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Gliomagenesis: a game played by few players or a team effort?

Stefania Mazzoleni[1, 2], Rossella Galli[1]

[1]Neural Stem Cell Biology Unit, Division of Regenerative Medicine, Stem Cells and Gene Therapy, [2]Vita-Salute San Raffaele University, San Raffaele Scientific Institute, Via Olgettina 58, Milan, Italy, 20132

TABLE OF CONTENTS

1. ABSTRACT

Glioblastoma multiforme (GBM) represents the most aggressive and deadliest brain tumor of adults. To date, cell heterogeneity within GBM has been explained by the "hierarchical" model of tumorigenesis, *aka* the "cancer stem cell" hypothesis. In agreement with this model, only rare tumor cells, namely the cancer stem cells (CSCs), are responsible for GBM initiation and, as such, are considered the favored target of therapy. However, multiple evidence has recently indicated that tumor-initiating cells (TICs) may not represent a restricted and infrequent GBM component; rather, they might constitute most of the cells within the tumor bulk. Here we review several studies that recently shed new light on the process of gliomagenesis. We critically analyze the methodological inconsistencies and drawbacks that are causing protracted controversy in the field. Finally, we discuss the clinical implications and the novel therapeutic scenarios that have been put forward by the presence of functionally and molecularly distinct subpopulations of GBM-initiating cells within the same tumor.

2. INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and aggressive brain tumor of adults (1). Clinical presentation and molecular profiles subdivide GBM into primary and secondary subtypes, which comprise 90 and 10% of all cases, respectively. Primary GBM arise in an acute *de novo* manner with no evidence of prior symptoms or antecedent low-grade pathology. In contrast, secondary GBM derive from the progressive transformation of low-grade gliomas. GBM is characterized by rapid growth characteristics and extreme invasive behavior, thus being inherently resistant to conventional therapy. As a consequence, median patient survival is approximately 14 months. Although standard treatment with surgery, irradiation, and chemotherapy postpones progression and extends survival, these tumors invariably recur and result in death. Therefore, improvement of treatment options for patients with GBM is imperative.

As the adjective "multiforme" implies, GBM is a highly heterogeneous tumor. Tumor heterogeneity may be

intended as the phenomenon by which, similar to other cancers, GBM comprise proper tumor cells and multiple non-tumor cell types (*i.e.* stromal cells, inflammatory cells, pro-angiogenic cells, etc.), which interact to promote and sustain tumorigenesis (2). On the other hand, tumor heterogeneity might also be intended as an additional level of cellular complexity in tumor organization, generated by intrinsic differences retrieved in proper tumor cells in terms of proliferation, invasion and specialization. This last definition of tumor heterogeneity has been explained through two main mechanistic models: the "cancer stem cell" (or "hierarchical" model) (3) and the "stochastic" (or "clonal evolution") model (4). According to the cancer stem cell model, tumor initiation and progression are sustained only by a rare subpopulation of tumor cells with "stem-like" properties and, thus, defined cancer stem cells (CSCs). These stem-like tumor cells are characterized by deregulated self-renewal, which leads to uncontrolled proliferation and aberrant differentiation. In this scenario, the majority of tumor cells, deriving by the differentiation of cancer stem cells, lacks self-renewal potential and, hence, does not contribute significantly to tumor perpetuation. Tumor heterogeneity is, therefore, generated by the coexistence of cancer stem cells (the real "players") and their not tumorigenic differentiated progeny within the same tumor. On the contrary, the stochastic model claims that most cells within a tumor are capable of self-renewal and contribute substantially to tumor initiation and maintenance. Tumor heterogeneity is ascribed mostly to genetic and epigenetic differences existing between different tumor clones. In this view, the distinct cell phenotypes retrievable within a tumor may reflect the presence of cellular clones at different stages of neoplastic transformation and malignancy. Most importantly, this mechanistic model endows all tumor cells with tumor-initiating ability, although to a different extent, thus resulting in the process of tumorigenesis being sustained by a cellular "team" effort.

3. CURRENT CONTROVERSIES IN THE CANCER STEM CELL FIELD

3.1 Tumor heterogeneity: cellular oligarchies or cellular confederations?

The field of CSC is currently animated by a vigorous debate concerning the applicability of the two different models to distinct type of tumors. In agreement with the CSC model, the cardinal property that allows defining a cancer cell as a *bona fide* cancer stem cell is the exclusive capability to generate tumors that recapitulate the main histo-pathological traits of the patient's tumor. This main characteristic has to be maintained also during serial transplantation and limiting dilution assay *in vivo*. Several evidences from various laboratories have shown that in many different types of cancer, such as hemopoietic malignancies, breast and colon cancer, only a rare subpopulation of tumor cells is endowed with the capacity to form new tumors, thus providing strong support to the "cancer stem cell" model. Historically, the first evidence of the existence of CSCs came from seminal studies in acute myeloid leukemia, in which a rare subset of cells, comprising 0.01–1% of the total cell population, was shown to induce leukemia upon experimental transplantation (5). Most recently, putative CSC populations have been identified in solid tumors, by exploiting either dye efflux assays or immuno-panning for distinct cell surface antigens. Among the latter, CD24, CD44 and ALDH1 have been used to identify CSC populations from breast tumors (6, 7), AC133 has been used to identify putative CSCs from brain (8, 9), colon (10,11), pancreatic (12) and lung (13) tumors, EpCAM and CD44 from colon tumors (14), and CD44, CD24 and ESA from pancreatic tumors (15).

However, very recent studies suggested that the cancer stem cell model might not fit to all types of cancers. In 2007, Kelly and colleagues demonstrated that, in contrast with previous findings, more than 10% of tumor cells isolated from three different mouse models of primary hemopoietic tumors readily induced tumor growth when injected into non-irradiated recipients (16). Likewise, in 2008, Shmelkov and colleagues reported that the AC133 negative cell fraction in mouse colon cancer was endowed with tumorigenic potential (17). Along this line, single cell transplantation assays indicated that the frequency of cancer-initiating cells in human melanoma could be higher than 25% (18). Similar conflicting results have been recently reported for brain tumors, in particular GBM. Indeed, multiple cohorts of tumor-initiating cells might be active in GBM, each identifiable based on the expression of specific markers and characterized by distinct functional (i.e. invasive, pro-angiogenic or proliferative) phenotypic features and molecular profiles (19, 20).

3.2 GBM CSC markers: better surrogate or functional?

The progress in brain cancer stem cell/tumor-initiating cell field is currently flawed by the inconsistency of results reported by different laboratories. One of the most likely reasons for this discrepancy might relate to the different experimental procedures exploited for the isolation and enrichment of tumor cell subpopulations (21). For many years, the most frequently employed methodology relied on magnetic bead-based cell sorting. Recently, the specificity and accuracy of this procedure have been put into question, thus explaining the seeming lack of tumor initiation capacity of marker-negative cell fractions (22). Another bias in the interpretation of controversial findings is generated by the use of xenografts obtained from the implantation of patient's tumor samples rather than the exploitation of the human samples as such. Although xenografting bears the significant advantage of increasing the availability of human-derived biological material to be subjected to many different analyses and of preserving the expression of specific molecules, e.g. mutant variants of the EGFR (23,24), it is also known that, in general, *in vivo* passaging of tumor cells might result in relevant modifications in their phenotypic and functional properties (25-27). Likewise, the purification of tumor cell subpopulations only from *in vitro* established cancer stem cell lines without any concurrent cell isolation directly from patient's tumor specimens might restrict the significance and the breadth of the findings (20, 28). Finally, culturing of tumor cells before sorting might alter the outcome of the subsequent analyses (8, 25).

Table 1. Meta-analysis of studies reporting on distinct prospective markers of tumor-initiating cells in human GBM

				Cell sorting by		Cell sorting from				CSC line(s) from	
Marker		**Functions**	**Refs**	**Magnetic beads**	**FACS**	**Patient's Tumor**	**Patient's Tumor Xenograft**	**CSC cultures**	**Additional Marker(s)**	**Patient's Tumor**	**Patient's Tumor Xenograft**
AC133	Surface glycoprotein	N.D.	8	+	-	+ *	-	-	None	-	-
			32	+	-	+	+	-	None	-	+
			28	+	-	-	-	+	None	+	-
			20	+	+	-	-	+	None	+	-
A2B5	Surface ganglioside	N.D.	34	-	+	+	-	-	AC133	-	-
			38	+	+	+	-	+	AC133	+	-
L1CAM	Surface transmembrane protein	Neural cell growth, migration, axonal outgrowth & neurite extension	47	-	+	+ **	-	-	AC133	+	+
SSEA-1	Surface carbohydrate	Adhesion	41	+	+	+	-	+	AC133	+	-
ITGA6	Surface receptor	Adhesion, cell-surface mediated signaling	49	+	+	+	+	+	AC133	+	+
EGFR	Surface receptor	Proliferation, invasion	19	-	+	+	-	+	AC133, SSEA-1	+	-

N.D. not defined *: pre-culturing before sorting; **: 12 hour-preculturing before sorting.

A variety of surface markers have been proposed to identify tumor-initiating cells (TICs) in GBM, the most commonly used being AC133, A2B5, L1CAM, SSEA-1/CD15 and, very recently, ITGA6 and EGFR (29) (Table 1). With the exception of ITGA6, L1CAM and EGFR, these markers do not exert a specific and clear biological function.

The widely used marker AC133 (also known in rodents as Prominin-1) is a pentaspan transmembrane glycoprotein, which specifically localizes to cellular membrane protrusions. This surface marker was used for the first time for the isolation of hematopoietic and neural stem cells (NSCs) (30, 31). Then, it has been employed for the identification of tumor-initiating cells with stem cell properties from different brain cancers, such as GBM and medulloblastoma (8, 32, 33). In the studies focused on GBM, AC133-positive cell fraction was characterized by exclusive tumorigenic potential after intracranial transplantation into NOD-SCID mice, whereas the AC133 negative cell fraction wasn't tumorigenic at all. However, several studies reported recently that AC133-negative GBM cells, either obtained directly from GBM specimens (26, 34) or from established GBM CSC lines (28) were endowed with tumor-initiating capacity, although to a lesser extent than AC133-positive cells. The finding that the $AC133^{neg}$ cell component isolated from fresh GBM specimens, without any *in vitro* culturing before and after cell sorting, could be tumorigenic, as shown in (34), might well be explained by the modality through which the distinct GBM subpopulations were purified, *i.e.* by fluorescence activating cell sorting (FACS). This method is different from that used in previous reports, in which cell enrichment was achieved by magnetic bead-based assays (Table 1). Notably, a very recent study, which compared the use of magnetic activated cell sorting with FACS in GBM fractionation, demonstrated that AC133-microbeads lacked of specificity, with no enrichment for AC133 expression in the isolated cell fractions (22). By this way, most tumor cells could have bound unspecifically to AC133-microbeads, thus explaining the apparently exclusive tumorigenic ability of $AC133^{pos}$ GBM cells. Another possible explanation for the contrasting results obtained by different laboratories might reside in the presence of contaminating $AC133^{pos}$ cells into the marker-negative cell fraction, although this possibility should be ruled out by standard assessment of cell purity or by the inclusion of internal controls, e.g. tumor tissues devoid of $AC133^{pos}$ cells in origin (19). Moreover, given that endothelial cells in the tumor stroma also express AC133, it might be hypothesized that the $AC133^{pos}$ cell fraction containing both tumor cells and stroma is endowed with a growing advantage over the $AC133^{neg}$ fraction. However, we recently reported that both FACS-purified $AC133^{pos}$ and $AC133^{neg}$ GBM cells were capable to promote tumor formation into nude mice even after exclusion of endothelial cells (19). Finally, also the type of anti-AC133 antibodies used for TIC isolation (e.g. anti-AC133/1 and anti-AC133/2, which recognize two different glycosylated epitopes of the transmembrane protein CD133), might affect the experimental consistency between laboratories (35). Notwithstanding the fact that AC133 could not be considered a universal marker, it remains helpful in association with other markers, given that its expression has been associated to fast proliferating cells (28, 36).

Another interesting marker is the cell surface ganglioside A2B5, known to be expressed in neural precursor cells in both the embryonic and adult human brain (37). In GBM, A2B5 expression identified a subpopulation of cells characterized by self-renewal, multipotency and tumor initiation capacity (34, 38). However, whereas the first study reported that both $A2B5^{+}CD133^{+}$ and $A2B5^{-}CD133^{-}$ subpopulations were endowed with tumor formation capacity, the latter suggested that $A2B5^{+}$ cells only were tumorigenic. As a matter of fact, these conflicting results might be due again to differences in the methodology employed for isolating GBM cells, with the use of FACS in the study documenting wider tumorigenic ability and the exploitation of magnetic bead-based sorting in the other study.

Very recently, a novel marker, CD15, has been proposed to specifically label tumor-initiating cell

subpopulations both in medulloblastoma (MB) and in GBM (39, 40, 41). CD15 is a carbohydrate expressed by glycoproteins, glycolipids and proteoglycans. Also known as SSEA-1 (stage-specific embryonic antigen-1), CD15 represents a useful marker for the identification of ES-derived and embryonic NSCs (42, 43). Notably, GBM-derived CD15 positive cells are characterized by self-renewal, multipotency and enhanced expression of stem cell associated genes, such as Sox2 and Bmi1. CD15-positive GBM subpopulations only are highly tumorigenic, with occasional tumor formation observed after the transplantation of CD15-negative cells, ascribed by the Authors to a possible cell contamination after FACS. However, others and we recently demonstrated that FACS-purified CD15-negative cells from GBM could also give rise to experimental tumors with 100% efficiency, putting into question the exclusive tumor-initiating ability of CD15-positive GBM cells (44).

In addition to the surrogate markers described so far, three functional markers have also been suggested as prospective GBM TIC markers. L1CAM, also known as L1 or CD171, is a transmembrane protein involved in neural cell growth, survival, migration, axonal outgrowth and neurite extension during central nervous system development (45). Based on these observations, L1CAM has been identified as a potential therapeutic targ*et al*so in neuro-oncology, given that it is overexpressed in gliomas (46). In 2008, Bao and colleagues observed that $L1CAM^+$ and $CD133^+$ cells co-segregated in glioma cells (47). Suppressing L1CAM expression in $CD133^+$ cells by RNAi negatively affected neurosphere formation and induced apoptosis, thus leading to GBM growth inhibition. The knockdown of L1CAM decreased Olig2 expression and upregulated the p21WAF1/CIP1 tumor suppressor in CD133+ glioma cells, thus indicating that L1CAM was required for maintaining the growth and survival of CD133+ glioma cells, both *in vitro* and *in vivo* (47).

Integrin alpha-6 (ITGA6) is a cell-surface protein that functions as receptor for the ECM protein laminin. Integrins are known to participate in cell adhesion as well as in cell-surface mediated signaling. Integrin a6 is highly expressed in embryonic, hematopoietic, and NSCs (48). Very recently, Lathia *et al.* proposed ITGA6 as an enrichment marker for GBM cancer stem cells (49). ITGA6 was coexpressed with the putative CSC marker AC133 and identified cancer cell with a stem cell phenotype and enhanced tumorigenic potential as compared to ITGA6-negative cells, which, however, could also give rise to experimental, tumors although at a very low frequency.

The third functional marker recently suggested playing a role in gliomagenesis, through the identification of highly malignant TICs is the epidermal growth factor (EGF) receptor (-R) (19). EGFR has been proposed as neural stem cells (NSC) marker (50) that plays a relevant role in regulating subevntricular zone (SVZ) NSC division and stemness maintenance (51,52). Most importantly, EGFR is one of the molecular markers that distinguish the two main GBM subtypes, being expressed in more than 60% of primary GBM and absent in secondary GBM (53). EGFR expression has both diagnostic and prognostic significance, although the prognostic value of the EGFR expression is still controversial (54). According to this study, EGFR is unevenly distributed among the cells of the same GBM patient's sample, thus proposing that EGFR might identify distinct subsets of GBM cells. Accordingly, different GBM cell subpopulations, FACS-purified from GBM patient's specimens and from cancer stem cell (CSC) lines based on the expression of EGFR and independent from the co-expression of AC133/CD15, were molecularly distinct and all endowed with tumorigenic potential (Figure 1). Notably, EGFR-expressing tumor-initiating cells (TICs) displayed the most malignant functional and molecular phenotype. Accordingly, modulation of EGFR expression by gain-of-function and loss-of-function strategies in CSC lines promotes and inhibits tumor formation, respectively. Remarkably, EGFR expression is also an obligated requirement for gliomagenesis. In fact, $EGFR^{neg}$ TICs re-express EGFR upon experimental tumorigenesis, thus implying that the expression of stem cell markers might be dinamically regulated (19). In agreement with this observation, the presence of dynamic subpopulations of tumor-maintaining cells has been recently proposed to explain tumor heterogeneity in melanoma (55) and glioblastomas as well (20).

In summary, it appears that a plethora of prospective TIC markers are already available, which can be exploited as enrichment markers, either alone or combined. However, the identification of markers that could simultaneously be used as enrichment marker and therapeutic target might be beneficial and highly desirable.

4. EXISTENCE OF MULTIPLE COHORTS OF TUMOR-INITIATING CELLS IN GBM.

4.1. Implications for therapy

Multiple evidence has convincingly demonstrated that, in solid tumors, CSCs may not represent a restricted and infrequent tumor component; rather, they might comprise the majority of the cells within the tumor bulk, all endowed with tumor-initiating ability, thus being better defined as tumor-initiating cells (TICs) (ref.17 eliminated). Accordingly, findings from different laboratories now indicate that a novel biological scenario, built upon the coexistence of different tumor-initiating cell (TIC) subpopulations within the same tumor and more in line with the stochastic model of tumorigenesis, might be in place also in GBM (19, 20).

Notably, the presence of functionally heterogeneous TIC subpopulations within the same tumor might affect clinical response to treatment and might be mainly responsible for treatment failure in GBM. In the case of EGFR, treatment by means of tyrosine kinase inhibitors (TKI), *i.e.* small-molecule compounds that inhibit the kinase domain of the EGFR, has been proposed as a possible therapeutic strategy in GBM (56). To date, several clinical studies have reported the only partial efficacy of single agent EGFR inhibition for GBM treatment (57). This failure has been ascribed primarily to the maintenance of a high level of Akt-dependent signalling

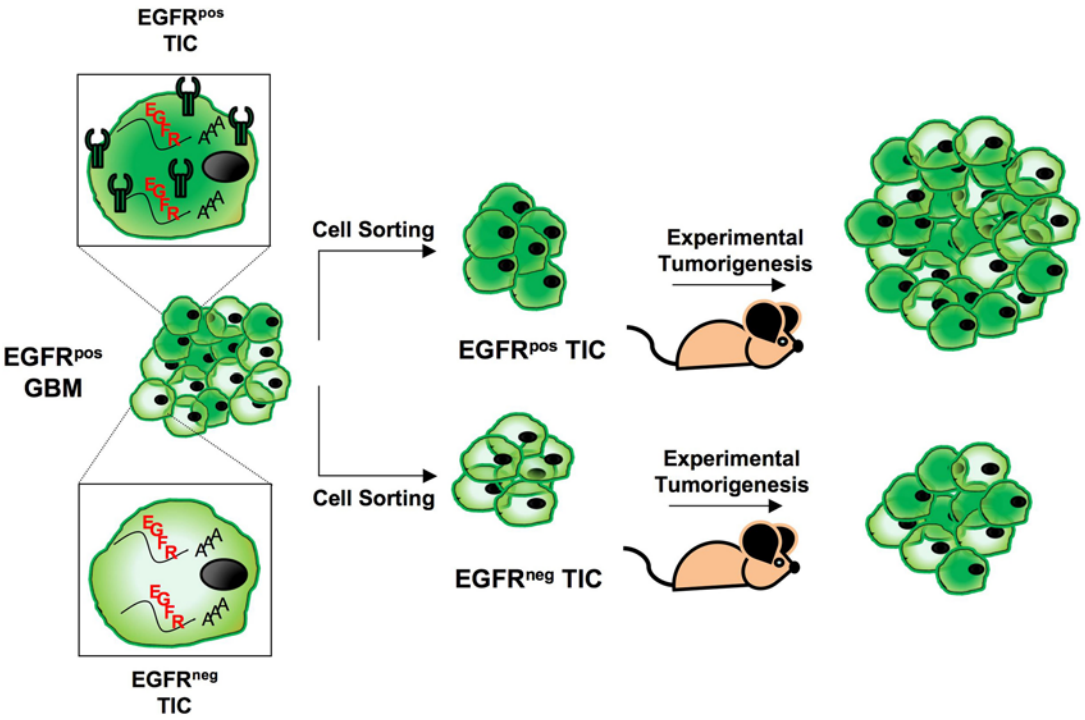

Figure 1. Model summarizing the role of EGFR in experimental gliomagenesis

in PTEN-mutated tumors (58, 59), to the co-activation in the same tumor cell of multiple RTKs (60), as well as to low drug concentrations in tumor tissue or drug inactivation mechanisms. In addition to these possible explanations, the unsatisfactory outcome of EGFR targeted therapies in GBM might also depend on the coexistence of distinct TICs within the same tumor. In fact, highly malignant EGFR-expressing GBM TICs did respond to tyrosine kinase inhibitors (TKIs), thus proving to be EGFR "oncogene addicted" and as such representing a valuable and rationale therapeutic target (19). On the other hand, EGFRneg GBM TICs, which were present within the same tumor together with EGFRpos cells, did not respond to treatment. Thus, although the EGFRpos cell component in EGFRpos GBM can be efficiently eliminated by TKI treatment (Figure 2A), the non-responsiveness of the EGFRneg TIC fraction to EGFR inhibitors might allow these cells to survive treatment, giving rise to tumor recurrence. Hence, to achieve complete tumor eradication, therapeutic strategies should take into consideration the GBM "minimal residual disease" constituted by non-responder GBM TICs, against which developing innovative and specifically targeted approaches (Figure 2B).

Importantly, a wide-ranging therapeutic strategy should include also GBM subpopulations isolated by exploiting other putative TIC markers. As an example, a combination therapeutic paradigm might be hypothesized in GBM by simultaneously targeting ITGA6-expressing GBM TICs with inhibitors of integrin-dependent signaling, such as Cilengitide (49), and EGFR-expressing TICs with EGFR-targeted biological drugs (19).

4.2. Implications for drug discovery

Molecularly targeted therapies hold the promise of providing new anticancer treatments that are more effective and less toxic than traditional cytotoxic chemotherapy. Unfortunately, results of first generation targeted therapy trials for malignant gliomas have been disappointing. Indeed, single-agent drugs have been largely unsuccessful when tested in recurrent disease clinical trials. Thus, only the development of multi-targeted strategies, inhibiting multiple molecular (and cellular) targets simultaneously, might overcome GBM non-responsiveness. To this end, novel therapeutic molecular targets should be identified, which might complement current treatment options. Gene expression studies might be instrumental not only for the understanding of the functional differences between tumor (initiating) cells, but also for identifying novel therapeutic targets. As a matter of fact, clinically relevant distinct molecular subtypes of GBM have been already identified by transcriptional profiling of large collections of GBM patient specimens (61).

However, in spite of novel promising findings (62), neither consensus gene expression profiles have been conclusively associated to GBM patient outcome, nor specific genes have been convincingly interpreted as prognostic factors or promising "druggable" targets. To overcome these limitations, it might be useful to identify molecular mediators that might act both as predictors of survival and therapy choice as well as potential therapeutic targets by exploiting the distinct TIC subpopulations identified in GBM. The identification of minimal gene signatures associated to distinct TICs in GBM will help optimizing and individualizing GBM therapy by

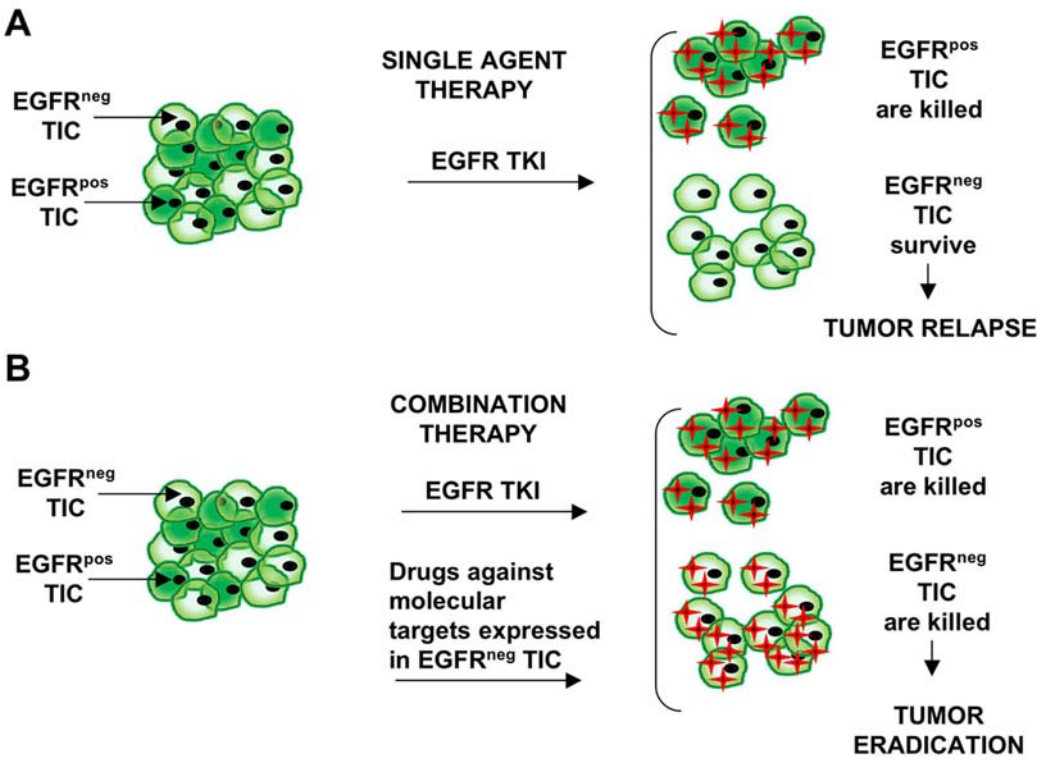

Figure 2. Model summarizing the contribution of distinct GBM TIC subpopulations to therapy response.

prospectively identifying those patients who will benefit most from a specific therapy and by pinpointing novel therapeutic targets for those patients refractory to standard therapy. In the end, a biomarker panel with predictive power to distinguish treatment-sensitive from treatment-refractory GBM patients might become available.

5. SUMMARY AND PERSPECTIVE

In conclusion, the biological landscape that many different studies are progressively building for GBM is complex and variegated.

Firstly, many evidences are suggesting that a stochastic model of tumorigenesis might be more appropriate than the CSC model to explain the intrinsic heterogeneity of GBM. These findings are in line with several recent studies supporting the same view for other types of cancer. However, this novel interpretation of gliomagenesis should not be envisioned as a dismissal of the CSC model. As a matter of fact, the CSC hypothesis proves to be well founded for many hemopoietic malignancies and for several solid tumors. It might be speculated that breast, colon, pancreatic cancers as well as medulloblastoma, all known to originate from tissues in which a rigorous defined cellular hierarchy is defined, *i.e.* the mammary gland, the intestinal crypts, the pancreatic glands and the external granular layer of the cerebellum, might maintain the same type of hierarchic cellular organization during tumorigenesis. On the contrary, solid cancers as melanomas and GBMs, which derive from tissues with a mesenchymal-like organization such as the neural crest and the mature brain, might be sustained by a stochastic and highly flexible model of tumorigenesis.

Secondly, the exploitation of a combination of different enrichment markers might be necessary to fully depict the complexity of GBM biology in terms of cell composition. In particular, the use of functional, rather than surrogate, markers might allow selective and efficient therapeutic targeting of the most malignant tumor-initiating cells. Advanced molecular studies are therefore required for singling out novel molecular targets that selectively identify each GBM subpopulation.

Finally, effective therapeutic strategy for GBM should take into consideration the presence of many different “actors” playing on the GBM stage. In this scenario, it should be reminded that the expression of stem cell markers in GBM might be dynamically regulated, given that stem cell marker-negative cells can become marker-positive. Hence, GBM heterogeneity should be considered a dynamic process mediated by temporarily distinct GBM cell subpopulations that need to be targeted timely and according to well-balanced treatment regimens and schedules.

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Send correspondence to: Rossella Galli, Neural Stem Cell Biology Unit, Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy, Tel: 39022643 4626, Fax: 390226434621, E-mail: galli.rossella@hsr.it

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