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Review

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# Overcoming therapeutic resistance in glioblastoma: the way forward

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**Glioblastoma is the most common and lethal primary malignant brain tumor in adults. Patients die from recurrent tumors that have become resistant to therapy. New strategies are needed to design future therapies that target resistant cells. Recent genomic studies have unveiled the complexity of tumor heterogeneity in glioblastoma and provide new insights into the genomic landscape of tumor cells that survive and initiate tumor recurrence. Resistant cells also co-opt developmental pathways and display stem-like properties; hence we propose to name them recurrence-initiating stem-like cancer (RISC) cells. Genetic alterations and genomic reprogramming underlie the innate and adaptive resistance of RISC cells, and both need to be targeted to prevent glioblastoma recurrence.**

## Glioblastoma therapeutic challenge: preventing and treating tumor recurrence

Patients with glioblastoma multiforme (GBM) have a median overall survival of approximately 15 months (1). Standard therapy for GBM encompasses maximally safe surgical resection followed by radiation and chemotherapy (1–4). While many patients' tumors initially respond to these treatments, no current regimen can overcome inevitable tumor recurrence, after which patient survival drops to less than 6 months. Personalized therapies against molecular targets that drive the growth of the bulk of primary tumors (5, 6) have so far also been unsuccessful in clinical trials, warranting new approaches.

GBM recurs locally around the surgical cavity without evidence of tumor growth into other organs, despite evidence for extracranial tumor cell dissemination (7, 8). Hence, the failure to control tumor growth at the primary site is the major cause of patient demise. GBM patients have poor prognosis due to tumor cells that survive initial therapies and cause tumor regrowth/recurrence. Tumor heterogeneity is an important reason for the failure of conventional and molecularly targeted therapies (9–12). Consequently, it is essential to identify and characterize which types of cancer cells can evade therapies and become recurrence-initiating cancer cells so that they can be targeted. New knowledge derived from studying the genetic evolution of cancer cell populations in response to therapy, as well as the ability of cancer cells to adopt stem-like characteristics, has provided us with unprecedented new insights to tackle this major challenge.

## Complexity of tumor heterogeneity in GBM

**Intertumoral heterogeneity.** Recent advances in genomic analyses provide us with a comprehensive view of the tumor-to-tumor complexity of GBM. Subgroups have been defined based on distinct

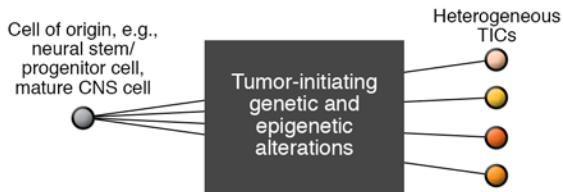
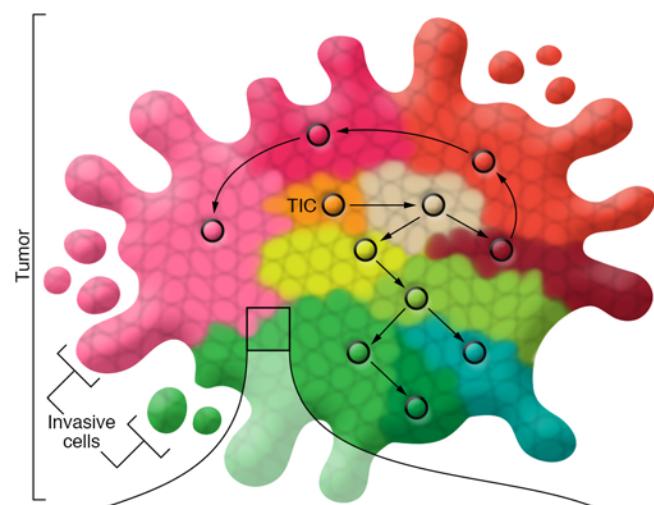
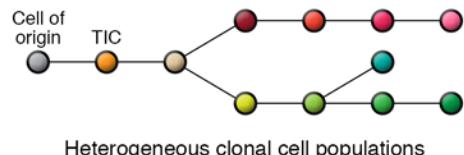
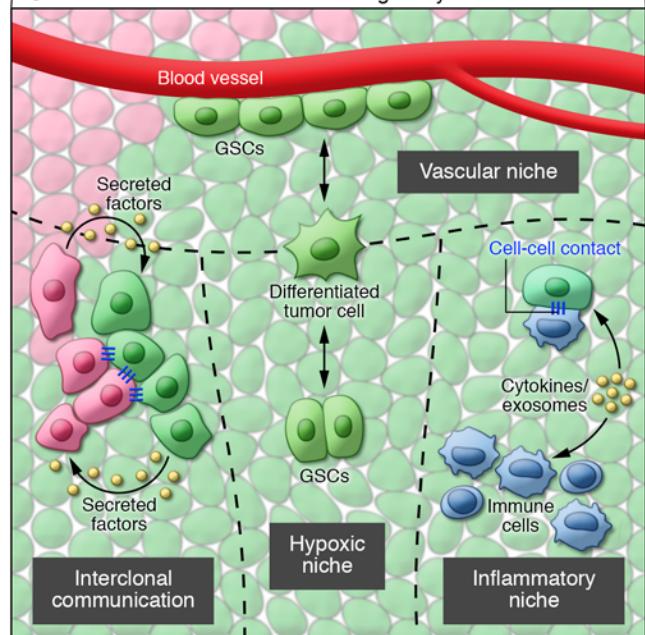
genetic and epigenetic alterations and gene expression profiles (13–16). Differences in cell of origin may further underlie intertumoral heterogeneity (17), and neural stem cells, several CNS progenitor populations, and even mature astrocytes and neurons have been proposed as cells of origin for GBM (18–22). The specific phenotypes of tumors may depend on both the cells of origin and subsequent genetic and epigenetic alterations to these cells (Figure 1A), but our understanding of these matters is still incomplete. The tumorigenic processes within GBM subgroups are being gradually unraveled, particularly in those carrying isocitrate dehydrogenase (*IDH*) gene mutations (23, 24). Gliomas that carry mutations in the *IDH1/2* enzymes produce excessive amounts of hydroxyglutarate (2-HG), which interferes with the function of several epigenetic enzymes, leading to genomic reprogramming (25, 26). Subgroup-specific therapeutic designs will need to be implemented based on the final biological phenotype, which integrates all sources of tumor heterogeneity. For example, drugs that inhibit epigenetic modifiers could reprogram the genomes of gliomas with mutant *IDH1/2* enzymes (25), while the use of temozolomide in such tumors may need to be reconsidered, as it induces a hypermutated phenotype (27–29).

**Clonal evolution of cancer cell populations drives intratumoral heterogeneity.** Pathological analyses of GBM have long provided evidence of extensive intratumoral heterogeneity (30–32), including variable amounts of necrosis (sometimes with perinecrotic pseudopalisading cells), evidence for intratumoral hemorrhage and thrombosis, glomeruloid microvascular proliferation, and pleomorphic tumor cells (30–32). Immunohistochemistry and molecular biology studies have shown heterogeneous patterns of tumor marker expression, and uneven cellular distribution of genetic alterations (33–35). Nonetheless, a deeper understanding of the complexity of intratumoral heterogeneity has remained elusive because of limitations in technology, which have restricted the ability to trace different tumor cell populations within a human tumor mass. New genomic analyses on separate surgical samples from the same tumors have revealed that multiple clones harbor-

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**A** Intertumoral heterogeneity**B** Intratumoral genetic heterogeneity**C** Intratumoral environmental heterogeneity

**Figure 1. The complexity of inter- and intratumoral heterogeneity in glioblastoma.** (A) Heterogeneity between individual patient tumors stems from both the cell of origin and the subsequent major epigenetic and genetic alterations. These variations produce different types of tumor-initiating cells (TICs). (B) TICs expand and establish genetically divergent clonal cell populations. During this clonal evolution process, cellular offspring acquire diverse genetic alterations and engender a variety of clones. Cells with similar types of genetic alterations exist in close spatial proximity, but their invasive properties will lead to clonal mixing and normal brain invasion. (C) Further heterogeneity at the cellular level is added by environmental factors. Proximity to blood vessels (vascular and hypoxic niches), paracrine signals between tumor cells, and immune responses (inflammatory niche), will influence individual tumor cell biology, including regulating stemness versus differentiation state of glioma stem cells (GSCs).

ing a variety of genetic alterations coexist within the same tumor. Segregating clones based on the presence of independent or shared mutations has revealed part of the tumor development process (27–29, 35–37). The clonal evolution model posits that tumor formation is initiated in a cell of origin and is followed by the accumulation of single or multiple somatic genetic alterations, leading to advantages in survival or growth (38). Knowledge derived from genetic syndromes (39) and GWAS studies (40, 41) has shown that an inherited genetic component may accelerate this process. The fittest cell populations likely establish precancerous clones, although we have little direct evidence to support this process in human GBM because of limitations in detecting the early steps in brain cell transformation (42, 43). Divergent genetic alterations in early transformed cells can subsequently give rise to a variety of clones under the selective pressure of the evolutionary ecosystem in the tumor (27–29, 35–37). A cell's spatial location in the tumor is related to its divergent genomic profile, and clones with similar types of genetic alterations are more proximal to each other than those with dissimilar profiles (Figure 1B and refs. 35, 37, 44).

A high capacity for dissemination is one of the defining features of gliomas (45, 46), and this invasion process renders tumors more complex. Invading tumor cells escape at the periphery of the tumor mass and diffusely infiltrate the normal brain parenchyma (47). Deeply infiltrated tumor cells are more likely to escape surgery, and we do not know whether infiltration is a property of a more resilient cell population that initiates and drives tumor recurrence.

There are still many unanswered questions related to what is the exact cell of origin, how fast individual clonal populations develop, and how clonal populations define intratumoral heterogeneity and societal interactions such as competition and cooperation between individual clones and stromal cells. To address these unknowns, further in-depth genetic analyses on hundreds of samples from single patient tumors are warranted. These analyses will define the relative distribution of cell populations in the tumor so that a 3D reconstruction and model of tumor formation can be envisioned and related to imaging (48, 49).

**Intratumoral heterogeneity at the single-cell level.** Molecular heterogeneity exists even at the cellular level between cells that carry similar genetic alterations (50, 51). Recently, single-cell transcriptional profiling of 430 cells from five different primary GBM tumors uncovered intratumoral heterogeneity at the cellular level. Individual cells displayed different transcription signa-

tures for genes that regulate cell cycle, hypoxia, immune response, and stemness (50). Expanding single cells into clonal populations further demonstrated unique functionality including proliferation, differentiation abilities, and different sensitivities to temozolomide (51). The complexity of intercellular heterogeneity also relates to variations in epigenetic or transcriptional programs, which define a hierarchy of cells showing variable degrees of differentiation and stemness. The molecular phenotype of each cell is further altered by its unique position relative to other cells and local environment. Hypoxia (52), vascular niche for maintaining stemness (53, 54), and secreted factors produced by other tumoral or stromal cells all influence the molecular phenotypes of tumor cells (55–57). Heterogeneity at the cellular level adds another layer of complexity (Figure 1C). Astoundingly, although these observations hint at each tumor cell being unique, tumor cells can be connected in a network that responds to a therapeutic insult in a coordinated fashion (47). Regional heterogeneity in molecular properties of tumor cells is thus governed by local variation in environmental selection forces.

### Glioma stem cells

Glioma stem cells (GSCs) are defined as tumor cells capable of forming heterogeneous glial tumors. They are endowed with specific properties including high tumorigenic ability, unlimited self-renewal potential, and capacity for multipotent differentiation, e.g., generating a diversity of progeny (58, 59). The existence of a hierarchy of cells within gliomas, including some with GSC characteristics, is recognized, although many questions related to their number, dynamics, and physiology remain, in part due to limitations in biomarkers.

Certain GSC populations display higher intrinsic chemo- and radioresistance than non-GSCs, indicating that a fraction of the primary tumor GSC population can survive the initial therapy and initiate recurrent tumor formation (58–62). GSCs can overcome the damage induced by chemotherapy and radiotherapy not only through innate properties (e.g., genetic heterogeneity), but also through adaptive resistance pathways (20, 55, 63). How well studies that are based on known cell surface markers represent GSC populations and behavior is being debated (58, 59). For example, CD133, a commonly used GSC marker, fails to identify all tumor cells capable of self-renewal and tumor-initiating ability (64, 65). The GBM single-cell analysis also revealed that a surprisingly large subpopulation of cells (~40%) had a stemness signature (50). How many of these cells may display self-renewal and tumor initiation is unknown, and they may encompass both GSCs and their hierarchical progeny, such as cancer cells with transit-amplifying or progenitor cell properties. This subpopulation had low expression of cell cycle genes, suggesting slower growth than the remainder of tumor cells. There is emerging evidence that GSCs vary in different GBM subtypes (66–68), between treatment-naïve and recurrent GBM (51, 55, 63, 69), and even within alternate niches in the same tumor (i.e., perivascular and hypoxic) (54, 66, 70). Which subpopulations of GSCs can initiate tumor recurrence needs further clarification.

We need to also continually reevaluate and refine the concept of GSCs, taking into account several unanswered questions: can we identify stem cell markers that define specific GSC subpopulations; is there plasticity between non-GSCs and GSCs, such as cell iden-

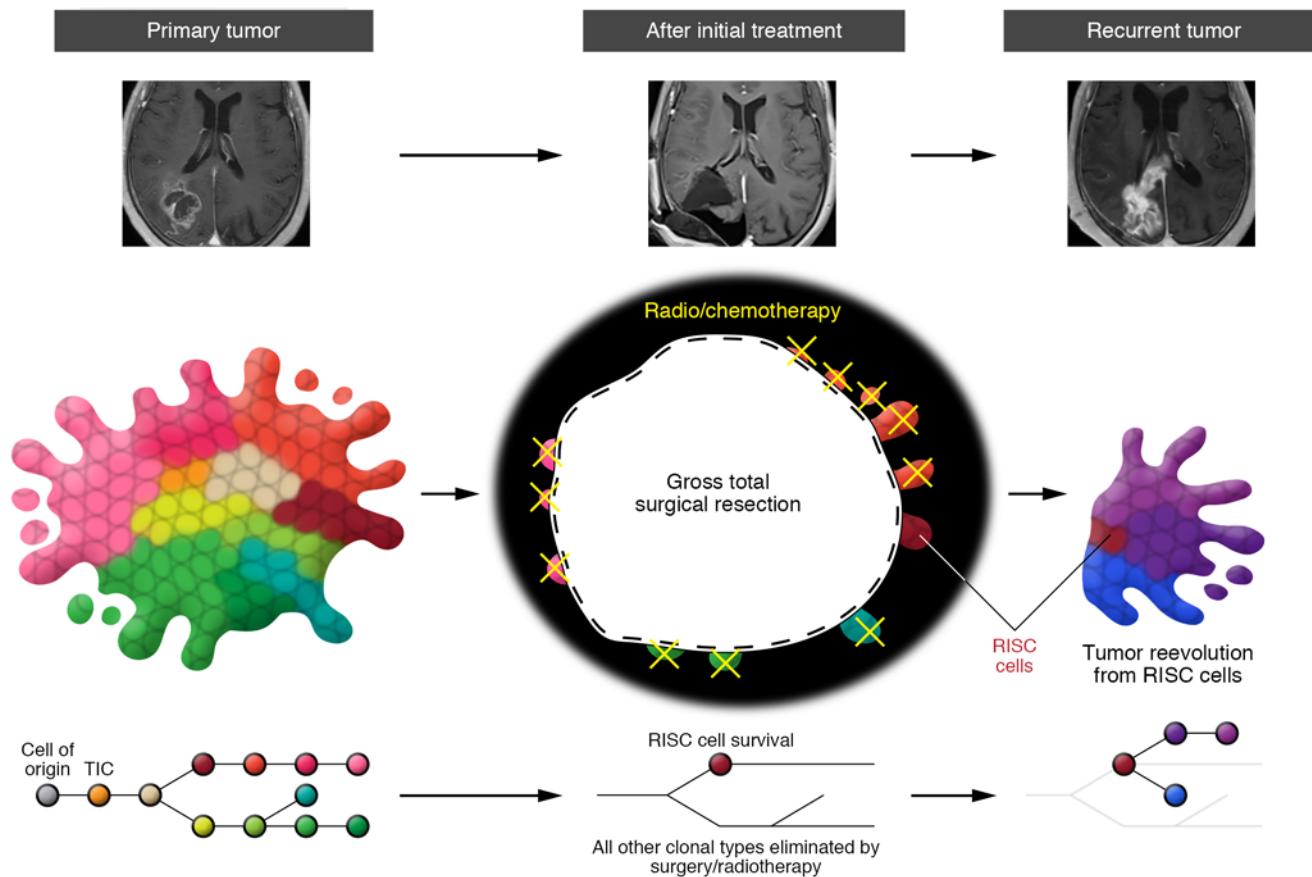
tity drift; and what are the molecular mechanisms underlying the maintenance of GSC stem properties? These limitations are obstacles in defining which tumor cell populations are the most important contributors to tumor recurrence. New technical capabilities in single-cell analyses (50, 51) will soon quantify and characterize cells in GBM that are capable of initiating tumor recurrence.

Because of their tumor-sustaining capacity and resistance to conventional therapies, GSCs represent an important target in the quest to find more effective therapies for GBM. New genomic analyses have uncovered how therapeutic intervention alters the dynamics of glioma cell populations (27–29, 35–37, 44), and an increase in the size of the GSC population after radiation or chemotherapy has been suggested (71, 72). Studies have identified important signaling pathways that are required for the biological maintenance of GSCs (58, 73), and they represent potential new targets to prevent tumor recurrence.

### The process of tumor recurrence in GBM

*Clinical treatment.* Although the clinical course of each GBM patient is unique, and influenced by tumor location, age, and complications, we present an outline of the standard of treatment. When possible, GBM patients receive a maximally safe surgical resection, and the best outcome is called gross total resection, where the entire tumor has been physically removed and only minimal residual disease remains that is invisible on postoperative contrast-enhanced MRI (ref. 74 and Figure 2, top). Thereafter, a standard protocol of radiotherapy (5 d/wk at 1–2 Gy/d) focused on the tumor mass and adjacent margin is delivered in combination with the alkylating chemotherapeutic temozolomide (75 mg/m<sup>2</sup>) over the course of 6 weeks (1–4). After this initial treatment phase, the subset of patients with gross total resection often exhibit stabilization of their disease, with no radiological evidence of further tumor growth (1, 3). Nearly all patients receive further adjuvant temozolomide during this radiographic progression-free phase, even though remnant tumor cells are likely undergoing active biological progression (75, 76). This intermediate phase is typically short-lived (a few months), and most patients develop radiological evidence of local recurrence around the surgical cavity (Figure 2, top and refs. 3, 44). Once the tumor grows back, patients may receive further therapies, including additional tumor resection, bevacizumab (an anti-VEGF antibody), different chemotherapy, or additional radiation therapy focused exclusively on the tumor site. Yet there is little evidence that such salvage treatments increase survival. Tumor cells resistant to multiple therapies persist in the brain parenchyma around the tumor cavity and underlie tumor repopulation, making them a critical target to overcome tumor recurrence.

*How do heterogeneous tumors respond to therapeutic intervention?* Genomic landscape analyses of pre- and post-treatment GBM pairs from the same patients have demonstrated that recurrent tumors display variable degrees of genetic relatedness to the original tumor (clonal evolution), but also have acquired new mutations (subclonal evolution) (27–29, 35–37, 44). Recurrence is a complex process, with a diversity of evolutionary trajectories broadly classified into linear recurrences that share extensive genetic similarity with the primary tumor, and branched evolution leading to the formation of divergent cell populations (subclones) (27–29, 35–37, 44). Recurrence shows a high degree of variability



**Figure 2. Treatment and tumor recurrence in glioblastoma.** Top: MRI scans of a patient with a primary glioblastoma before treatment, after initial gross total resection followed by chemo- and radiotherapy, and after tumor recurrence. Middle: A cartoon rendering of the associated changes in clonal populations in the tumor at each stage. Surgery successfully removes the tumor and eliminates many subclones. Postoperative chemo- and radiotherapies can further reduce tumor burden around the surgical cavity. However, a small fraction of tumor cells survive and initiate the formation of the recurrent tumor. The length of each line is proportional to the number of mutations acquired between each clone and branching indicates acquisition of divergent mutations. We have proposed calling these surviving cells recurrence-initiating stem-like cancer (RISC) cells. Bottom: Phylogenetic tree showing the process of clonal evolution in the primary tumor, the survival of RISC cells that have acquired adaptive resistance to therapy after initial treatments, and their evolution into a recurrent tumor. The length of each line is proportional to the number of mutations acquired between each clone, and branching indicates acquisition of divergent mutations.

and can originate from one subpopulation that branched off early during tumorigenesis or much later. In most patients, 50–200 clonal or subclonal mutations are found at relapse (27, 29, 44), and this number can increase to more than 1,000 in cases with mismatch repair gene alterations (hypermutated tumors). This information provides for the first time a detailed genetic portrait of the impact of therapies on GBM.

These data further suggest that the efficacy of surgery in prolonging patient survival (74, 77, 78) occurs through both a reduction in the physical burden of tumor and an alteration in the dynamics of tumor cell populations. Surgical debulking reduces intratumoral heterogeneity by removing many subclones from the tumor. This conclusion is based on two observations: first, there is regional diversity in primary tumor clones, suggesting limited cell mixing (27–29, 36, 37, 44), and second, comparison of primary/recurrent tumors of patients having only received surgery shows that the recurrences diverge early from the primary tumor and lack the end mutations found in the primary tumor (27, 36). Post-operative chemo- and radiotherapies can likely also reduce clonal diversity when only biopsy or partial surgical resection is possible,

but the main documented clinical benefit of these treatments after tumor debulking is probably the reduction of the infiltrative tumor cells left by surgery. Despite these treatments, small fractions of clones originating from beyond the surgical margin survive and lead to recurrence (Figure 2, middle).

**What type of clone can initiate tumor recurrence?** Of the many primary tumor clones, it is important to determine which can engender recurrence-initiating cells. Deep sequencing of multiple sectors of primary/recurrent GBM pairs has shown that recurrent clones can variably originate from a clone positioned early, in the middle, or at the end of the clonal evolution process of the primary tumor (Figure 2, bottom, and refs. 27, 44). While the branching position for the start of each recurrent clone was variable in each patient, it was surprising that recurrent clones did not all originate from clones at the end point of the clonal selection process as was observed in other cancers (79, 80). The dominant clone at recurrence usually was not a lineal progeny from the main clone in the primary tumor. It is unclear whether this is because the dominant clones in the primary tumor were removed or killed by therapy, whether not all tumor cells can act as recurrence-initiating cells,

or whether recurrent cells are more invasive and deeply infiltrated in the surrounding normal brain, protecting them from surgical removal. Thus, the number of genomic alterations is not simply correlated with therapeutic resistance in GBM, and recurrences either share most primary tumor mutations with accumulation of additional genetic alterations or diverge early genetically and evolve rather separately, with little in common with the primary tumor. Consequently, targets identified based on the analysis of the primary tumor may not be informative in treating the recurrence. Moreover, analyses combining the branching pattern with estimates of evolutionary rate suggest that subclones associated with recurrence were already present years before diagnosis, which implies that many of the unique genetic alterations found in the cells initiating recurrence were not caused by the treatment (29).

The above findings demonstrate that the tumor cells leading to recurrence differ from GSCs that initiated and maintained the primary tumor: they took a divergent evolutionary path and need to be studied separately. We hypothesize that recurrence-initiating cancer cells emerged from the residual tumor cell population that survived therapy and have stem-like properties, because they can initiate a recurrent GBM with a diversity of tumor cells. Therefore, we propose to call them recurrence-initiating stem-like cancer (RISC) cells. Early studies support this model. Cells with GSC properties can be isolated from recurrent GBM, and can generate heterogeneous GBM when transplanted in mice (66). Such cells are more aggressive than primary tumor-derived GSCs (51, 63, 69), consistent with the shorter survival of patients with recurrent GBM (81), and display different markers (loss of CD133 and gain of CD15, BMI1, and SOX2) (63). Human and mouse GSCs acquire therapeutic resistance following repeated chemo- and radiotherapy (55, 63), and temozolomide treatment in a transgenic mouse model of glioma showed that recurrent tumors originated from quiescent glioma cells with stem cell features (20). Therefore, we further hypothesize that RISC cells are a subset of GSCs that developed increased innate resistance to treatment through further genetic mutations, and acquired further adaptive resistance through epigenetic alterations during the course of therapy. Whether RISC cells are direct descendants from the GSCs in the primary tumor or have emerged from more differentiated progeny remains to be established (82). Comparisons of GSCs from the primary tumor with RISC cells from the recurrent tumor of the same patients will further help in defining the ontology of RISC cells. Understanding the genomic alterations and molecular architecture of RISC cells is critical for the development of successful therapies that could be deployed immediately after surgery, thereby preventing their adaptive resistance and expansion into a new tumor mass.

### Molecular pathways implicated in therapeutic resistance of RISC cells

A growing number of molecular pathways have been associated with therapeutic resistance in GSCs and should be particularly relevant to treatment of RISC cells (58, 73).

**Extracellular signaling pathways.** These signaling pathways are activated through autocrine or paracrine secretion of growth factors/cytokines, as well as homotypic tumor cell contacts and heterotypic tumor-stroma interactions, involving tumor cells, endothelial cells, and immune cells (Figure 1C). The most studied

include the Wnt/β-catenin (83–85), Notch (86), receptor tyrosine kinase (RTK)/PI3K (87–92), NF-κB (93), SHH/GLI (94, 95), and JAK/STAT signaling pathways (96, 97), and others were recently reviewed (58, 73). These pathways maintain stemness in several types of normal and cancer stem cells (58, 73).

**Transcription factors implicated in GSC maintenance.** The above signals are integrated through the activation of a limited number of transcription factors that control a variety of functions underlying GSC maintenance, including survival, self-renewal, proliferation, metabolism, and stemness state. They include OLIG2 (98, 99), c-Myc (100, 101), BMI1 (102, 103), SOX2 (104), NANOG (105), OCT4 (106), and ID1 (107). Some transcription factors were already known to maintain several types of normal stem cells (108–110), and c-Myc and OCT4 can help induce the formation of GSCs from astrocytes (111). Expression of all these transcription factors can be increased in GSCs (101–104), and is controlled by extracellular signaling pathways, superenhancers (112, 113), epigenetic regulation (102), and microRNAs (114, 115). They also activate DNA damage repair pathways that contribute to the therapeutic resistance of GSCs (58).

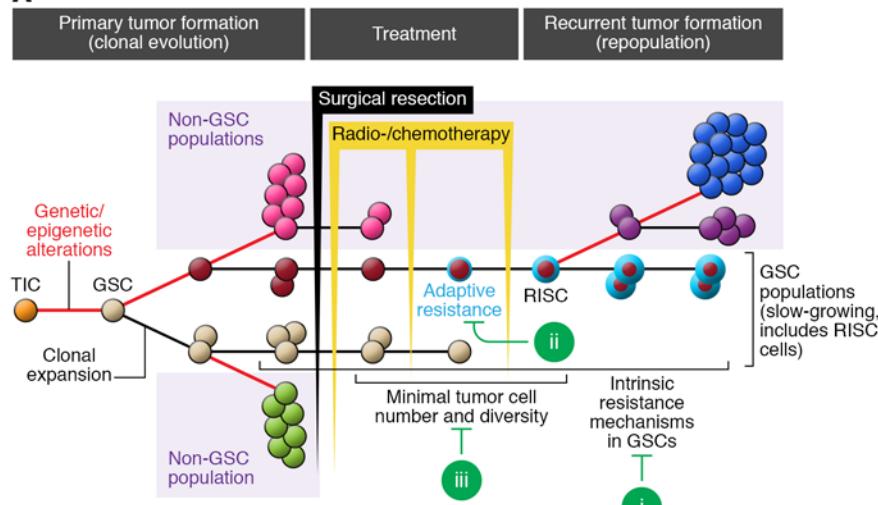
**DNA damage repair and other resistance mechanisms.** The activation status of intrinsic or adaptive DNA damage pathways is an important determinant in chemo- and radioresistance of cancer cells. DNA damage checkpoint proteins can render human GSCs more resistant to radiation-induced apoptosis through increased efficiency in repair of damaged DNA. GSCs display increased expression of ataxia telangiectasia mutated (ATM), the cell cycle checkpoint protein RAD17, and the checkpoint kinases CHK1 and CHK2 (61). O-6-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme whose expression level is regulated epigenetically at the gene promoter, correlates with resistance to chemotherapies (116), and renders GSCs resistant to temozolomide (62). Secretion of exosomes, activation of autophagy (117), cell metabolism (66, 118), ROS production (119, 120), drug efflux (121), and microRNA expression (114, 115) are also altered in GSCs and can further enhance therapeutic resistance.

### Which molecule should be the next target for clinical trials?

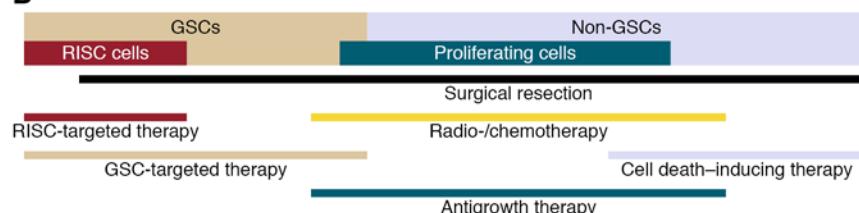
Mining resistance pathways is an important step to identify the next set of clinical targets, and several clinical trials have already been conducted to identify GSC resistance pathways. However, the antitumor effects observed in these trials were limited and did not prevent tumor recurrence (122–126). Further understanding of the role of these signaling pathways in the different tumor cell populations is needed to optimize their targeting. For example, adaptive radioresistance in mouse GSCs is associated with autocrine IGF-1 receptor activation and downregulation of Akt/ERK signaling, leading to a slow-growth/high-self-renewal phenotype (55). Additional targets for clinical testing need to be identified as well.

**Targeting transcription factors for GSC maintenance.** Most clinical trials have targeted the ligand or receptors that initiate extracellular signaling. In contrast, the targeting of important transcription factors for GSC maintenance has not been achieved, largely because of inherent difficulties in designing small molecules to target them. Because multiple extracellular signaling pathways regulate the transcription factors that maintain GSCs, if one major

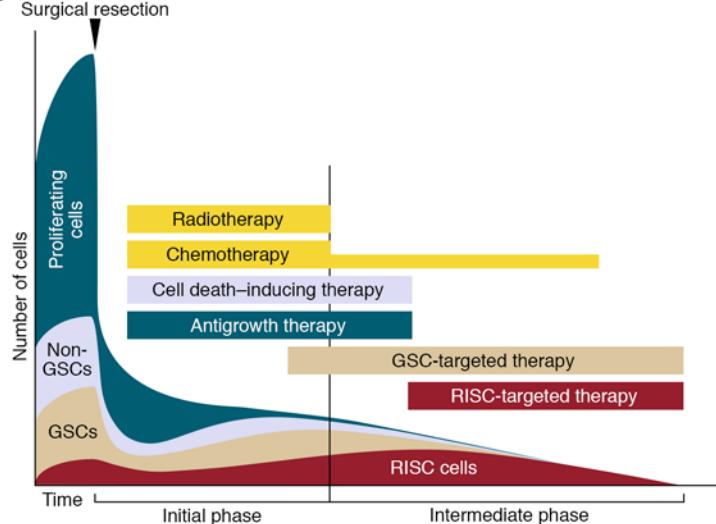
A



B



C



pathway is inhibited, alternative pathways can substitute for their activation and lead to resistance (127–129). Soon, advances in drug design or different approaches (e.g., RNAi, stapled peptides, ref. 130; or artificial transcription factors, ref. 131) will allow targeting of the transcription factors that control developmental signaling pathways that GSCs hijack for self-maintenance.

**Slow-growth state.** The effectiveness of radiation and chemotherapy is in part dependent on cell proliferation rate, which underlies the increased sensitivity of cancer cells over normal cells (132, 133). Ergo, GBM cells in a state of slow growth could play an important role in recurrence as suggested by human and mouse studies (20, 55, 134, 135). About 40% of the tumor cells

in human GBM have a high-stemness and low-proliferation gene expression profile (50), yet the molecular mechanisms that maintain the GSC population and control their state of slow growth are still unknown. In normal stem cells, this is regulated by niche factors that ensure balanced self-renewal and differentiation through asymmetrical cell division, but this process is disrupted in gliomas (136–138). Studying and finding an effective therapy for slow-growing clones is challenging, because they are dispersed within the bulk of the tumor. Yet inroads into the slow-growing mechanisms of GSCs are being made (20, 55). Improved knowledge of the signaling mechanisms maintaining the slow-growth status will unveil new RISC cell targets.

**Figure 3. Opportunities for future molecularly targeted therapy for glioblastoma.** (A) The primary tumor is heterogeneous, and composed of several abundant cell subpopulations: cells with variable proliferation properties (pink and green), glioma stem cells (GSCs) (tan), and recurrence-initiating stem-like cancer (RISC) cells (red). Initial treatments successfully reduce the bulk tumor volume and its heterogeneity. However, subpopulations of RISC cells survive therapeutic intervention through intrinsic and adaptive resistance mechanisms (indicated by a blue ring). RISC cells initiate tumor recurrence through a second round of clonal evolution that repopulates the tumor. The current clinical strategies for GBM could be strengthened by adding molecular therapies to target: (i) initially resistant clones, (ii) adaptive resistance mechanisms, and (iii) the tumor when its population diversity and cell numbers are at their lowest. (B) Schematic showing the 4 proposed different cancer cell populations in the tumor (GSCs [tan], RISC cells [red], non-GSCs [light blue], and proliferating non-GSCs [dark blue]) and putative ways to target them (see also Table 2). RISC cells likely represent a subset of the GSC population with both innate and acquired resistance. The precise proportion of each cell population remains to be established. (C) Proposed optimal timing for each therapeutic option during the initial (primary treatment) and intermediate (stabilization/remission period before recurrence) phases of therapy.

**Table 1. Working model to define different cell populations in the primary GBM (IDH1 wild type)**

Cell populations in GBM:	GSC-like		Non-GSC					
	RISC cells	GSCs	Proliferating cells	Resting cells				
<b>Definition</b>	Are cancer stem cells (self-renew and give rise to diversity of progeny)		Do not meet cancer stem cell definition (do not generate diversity of progeny)					
Initiate recurrent tumor								
Initiate and maintain primary tumor								
<b>Properties (hypothetical):</b>								
Growth	Slow	Slow	Fast	Limited				
Differentiation ability	Multipotent: can give rise to diversity of progeny	Multipotent: can give rise to diversity of progeny	Unipotent: progeny with limited diversity	Differentiated (potential to dedifferentiate)				
Markers	Stem cell markers (CD15, etc.), mesenchymal GSC markers (CD44, CD109), adaptive resistance markers (IGF1R)	Stem cell markers (CD133, L1CAM, etc.)	Differentiation and progenitor cell markers?	Differentiated cell markers				
Tumorigenic ability (in mice)	High	Moderate	Low	No				
<b>Response to conventional therapies (hypothetical):</b>								
Innate resistance	High (slow growth and mutations)	Moderate (related to slow growth)	Low (sensitive due to fast growth)	Moderate (related to slow growth)				
Adaptive resistance	High	Low	Low	Low				
Sensitivity to radiotherapy	Low	Moderate	High	Low				
Sensitivity to chemotherapy	Low	Moderate	High	Low				

**Adaptive resistance to therapeutic intervention.** Prospective analysis of the molecular features of RISC cells in human tumors is challenging. The tissue surrounding the resection cavity has the appearance of normal brain parenchyma, and although autopsy studies have shown that it contains infiltrating cells (135, 139), their numbers are small and further surgical removal cannot be justified. Thus, we lack a sample of the tumor cells from the location of the recurrent tumor, and just have cells from the resected tumor bulk. This is important, as the biological features of deeply infiltrated cells may be different from those from the resected primary tumor (134, 135). To overcome this limitation, mouse models have been used to garner data on the molecular changes associated with radio- (55) and chemotherapies (20). Mouse GSCs can overcome the damage of repeated irradiation through gradual activation of IGF1R-dependent resistance pathways (55), and repeated chemo- and radiotherapies render human GSCs more aggressive and enrich their stem cell features (63, 140). GBM recurrence is also associated with a transition from glial to mesenchymal phenotype and is related to poor outcome (141, 142). Autophagy is another player in adaptive radioresistance mechanisms in GSCs (117). Such adaptive processes are driven by molecular alterations induced by epigenetic or genetic cues. These data demonstrate that adaptation mechanisms represent an important strategy for tumor cell survival and repopulation in response to therapeutic stress.

**Tumor-stroma interactions and microenvironment.** Disrupting the tumor-stroma interactions that support GSC survival is another potential approach for antagonizing GSCs. Self-renewing GSCs are known to interact closely with endothelial cells (53, 143) and

pericytes (144) in the perivascular niches. Microenvironmental changes such as hypoxia can also render tumors more resistant to conventional therapies. Hypoxic cells display increased radiation resistance because oxygen radicals play a major role in the damage generated by irradiation and because hypoxia-inducible transcription factor (HIF) alters the DNA damage response (145–147). Cytotoxic chemotherapies delivered via the bloodstream diffuse into the tumors from functional blood vessels, and the hypoxic areas are the furthest removed from these vessels. The hypoxic niche further promotes the self-renewal capacity of GSCs through HIF-mediated activation of the inducible nitric oxide synthase (iNOS) (148). Both HIF-1 and HIF-2 are important for GSC maintenance and tumor angiogenesis (52). GSCs display plasticity in the metabolic pathways they use to adapt to nutrient limitations in their microenvironment (66, 149, 150). Although the complexity of metabolic alteration in GSCs is not fully understood, metabolic inhibitors could be developed to target them in the future. Some studies suggest that GSCs preferentially use oxidative phosphorylation while the rest of the tumor is glycolytic, suggesting targeting with mitochondrial inhibitors (149, 150).

**Immune suppression of tumor cells.** Cancer cells can also evade the immune response by activating immune checkpoint receptors (PD-1 and CTLA-4) on effector T cells, thus blocking the antitumor immune response (151, 152). Programmed death-1 (PD-1) is a T cell receptor that, upon activation by its ligand (PD-L1), can negatively regulate the T cell-mediated immune response. Tumor cells, including GBM, overexpress PD-L1 (153). Blockade of the PD-1 pathway in mice also promotes killing of GSCs by NK cells

**Table 2. Proposed therapies for different GBM cell populations**

RISC cell–targeted therapy	Stem cell–targeted therapy	Antigrowth therapy	Cell death–inducing therapy
Adaptive resistance targeting (IGF1R signaling inhibitor, other)	Stem cell signaling pathways (Wnt, SHH, Notch signaling inhibitors)	Growth pathway targeting (PDGFR, PI3K, EGFR signaling inhibitors)	Apoptosis pathway targeting (BCL-2 family protein, p53 target drugs)
Immunosuppression targeting (anti-CTLA-4 and anti-PD-1 inhibitors)	Epigenetic reprogramming (alteration of DNA methylation and histone modification)	Metabolic targeting	Autophagy pathway targeting
Virotherapy	Stem cell niche therapy (HIF inhibition, antiangiogenic therapy)	Immunotherapy (vaccines)	

(140). Anti-PD-1 and -CTLA-4 antibodies have shown impressive efficacy with high response rates and tumor remissions in several cancer types in the clinic (154–156), and clinical trials are currently ongoing in GBM (NCT02667587, NCT02017717, NCT02311920; ClinicalTrials.gov). Patients with hypermutated tumors may benefit from immune checkpoint inhibitors because of increased neoantigen load and have slightly longer survival (27, 29, 157). GSCs also modulate the immune system by recruiting microglia/macrophages, modulating their function toward tumor growth and generating an immunosuppressive phenotype through the secretion of IL-10 and TGF- $\beta$  (57, 158). These new findings support targeting immune checkpoints to activate an immune response against RISC cells.

### Future therapeutic strategy

With our improved understanding of resistance mechanisms in the recurrent tumor (Figure 2), we can now envision new therapeutic strategies. At the risk of oversimplifying, we propose that current clinical therapy has three major shortcomings (Figure 3A), and we outline new strategies to overcome them (Figure 3, B and C). We also propose that future molecularly targeted therapies should be designed for at least four types of cancer cells based on their different properties and response to therapies: primary GSCs, RISC cells, and both the proliferating and the postmitotic fractions of non-GSCs. To facilitate this process, we have attempted to summarize their known and hypothetical properties (Table 1) and suggested ways to target them (Figure 3, B and C, and Table 2).

**Shortcomings of current clinical therapy.** Improved tumor targeting during the intermediate phase that follows the primary treatment period and ends at evidence for tumor recurrence could be developed. In this phase of a few months, imaging often provides evidence for remission/stabilization, but resistant cancer cells are left behind untreated, given ample time to recover from the initial treatment, and provided with the opportunity to grow and progress into a new tumor. Currently, aggressive treatment is not delivered in this phase, except for maintenance temozolomide (75, 76). Within this therapeutic window, the tumor is most vulnerable, as the heterogeneity and number of tumor cells are most reduced and they might not yet have acquired full resistance.

A second opportunity lies in targeting the intrinsically resistant subpopulation in the initial tumor. The basic effect of radiation and alkylating chemotherapy is DNA damage, and this effect is mainly (but not exclusively) dependent on the speed of cell

division (132, 133). Slow-growing tumor cells are important targets to prevent chemo- and radioresistance (20, 55, 132, 133, 159–161). However, in the majority of molecularly targeted therapies, the targets of interest were selected based on highly expressed molecules found on a large fraction of tumor cells, such as the tyrosine kinase receptors, and their downstream signaling effectors (162–164). While these therapies are efficient in controlling the initial tumor, they may do little to prevent recurrence because they target mainly proliferating cells.

The third point that merits considerable further attention relates to acquired resistance mechanisms. Prior attempts to develop new molecular therapies aimed at targeting cancer cell resistance mechanisms focused on intrinsic resistance (162–165). However, the precursors to RISC cells can also become radioresistant through several acquired resistance mechanisms during the intermediate phase of disease (20, 55, 63). Identifying novel targets of adaptive resistance is currently a challenge, as there is a paucity of experimental models specifically addressing adaptive resistance in GBM (20, 55, 63). New models need to be developed to discover novel targets and validate appropriate targeting agents. While the brain has remained hermetic to most chemotherapies, novel approaches that open the blood-brain barrier in a sustained manner are becoming available, thus making the CNS accessible to a plethora of drugs already in use for other cancers (166).

**Future molecularly targeted therapies: concepts and timing.** Future molecularly targeted therapies should be designed for all cancer subpopulations within the tumor, RISC cells being the most important to prevent tumor recurrence. Appropriate therapies are needed for each cell type (Figure 3B), and they need to be delivered at the right time (Figure 3C). A number of candidate inhibitors for these cellular targets are already being used in clinical trials for other cancers and could be rapidly tested in GBM.

Radiation and chemotherapy are appropriate therapies for proliferative non-GSCs that are sensitive to DNA-damaging agents. Proapoptotic agents could be evaluated for the slower-growing, terminally differentiated non-GSCs (167). To further reduce the dividing non-GSC population, radio- and chemotherapies should continue to be delivered as initial treatments following surgery. Adjuvant chemotherapy is one option to maintain the suppression of cycling non-GSCs in the intermediate/remission phase, as are molecular therapies targeting a mosaic of growth-signaling pathways.

Existing Wnt, SHH, and Notch pathway inhibitors are good candidate therapies for all GSCs, and in the future, transcription

factors sustaining stemness could also be targeted. Molecular therapies targeting epigenetic reprogramming (DNA methylation and histone modification) (13, 168), hypoxia-activated pathways, angiogenesis, and metabolic rewiring/reprogramming all hold promise for GSCs (66, 149, 150). They should be applied starting at the end of the initial therapeutic phase and continuously during the intermediate phase, when the population of RISC cells is emerging.

A further type of targeted therapy is needed to eliminate RISC cell populations. This should start early to eliminate intrinsically resistant RISC precursor cells. Targeting of adaptive resistance mechanisms and blocking of immune suppression can be accomplished in the intermediate phase (27, 29, 157). Creatively engineered virotherapies can also be considered to target all GSCs, including RISC cells (169–171).

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