on WNT5A signaling to promote cellular lineage infidelity and acquisition of endothelial-like phenotypes, which promotes tumor neo-vascularization and invasion (Hu et al. 2016).

Signals derived from neuronal components of the tumor microenvironment are similarly critical for maintaining GSCs. Using optogenetic control of neuronal activity in xenograft models, neuronal stimulation was found to promote glioma growth via the soluble mitogen neuroligin-3, which is liberated from neurons (as well as OPCs) by ADAM10 sheddases and functions to support FAK and PI3K–mTOR signaling (Venkatesh et al. 2015, 2017). Dopamine signaling, which presumably originates from neurons, maintains GSCs, as inhibition of the dopamine receptor DRD4 inhibits GSC growth and stem properties through disrupting autophagy and ERK–mTOR signaling pathways (Dolma et al. 2016). Neural precursor cells in the SVZ provide chemoattractant and proinvasive signals to DIPG cells through secretion of pleiotrophin, which maintains RhoA and ROCK signaling (Qin et al. 2017). Jagged, a NOTCH ligand expressed along axons, supports GSC invasion along unmethylated white matter tracts through up-regulating SOX9 and SOX2 in GSCs (Wang et al. 2019). Furthermore, GSCs adapt to conditions outside of their typical environmental niche. Deletion of the RBP Qki promotes stemness by down-regulating endolysosome-mediated receptor degradation, allowing for limited signaling factors to stimulate stem signaling pathways, including Wnt and Notch signaling (Shingu et al. 2017).

Interactions between tumor and stromal components are also important for maintaining GSCs. In IDH mutant gliomas, high expression of the extracellular matrix glycoprotein tenasin C (TNC) supports increased extracellular matrix stiffness, which increases mechanosignaling through the oncogenic FAK pathway (Miroshnikova et al. 2016). In glioblastoma, TNC supports the maintenance of stemness through activating Notch signaling through binding to cell surface integrins (Sarkar et al. 2017). Extracellular matrix stiffness driven by high levels of glycoproteins in mesenchymal subsets of glioblastomas promotes stemness through integrin mechanosignaling pathways (Barnes et al. 2018). Integrin signaling, particularly mediated by integrin α7, maintains GSC proliferation and invasiveness, serving as a therapeutic target (Haas et al. 2017). Additionally, glioma-associated mesenchymal stem cells, a nontumorigenic stromal component of human glioblastomas, support GSCs through an IL-6/STAT3 axis (Hossain et al. 2015) and secretion of microRNA-containing exosomes (Figueroa et al. 2017).

Tumor tissues are composed of a heterogenous population of tumor cells with distinct functional properties. Transcriptomic and genomic mapping efforts of histologically defined anatomic regions of glioblastoma tumors provide a resource to begin to functionally characterize this intratumoral heterogeneity (Puchalski et al. 2018). Intercellular communication plays important roles in the maintenance of the complex cellular systems that compose a tumor. In vivo multiphoton microscopy of glioblastomas in xenograft hosts reveals multicellular anatomic networks composed of “tumor microtubes” connecting glioblastoma cells. These microtubes permit long-range coordinated communication between cells via connexins, providing tracts for invasion, and are required for maintenance of the tumor cell network following therapeutic interventions (Osswald et al. 2015). Functional connexin-mediated gap junctions are essential for GSCs (Hitomi et al. 2015). Communication via secreted factors is also important, as differentiated glioblastoma cells produce BDNF to support the growth and survival of their stem cell counterparts (Wang et al. 2018).

The critical role of the immune system in regulating tumor biology represents one of the most rapidly evolving microenvironmental dependencies. Immune editing of developing tumors by both innate and adaptive arms of the immune system drives the evolution of GSCs as they evade immunosurveillance, while signaling from GSCs can shape their local immune environment. Tumor-associated macrophages [TAMs] stimulate GSC maintenance and tumorigenicity through a pleiotrophin–PTPRZ1 signaling axis (Shi et al. 2017) and promote glycolytic metabolism through an IL6–PGK1 axis (Zhang et al. 2018). Inhibition of this GSC–TAM cross-talk has been explored as a therapeutic avenue through targeting CSF-1R on macrophages [Pyonteck et al. 2013; Quail et al. 2016]. GSCs down-regulate expression of innate immune sensors (namely, the toll-like receptor TLR4) to prevent negative modulation of stem-like properties by the immune system [Alvarado et al. 2017] and recruit TAMs that enhance tumor growth and block tumor rejection (Zhou et al. 2015; Otvos et al. 2016). Engineered mouse models of low-grade IDH mutant gliomas demonstrate lower immune infiltration and leukocyte chemotaxis than in IDH wild-type models, suggesting that differential immune activation between low- and high-grade tumors may partially explain the prognostic disparity between these two diseases (Amankulor et al. 2017). Glioblastomas, along with other cancers localized to the brain, mediate profound systemic T-cell deficiency, with T cells accumulating within bone marrow and unable to traffic to the site of the tumor due to loss of cell surface S1P1 (Chongsathidkiet et al. 2018). Glioblastomas also generate both local and systemic immunosuppression by inducing M2 macrophage polarization and Th2 reactivity that impairs antitumor responses [Prosnak et al. 2013; Harshyne et al. 2016]. GSCs evade T-cell killing by secretion of extracellular vesicles containing the T-cell checkpoint molecule PD-L1 (Ricklefs et al. 2018). GSC-derived exosomes may suppress T cells through monocyte maturation [Domenis et al. 2017]. Thus, GSCs use a number of mechanisms to avoid immune-mediated destruction and derive growth benefit from factors derived from the immune microenvironment.
Targeting GSCs: applying basic research to clinical therapy

The promise of the cancer stem cell hypothesis is that a deeper understanding of this unique cell population that initiates tumors and mediates recurrence following therapy will allow for efficient therapeutic targeting of these cells and survival benefits for patients. Over the past few years, research in preclinical application of these findings has used several therapeutic modalities, including molecular targeting, cell-based drug delivery methods, viral therapy, and immunotherapy, among others [Fig. 5]. Telomerase reverse transcriptase (TERT) represents an attractive therapeutic target, as its promoter contains the most recurrent single nucleotide mutation in glioma and many other cancer types [Killela et al. 2013; Vinagre et al. 2013]. More recently, binding of the GABP transcription factor to the mutant promoter has been demonstrated as a mechanism for TERT reactivation in glioblastoma [Bell et al. 2015], and this can be targeted with inhibition of this interaction [Mancini et al. 2018]. Targeting of the telomere-protecting factor TRF1 by chemical and genetic means can also reduce GSC viability and tumorigenicity and lead to regression of established tumors in mouse models [Bejarano et al. 2017]. Targeting of circadian rhythm regulators in the REV-ERB family has also demonstrated efficacy against GSCs and cells from other cancer types in vivo associated with alterations in lipid metabolism and autophagy pathways [Sulli et al. 2018], although the precise roles of the circadian clock remain undefined in GSCs. Autophagy is an important cellular survival mechanism, particularly following exposure to extreme stresses. Targeting a key autophagy regulator, ATG4B, with a small molecule inhibitor enhanced the efficacy of radiation therapy in orthotopic xenograft models [Huang et al. 2017]. Secondary glioblastomas contain a high frequency of MET alterations and are sensitive to MET inhibitors, which displayed some efficacy in early clinical trials [Hu et al. 2018].

Oncolytic viral therapy represents a new therapeutic modality with increasing promise. Improved oncolytic adenovirus treatment regimens have demonstrated efficacy in mouse models through stimulating an influx of T cells [Jiang et al. 2017] and in human patients through altering the composition of TAMs [van den Bossche et al. 2018]. Modified herpes simplex viruses combined with immune checkpoint inhibition increase CD8+ killer T-cell infiltration and transition to a more inflammatory antitumor macrophage phenotype [Saha et al. 2017]. Phase 1 trials of modified poliovirus have demonstrated some efficacy in the treatment of recurrent glioblastoma, with evidence that a subset of patients may gain relatively long-term survival benefit [Desjardins et al. 2018]. The highly neurotropic flavivirus Zika virus selectively ablates GSC populations in organoid and mouse models, although concerns surrounding biosafety limit widespread adoption of this virus in clinical trials at the present time [Zhu et al. 2017].

In addition to viral therapies, cell-based therapies are emerging as viable treatment options. Treatment with dendritic cell vaccines containing glioblastoma-specific cytomegalovirus antigens in combination with immune adjuvants extended survival in a small cohort of patients...
(Mitchell et al. 2015). Treatment with autologous tumor lysate-containing dendritic cells may also be a feasible approach [Prins et al. 2011]. In two early clinical trials, administration of personalized vaccines containing tumor neoepitopes elicited strong T-cell immunologic responses with evidence of immunologic memory and tumor-infiltrating capacity [Hilf et al. 2019; Keskin et al. 2019]. Neural stem-like cells engineered from autologous patient-derived fibroblasts home to glioblastoma tumors and deliver cytotoxic compounds to tumor cells in mouse models [Bagó et al. 2017] and have been investigated in early phase clinical trials [Portnow et al. 2017]. Chimeric antigen receptor (CAR) T-cell therapy against tumor-specific targets, including IL13RA2 [Brown et al. 2016] and EGFRvIII [O’Rourke et al. 2017], display some efficacy in patients with recurrent glioblastoma without major limiting toxicities in phase 1 clinical trials. In preclinical studies, CAR natural killer (NK) cells targeting EGFR appear to also prolong survival in xenograft-bearing mouse glioblastoma models [Han et al. 2015]. While immune checkpoint inhibitors targeting CTLA-4 and the PD-1/PD-L1 axis demonstrate remarkable efficacy in other cancers, their role in glioblastoma remains to be determined pending completion of clinical trials. Thus, utilization of innate and adaptive immune responses to target GSCs and glioblastoma remains an active and fruitful area of ongoing investigation.

Emerging directions and GSC themes

Over the past 5 yr, progress has been made in understanding the epigenetic, metabolic, microenvironmental, and developmental underpinnings of GSCs. These findings have been driven by new technological advances, including the rise of single-cell genomics, which has allowed for an unprecedented characterization of tumor heterogeneity at the epigenomic and transcriptomic levels. The emergence of CRISPR–Cas9 genome-editing tools and derivatives has enhanced our ability to precisely perturb our cellular models and read out effects in a high-throughput manner. Thus, the burden of screening large sets of genes or genomic regions in a variety of cellular contexts has been dramatically reduced. The role of the immune system as a critical regulator of tumor biology and an essential component of therapeutic intervention has come into sharper focus. Immune checkpoint inhibition and cell-based therapies, including CAR-T cells, must be investigated further in the coming years.

However, despite the deluge of basic science publications purporting to uncover the next greatest molecular target for glioblastoma therapy, few targets have been effectively translated into clinical care. Glioblastoma patient survival has increased only marginally since the addition of temozolomide and radiation, and the disease remains uniformly fatal. Many obstacles remain that are beyond the scope of this review, including scientific reproducibility issues, poorly aligned incentive structures that do not always reward the most high-impact investigation, and the immense cost and time required to translate a potential target into clinical practice [Kaelin 2017]. We focused on several outstanding challenges here, which revolve around a single major issue: our current inability to effectively model the heterogenous patient disease. First, a large proportion of glioblastoma studies continue to use cell lines that have been cultured in artificial conditions. These experiments almost certainly fail to recapitulate the patient disease, as one prominently used model, U87MG, has an unknown origin [Allen et al. 2016]. Second, even if low-passage patient-derived xenograft models are used for study, clonal selection following in vitro culture rapidly depletes cellular heterogeneity. This prevents the field from effectively modeling tumor heterogeneity and may explain why certain therapies perform well in relatively homogenous tumor models but fail in real-world heterogenous tumors in patients. Third, in vitro studies are frequently conducted in hyperoxic, hyperglycemic, and otherwise nonphysiologic conditions that are devoid of normal cell–cell interactions. Increased use of organoid model systems and other tools to perform high-throughput and high-fidelity tumor modeling will be essential to overcome this challenge. Fourth, modeling of the specialized immune–tumor cell interactions has been limited by the field’s inability to study the effects of a human immune system on human tumors in a model organism. These challenges, while difficult, are not insurmountable and must be addressed to ensure that basic science research can be relevant beyond the benchtop and inform patient management.

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GENES & DEVELOPMENT 603
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