

Glioblastoma stem cells

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Abstract Glioblastomas are highly malignant primary brain tumors with one of the worst survival rates among all human cancers. With a more profound understanding of the cellular and molecular mechanisms of tumor initiation and acquired resistance to conventional radio- and chemotherapy, novel therapeutic targets might be discovered to optimize therapeutic approaches. In this regard, the identification of a small cellular subpopulation, called glioblastoma stem cell or stem-like cells or glioma-initiating cells or brain tumor propagating cells, has gained attention. In this article, we briefly summarize the current state of knowledge about this tumor cell population and discuss future directions for basic and clinical research.

Keywords Neurooncology · Glioblastoma · Tumor initiation · Resistance to therapy · Targeted therapy

Stem cell and cancer biology

A histological key feature of glioblastomas is the cellular and morphological heterogeneity and the parallel detection of cell populations of apparently different grades of differentiation. Moreover, glioblastomas harbor a variety of genetic abnormalities. This tumor heterogeneity may pose a therapeutic challenge because different cell populations within the tumor tissue might respond differently to therapies. One strategy to overcome these problems is to identify and characterize distinct subpopulations that drive

specific disease pathologies, e.g. tumor initiation or tumor recurrence or both.

In this regard, overlaps have been defined in stem cell biology and oncology, particularly because of the growing evidence demonstrating that genes with important roles in stem cell biology also play a role for cancer biology. Consequently, the existence of a stem-like tumor cell was hypothesized, i.e. a relatively small percentage of cells that drive tumorigenesis and maintain tumor viability despite multimodal anticancer therapy. Candidate cancer stem cells should display several features that signify stemness including the unique ability to morphologically recapitulate the tumor of origin in xenografts.

The first experimental evidence for the actual existence of cancer stem cells came from hematological malignancies. Studies of acute myeloid leukemia (AML) revealed that a single cell initiated the disease after transplantation in mice. Cell isolation depending on cell surface marker expression revealed that CD34⁺CD38⁺ cells initiated AML, whereas CD34⁺CD38⁻ did not. After development of AML in mice, the frequency of these leukemia-initiating cells in peripheral blood was about 1,000-fold lower than the frequency of other leukemic cells. These findings suggested that a hierarchy of leukemic stem cells existed in human AML (Lapidot et al. 1994).

This hierarchical model implies that the majority of cancer cells within a tumor are derived from a few cancer stem cells. Thus, the tumor consists in a small subset of cancer stem cells and a large population of bulk cells that cannot per se form new tumors and might represent a mix of partially and terminally differentiated cancer cells with limited proliferative capacity.

This concept was also investigated in brain tumors. The seminal study in the field of neurooncology demonstrated that as few as 100 glioma stem-like cells, defined as

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CD133⁺ cells, were able to recapitulate the heterogeneity of glioblastomas in immunocompromised mice whereas CD133⁻ tumor cells could not. Notably, 100 CD133⁺ cells sufficed to recapitulate the original tumor whereas 10,000 CD133⁻ cells were not tumorigenic (Singh et al. 2004). Therefore, a hierarchy was postulated in gliomas, i.e. a tumor cell of origin that upon differentiation might lead to the establishment of astrocytomas, oligodendrogliomas or ependymomas (Fig. 1).

Several other groups have also isolated brain tumor stem cells from primary glioblastomas, based on cell surface marker expression or the ability to form neurospheres (also called gliospheres) as do normal neural stem cells (Table 1).

The identification of somatic mutations of the isocitrate dehydrogenase 1 gene (IDH1) most frequently found in glioblastomas which has evolved from lower-grade gliomas, so-called secondary glioblastomas, might be of relevance for further characterization of a putative cell of origin in gliomas, i.e. a cell of origin in gliomas that upon differentiation might lead to the establishment of astrocytomas or oligodendrogliomas (Fig. 1). Low-grade gliomas (astrocytomas and oligodendrogliomas) may carry IDH mutations in the absence of any other genetic aberration, suggesting that IDH mutations occur early, e.g. in a stem or precursor cell that can give rise to both, astrocytes and oligodendrocytes (Yan et al. 2009). Interestingly, IDH mutations never occur in ependymomas.

The nomenclature

The nomenclature of stem cells in glioblastoma has remained controversial. This is due to the lack of standardized criteria for identifying them. The terms glioblastoma stem cell, glioblastoma stem-like cell or glioblastoma-initiating cell are commonly used interchangeably but whether cells identified by the methods defined below are the only cells that can initiate tumors has remained unclear. This terminology reflects some shared characteristics with normal stem cells, especially adult somatic stem cells, including the capacities for self-renewal, differentiation and maintained proliferation. Yet, whether the cell of origin in glioblastomas is sometimes or always a stem cell (Fig. 1), as the terminology glioblastoma stem cell implies, is unclear at the moment. The term tumor-propagating cell has been alternatively proposed, because the defining bioassay involves tumor propagation in secondary hosts, e.g. mice (Kelly et al. 2007). In this article, we use the term glioblastoma stem-like cell (GSC) and are fully aware of controversies regarding its limitations.

Isolating and culturing GSC

GSC have been identified based on different cell surface markers, culture conditions or functional criteria. Currently used approaches are summarized in Table 1. The most

(Somatic) stem cell?

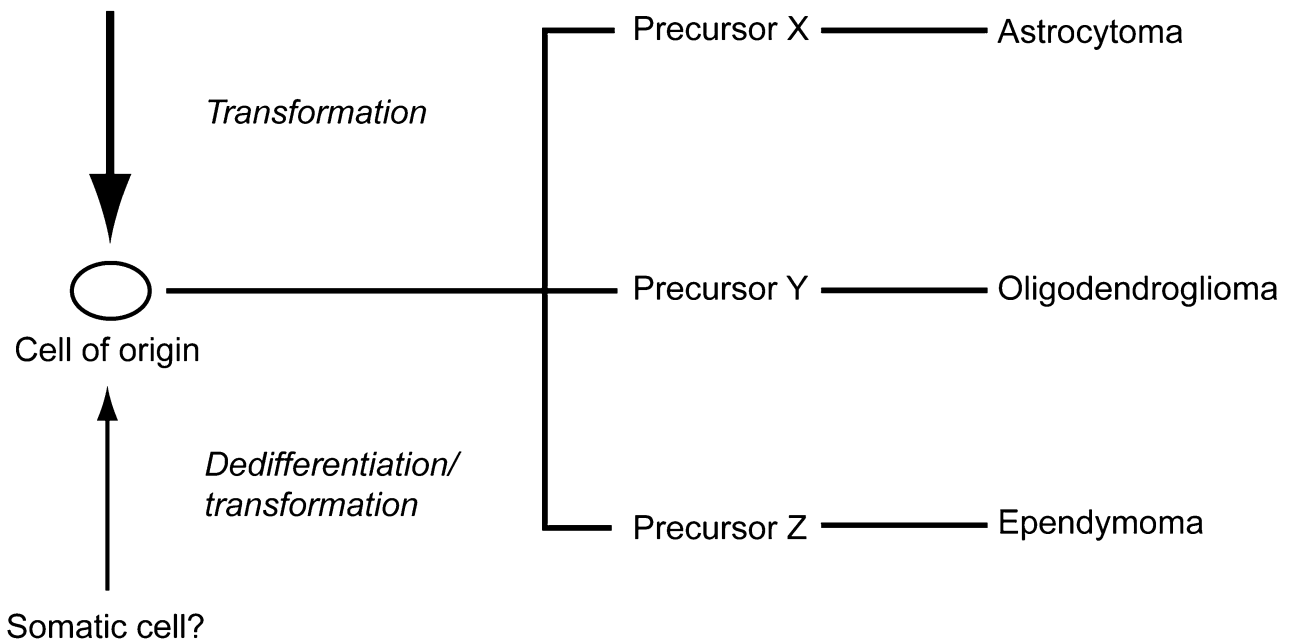


Fig. 1 Glioma stem-like cells might serve as a “cell of origin” either derived from a transformed stem cell or a transformed somatic cell. Further differentiation of this cell of origin might then lead to different

cellular lineages resulting in the development of astrocytomas, oligodendrogliomas or ependymomas

Table 1 Cell surface markers and functional assays for the isolation of glioma stem-like cells

Parameter	Assessment	Functional correlate	Key reference
CD 133	Magnetic sorting or flow cytometry sorting for CD133 expression	Self renewal Multipotency Tumorigenicity <i>in vivo</i>	Singh et al. 2004
Alpha 6 integrin	Sorting for alpha 6 integrin expression alone or in combination with CD133 expression by flow cytometry	High enrichment for cells with self-renewal Sphere formation Tumorigenicity <i>in vivo</i>	Lathia et al. 2010
Culture condition	Culture condition: serum-free medium supplemented with EGF, FGF	Self renewal Multipotency Tumorigenicity <i>in vivo</i>	Galli et al. 2004 Günther et al. 2008
Side population	Flow cytometry sorting based on exclusion of Hoechst 33342 (side population)	Tumorigenicity <i>in vivo</i> only in side population	Kondo et al. 2004
A2B5	Flow cytometry sorting for A2B5 and CD133 expression	Tumorigenicity	Ogden et al. 2008
ALDH1 activity	Flow cytometry sorting for ALDH1 activity	High ALDH1 levels keep glioma cells in an undifferentiated state High ALDH1-expressing glioma cells grow in neurospheres ALDH1 inhibition induces differentiation and reduces clonogenicity	Rasper et al. 2010
Autofluorescence	Intrinsic autofluorescence and morphology	Self renewal Multipotency Tumorigenicity <i>in vivo</i>	Clement et al. 2010

mentioned and controversial cell surface marker for GSC isolation is CD133, a cholesterol-binding membrane protein of unknown biological function.

CD133⁺ progenitor cells promoted the healing of ischemic ulcers through stimulating angiogenesis and activating the Wnt pathway (Barcelos et al. 2009). Recently, CD133 expression was demonstrated to increase in response to hypoxia and to mitochondrial dysfunction. This questions CD133 as a reliable marker for GSC.

One reason explaining these inconsistencies between various groups could be the lack of standardization regarding cell sorting methods and assays to confirm “stemness” across laboratories.

Recently, a marker-independent approach has been demonstrated exploiting cellular phenotypes. Combining morphological aspects and autofluorescence emission were used to enrich a subpopulation with self-renewal ability *in vitro*, tumor-initiating and propagating capacity *in vivo* and expression of stem cell-related genes (Clement et al. 2010).

Another ongoing debate exists on how to best culture GSC to facilitate their study *in vitro*. The first studies applied techniques used for culturing normal neural stem cells. In neurobasal medium supplemented with growth factors such as epidermal growth factor and basic fibroblast growth factor, neural stem cells can be propagated and expanded indefinitely, whereas most differentiating or differentiated cells rapidly die (Reynolds and Weiss 1996).

This approach allowed the prospective GSC isolation from fresh glioblastoma specimens, i.e. a subpopulation of cells capable of proliferation, self-renewal and differentiation (Singh et al. 2004). Some studies, on the other hand, have proposed protocols for adherent cultures of GSC in serum-free medium using attachment factors (Al-Mayhany et al. 2009; Pollard et al. 2009). The latter studies claim that adherent monolayer cultures are superior to non-adherent sphere cultures because of the possibility to ensure uniform exposure to growth factors, oxygen and nutrients leading to a more homogenous cell population.

The high relevance and the potential impact of *in vitro* culturing conditions on cellular characteristics are underscored by a study describing the emergence of cancer stem-like cells from *in vitro* cultured adult rat subventricular zone stem cells. After expansion *in vitro*, neural stem cells transformed into tumorigenic cell lines that acquired multiple chromosomal aberrations. They maintained characteristic stem cell expression markers like Nestin, Musashi-1 and CD133 but continued to proliferate upon differentiation induction and displayed tumorigenicity *in vivo* (Siebzehnrubl et al. 2009).

Functional evaluation of GSC *in vitro* and *in vivo*

The gold standard assay for investigating self-renewal and tumor initiation of GSC is the ability to form tumors in

animals after orthotopic transplantation even of very low cell numbers. These tumors should resemble the morphological and immunohistochemical phenotype of the original tumor. Further, serial dilutions should be performed, i.e. the definition of the smallest cell number capable of generating tumors *in vivo*. Finally, serial passaging, i.e. harvesting of an *in vivo* established tumor and implantation into a secondary mouse, should occur. Regarding these criteria, GSC should display much greater tumorigenic potential than corresponding non-stem tumor cells *in vivo* (Hemmati et al. 2003; Galli et al. 2004; Singh et al. 2004).

Of note, the role of GSC beside tumor initiation has also been studied *in vivo*: many key malignant features of glioblastomas have been attributed to GSC, e.g. elevated levels of vascular endothelial growth factor (VEGF) to promote angiogenesis (Bao et al. 2006a, b), expression of stromal-derived factor-1 (SDF-1) (Salmaggi et al. 2006), and resistance to radiation therapy (Bao et al. 2006a, b).

In vitro, GSC can be induced to differentiate into astrocytes, oligodendrocytes and neurons by addition of serum and withdrawal of the culture conditions outlined above (Hemmati et al. 2003; Galli et al. 2004; Singh et al. 2004). Given these standard functional assessments, it remains to be clarified, however, how stable a GSC phenotype is. This is of particular interest in the face of studies suggesting the isolation of stem-like cells from

established glioma cell lines, e.g. GL-261 or C6 (Pellegatta et al. 2006; Kondo et al. 2004).

Resistance of GSC to therapy

CD133 cell surface expression has been examined in glioblastoma specimens from ten patients who had undergone surgical resection before and after high-dose irradiation with gamma knife surgery and external beam radiation therapy. Comparative analysis of histological sections before and after radiation revealed that CD133⁺ cells were very infrequent in primary tumor sections. In post-irradiated tumors, however, the percentage of CD133⁺ cells was significantly higher. The authors concluded that this confirms the hypothesis that GSC are capable of escaping high doses of radiation (Tamura et al. 2010). However, studies showing that cellular stress induces CD133 must be taken into account in interpreting these data. Probably, CD133 increases after radiation therapy were due to CD133 induction on previously CD133⁻ tumor cells, because—as demonstrated by Griguer and colleagues—CD133 can be induced by hypoxia and cellular stress (Griguer et al. 2008).

Preclinical evidence for resistance to therapy were provided by Bao et al. (2006a, b). They performed colony formations assays using untreated or irradiated CD133⁺ and

Table 2 Signaling pathways and molecules in glioma stem-like cells

Signaling molecule	Biological role	Evidence for therapeutic targeting	Key reference
Chk1 and Chk2 kinases	DNA repair	Inhibitors enhance radiosensitivity of GSC	Bao et al. 2006a, b
EGFR	Growth / proliferation signal	EGFR kinase inhibitors inhibit proliferation and self-renewal	Soeda et al. 2008
Akt		Inhibitor blocks neurosphere formation, induces apoptosis, reduces invasive phenotype <i>in vivo</i> , impairs tumorigenicity <i>in vivo</i>	Gallia et al. 2009
Notch	Neural development	Inhibition blocks self-renewal and promotes radioresistance	Fan et al. 2010
TGF-beta	Numerous cellular processes in normal and neoplastic cells Maintenance of GSC by induction of leukemia inhibitory factor and the Sox family members	Inhibition of TGF-beta interferes with maintenance of GSC	Ikushima et al. 2009
BMP	Neural development	BMP 4 treatment induces differentiation	Piccirillo et al. 2006
miR-124	?	Decreases proliferation and promotes differentiation	Godlewski et al. 2010 ^a
miR-137	?	Decreases proliferation and promotes differentiation	Godlewski et al. 2010 ^a
miR-128	?	Blocks self renewal and decreases proliferation	Godlewski et al. 2010 ^a
miR-7	Neural development muscle differentiation	Inhibits migration and decreases proliferation	Godlewski et al. 2010 ^a

Chk Checkpoint kinase, *EGFR* epidermal growth factor receptor, *TGF* transforming growth factor, *BMP* bone morphogenetic protein

^a For further details refer to this review article

CD133⁻ cells derived either from a biopsy specimen or from glioma xenografts. These assays revealed a relative radioresistance of CD133⁺ cells. Beier et al. (2008) demonstrated on the other hand that temozolomide preferentially depleted CD133⁺ cells in glioblastoma cancer stem cell lines.

Therapeutic targets in GSC

Cell surface molecules differentially expressed in glioma stem-like cells and functionally associated with the maintenance of glioma stem-like cells may be ideal targets for therapeutic intervention. Several molecules including CD133 and A2B5 (Singh et al. 2004; Son et al. 2009; Tchoghandijan et al. 2010) have been discovered on the cell surface of GSC (Table 1). A2B5 monoclonal antibodies recognize GT3 gangliosides, which characterize a small fraction of cells in the subcortical white matter with neural stem cell properties. In glioblastomas, A2B5⁺ cells display

important features including migration and differentiation into oligodendroglia and astroglial cells. Moreover, A2B5⁺ cells but not A2B5⁻ cells form tumors in nude mice. Interestingly, combining A2B5 and CD133 for cell isolation revealed that both A2B5⁺/CD133⁺ and A2B5⁺/CD133⁻ cells formed tumors in nude mice (Tchoghandijan et al. 2010).

Further, identification of specific signaling pathways involved in the maintenance and functions of GSC may also identify useful therapeutic targets for novel strategies to improve the treatment for glioblastoma. The signaling pathways associated with GSC are summarized in Table 2. Disruption of the Notch pathway by Notch shRNA sensitized GSC to radiation therapy (Wang et al. 2009). Differentiation therapy forcing GSC to differentiate might be another promising and notably non-cytotoxic strategy for GSC targeting. In this regard, bone morphogenetic protein (BMP) 4 has been reported to activate BMP receptors, triggering Smad signaling cascade in GSC and leading to a reduction in GSC proliferation and increased

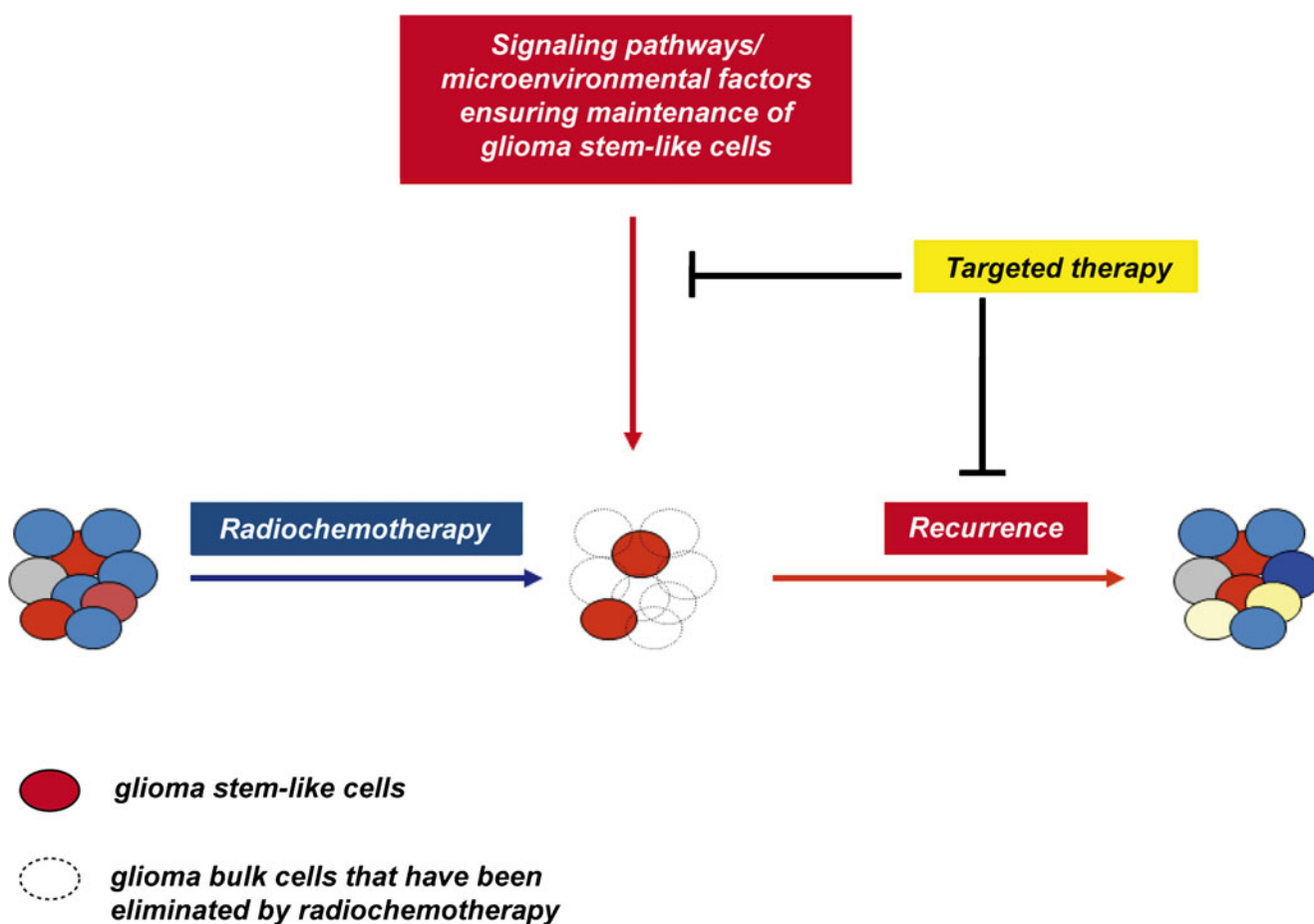


Fig. 2 Conventional radiotherapy might only hit glioma bulk cells while glioma stem-like cells survive and are maintained by key components of the tumor microenvironment. For successful therapeutic targeting, it will be important to design targeted therapies to interfere

with the signaling pathways maintaining glioma stem-like cells. Combining radiotherapy with targeted anti-GSC therapy might then diminish tumor recurrence

expression of differentiated neural markers in CD133⁺ cells (Piccirillo et al. 2006).

Novel important players include microRNAs, which have a central role in gene expression. A role for microRNAs has recently been established for cancers of different types, including glioblastomas. A very comprehensive and detailed overview on the role of miRNA in glioblastomas has recently been published (Godleswski et al. 2010) (Table 2).

The preferential distribution of GSC in the perivascular area has led to the identification of a GSC niche (Calabrese et al. 2007). Disrupting the niche could therefore be another therapeutic strategy. Thus, it is important to identify specific elements in these regions that maintain GSC. A role for endothelial cells as one niche component seems to be established: Co-implantation of GSC with endothelial cells accelerated tumor initiation and progression (Calabrese et al. 2007). Apart from the obvious interaction with endothelial cells, GSC are in close contact with extracellular matrix components that are preferentially expressed in the perivascular niche. Integrin alpha 6 has been recently identified as an important molecule for GSC in the perivascular niche (Lathia et al. 2010). It ensures interaction with laminin-expressing endothelial cells in the microenvironment. Sorting by flow cytometry based on alpha 6 expression alone or alpha 6 integrin/CD133 double-expression led to enrichment of cells with a self renewal capacity *in vitro* and tumorigenicity *in vivo*. Targeted depletion of alpha 6 integrin by lentiviral short hairpin RNA inhibited GSC growth and sphere formation. Moreover, treatment with an alpha 6 integrin-blocking antibody reduced tumor formation *in vivo*. Targeting integrin alpha 6 might be a promising strategy to interfere with the niche and to impair self-renewal, proliferation and tumor initiation capacity (Lathia et al. 2010).

Further, it will be desirable to identify candidate targets, e.g. for immunotherapy. It should be taken into account, for example, that the human AC133 gene, encoding CD133, has at least seven alternatively spliced 5'-UTR isoforms of AC133 mRNA, which are expressed in a tissue-dependent manner. The transcription of these AC133 isoforms is driven by five different promoters, P1–P5, depending on their location relative to different exons. Finding the brain-specific isoform of AC133 and targeting this specific isoform (Shmelkov et al. 2004), e.g. by immunotherapy, will also increase the specificity of these targeted approaches and decrease potential toxicity.

Future perspectives

Selective GSC targeting will be challenging, even if highly specific therapeutic targets are presently available, because

GSC may change and differently adapt *in situ* during the course of the disease within a patient. Because of many overlapping pathways and cell surface markers shared by neural stem cells and GSC, however, one challenge will be to identify therapeutic approaches that avoid potential toxicities to normal neural stem cells.

Taken together, the identification of GSC has increased the complexity of glioblastoma biology.

It remains to be determined to what extent the cells defined by the current methodology give rise to gliomas initially, maintain tumor viability during treatment and promote clinical recurrence. Successful future therapeutic approaches against glioblastoma will require a combination of standard radiochemotherapy with tailored personalized therapies targeting the unique molecular profile and GSC profile of every patient (Fig. 2).

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