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Cell of Origin and Cancer Stem Cells in Tumor Suppressor Mouse Models of Glioblastoma

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Abstract

The cellular origins and the mechanisms of progression, maintenance of tumorigenicity, and therapeutic resistance are central questions in the glioblastoma multiforme (GBM) field. Using tumor suppressor mouse models, our group recently reported two independent populations of adult GBM-initiating central nervous system progenitors. We found different functional and molecular subtypes depending on the tumor-initiating cell lineage, indicating that the cell of origin is a driver of GBM subtype diversity. Using an in vivo model, we also showed that GBM cancer stem cells (CSCs) or glioma stem cells (GSCs) contribute to resistance to chemotherapeutic agents and that genetic ablation of GSCs leads to a delay in tumor progression. These studies are consistent with the cell of origin and CSCs as critical regulators of the pathogenesis of GBM.

Glioblastoma is the most aggressive and prevalent primary malignancy in the central nervous system (CNS). However, despite numerous advances in cancer therapy, these tumors remain incurable (Stupp et al. 2005). In recent years, large-scale genomic analyses have been used to stratify glioblastoma multiforme (GBM) based on gene expression signatures, driver mutations, copy number changes, and other molecular alterations that could reflect differing pathologic characteristics (TCGA 2008). The Cancer Genome Atlas (TCGA) analyses based on the above-mentioned criteria resolve GBM into four subtypes denoted mesenchymal, proneural, classical, and neural (Verhaak et al. 2010). Several groups have since reported various glioma subtyping schema based on newer molecular data and other criteria (Eckel-Passow et al. 2015; Ceccarelli et al. 2016). The clinical implications for therapeutic response of these genomic subclassifications, however, remain unclear.

GBM-INITIATING LINEAGES

The extensive cellular heterogeneity found within the adult mammalian brain affords a variety of cell types that could, in principle, give rise to malignant tumors. Our studies have progressively focused on stem and progenitor cells in the adult brain as attractive candidates for tumor origin based on self-renewal and proliferative potential that would provide an opportunity for the stochastic accumulation of the essential mutations required for malignant

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transformation. In the mouse, adult neural stem cells reside in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus, where they produce neuronal progenitor cells (NPCs) that primarily give rise to new neurons (Ming and Song 2011). In contrast, the widely dispersed oligodendrocyte progenitor cells (OPCs) represent a significant proportion of dividing cells in the adult brain and are the primary source of oligodendrocytes in both the developing and mature CNS (Nishiyama et al. 2009).

A TALE OF TWO TUMORS

Various genetically engineered mouse models (GEMMs) of brain tumors have been generated by oncogene overexpression and/or deletion of tumor suppressors in various cell types of the brain. Our laboratory has applied the GEMM technology to drive GBM formation through ablation of three of the most commonly mutated genes in human GBM: *NF1* (neurofibromin 1), *TP53* (tumor protein P53), and *PTEN* (phosphatase and tensin homolog). Initially, we used a human glial fibrillary acidic protein (GFAP) promoter transgene construct (*hGfap-cre*) to drive Cre recombinase expression in the embryonic and adult stem cell niche to generate compound heterozygotes at the tumor suppressor loci. Fully penetrant GBM formation was accompanied by loss of heterozygosity for the tumor suppressors in the malignant tissue (Zhu et al. 2005). These studies were further refined by targeting the adult neural stem cell compartment using a *Nestin-creER*^{T2} transgene to ablate tumor suppressors and also generated GBM with 100% efficiency (Alcantara Llaguno et al. 2009). More recently, we used an *Ascl1-creER*™ transgene that targets two different populations of adult lineage-restricted CNS progenitors, the NPCs and OPCs. Adult bipotential progenitor-directed loss of *Nf1*, *Trp53*, and *Pten* in mice led to the development of two histologically and molecularly distinct GBM subtypes (Alcantara Llaguno et al. 2015). The type 1 GBM is a more infiltrative, Gfap^{hi} tumor and is preferentially found in the dorsal brain. The type 2 GBM has a Gfap^{lo} signature and better defined tumor borders and is most frequently found in the ventral brain (Fig. 1). Histologic and genomic analyses showed that *Ascl1-creER*™-driven type 1 GBMs resemble *Nestin-creER*^{T2}-driven tumors. In contrast, the type 2 GBMs resembled tumors that were generated through mutation of the three tumor suppressors with an OPC-specific Cre driver (*NG2-creER*™).

Thus, at least two independent populations of GBM-initiating adult progenitors exist that, in the setting of identical driver mutations, give rise to GBM as determined by classic histopathological criteria. However, additional pathological, molecular, and morbidity assays reveal differing features for the two GBM types, indicating that the cell of origin contributes to GBM genomic profiles and resultant phenotypes. This was further illustrated when GBM derived from different tumor suppressor mouse models were found to be molecularly separable based on the lineage of the tumor-initiating cell, indicating that tumor cells have cell-of-origin memory and thus supporting the idea that the cell of origin is a determinant of GBM subtype (Alcantara Llaguno et al. 2015). Given these results, the variety of cell lineages and progenitor populations in the adult mammalian brain (Ming and Song 2011; Merkle et al. 2014) present the plausible scenario that yet additional to-be-defined GBM subtypes may exist (Fig. 1). Thus, the various populations of tumor-initiating cells may underlie a diversity of functional subtypes found in human GBM.

It has been reported that more differentiated cell types can give rise to GBM in mouse models (Bachoo et al. 2002; Friedmann-Morvinski et al. 2012). Although in the experimental setting this may be possible, it is unclear how fully differentiated, nondividing neurons or astrocytes might acquire successive transforming mutations in the natural setting. To date, the occurrence of dedifferentiation in adult postmitotic CNS cells and its physiologic relevance remains controversial. Additional work on this topic will be essential to understand these issues.

DEVELOPMENTAL MEMORY OF GBM CELLS

How does a divergent lineage, such as the OPC lineage, give rise to a CNS tumor that exhibits predominantly histologic astrocytic features? Recent work suggests a common developmental origin for stem cells and progenitors. For example, embryonic OPCs have been shown to generate not only oligodendrocytes but also protoplasmic astrocytes in the gray matter of the ventral forebrain (Nishiyama et al. 2009). This raises the possibility that embryonic identity can be reactivated during adult glioma development, which may allow previously bipotential OPCs to give rise to type 2–like astrocytomas. Moreover, recent data show that adult SVZ neural stem cells share a common lineage with embryonic stem cells that give rise to the cells of the cortex, striatum, and septum (Fuentealba et al. 2015), suggesting that transformed stem cells or progenitors may exhibit a developmental memory that preferentially leads them to migrate to these dorsal forebrain regions, where type 1 tumors are prevalent.

HUMAN GLIOMAS AND THE CELL OF ORIGIN

When we transplanted type 1 and type 2 GBMs as intracranial allografts, the engrafted tumors retained their original primary histological features, and type 1 tumor-transplanted mice showed significantly shorter median survival compared to type 2 tumor-transplanted mice (Alcantara Llaguno et al. 2015). This raises the possibility that GBM prognosis may be defined not only by the sum of pathological characterization and molecular driver genes but equally or more so by the cell of origin in which the mutations arise.

Our results show that GBM can be stratified into functional subtypes in mouse models through initiation in different progenitor cells. Expansion of such approaches to include the larger spectrum of possible initiating driver mutations and additional progenitor lineages integrated with existing genomic and clinical data should provide a more robust and complete picture of GBM. Should this lead to discovery of lineage or subtype-specific pathways that can be therapeutically targeted, it will allow functional stratification of patients into subgroups that may respond to more tailored therapies.

CANCER STEM CELLS IN GBM

The concept of the cancer stem cells (CSCs) relates to the hypothesis that some solid tumors may propagate in a hierarchical fashion in which a relatively quiescent tumor cell lies at the apex of the hierarchy. In such a model, the highly proliferating derivative tumor cells are limited in their self-renewal capacity and are not responsible for therapeutic resistance or

tumor relapse. The CSC model was led by research in the hematopoietic system, where in-depth characterization has resulted in a toolkit of cell surface markers that distinguish between hematopoietic stem cells, progenitors, and differentiated cells, as well as their malignant derivatives (Bonnet and Dick 1997). In solid tumors, CSCs have been described in different organ cancers, including breast, prostate, pancreas, colon, skin, and brain (Hermann et al. 2007; Wang et al. 2009a; Pece et al. 2010; Chen et al. 2012a; Driessens et al. 2012; Schepers et al. 2012). However, faithful markers for CSCs in solid tumors are in infancy and most CSC assays for solid tumors rely on indirect surrogate approaches such as the limited repertoire of enrichment antibodies or transplantation assays that involve tissue dissociation, removal from microenvironment, and, in some cases, incubation with growth factor cocktails in vitro.

For GBM patients, survival has not improved significantly in the last several decades, suggesting that current treatment regimens fail to target the critical cells responsible for tumor growth. One explanation for this would be that GBM development and growth is hierarchical and that CSCs are impervious to current therapies. We have examined glioma stem cells (GSCs) in the context of a possible relationship with wild-type neural stem and progenitor cells, and their possible roles not only in tumor initiation and maintenance but also in therapy resistance and tumor recurrence.

Through genomic landscape studies, GBM core signaling pathways that may contribute to gliomagenesis have been proposed. Among them, the ARF/P53 pathway, the INK4A/RB pathway, the RTK pathway, and the NF-κB pathway are frequently dysregulated (Chen et al. 2012b). These same pathways are known to play essential roles in wild-type neural stem cell self-renewal, proliferation, and survival (Groszer et al. 2006; Meletis et al. 2006; Hegedus et al. 2007). A variety of mouse models manipulating these pathways in neural stem/progenitor cells have been shown to efficiently form high-grade gliomas (Zhu et al. 2005, 2014; Ligon et al. 2007; Alcantara Llaguno et al. 2009; Wang et al. 2009b; Zheng et al. 2013). These studies pointed to a critical role for neural stem/progenitor cells in glioma formation. However, whether GSCs exist in most or all forms of GBMs remains unknown.

GSCs were first isolated from human GBM samples by fluorescence-activated cell sorting (FACS) using the CD133/Prominin1 antibody (Singh et al. 2003). However, subsequent studies reported contradictory results showing that CD133-negative cells also possess similar properties as CD133-positive cells, such as neurosphere formation in defined culture media and the ability to form transplanted tumors (Nishide et al. 2009; Holmberg Olausson et al. 2014). Other markers have also been reported to enrich for GSCs, including integrin α6, CD44, CD15, and EphA3 (Son et al. 2009; Anido et al. 2010; Lathia et al. 2010; Day et al. 2013). Nevertheless, a thorough characterization of these potential GSC markers is still lacking. There are also a host of unanswered questions. Are these different GSC markers expressed in the same cells? How are they functionally related to GSC features? How do these markers relate to the traditional (primary versus secondary) and genomic (neural, proneural, mesenchymal, classical) classifications of GBM? Furthermore, different assays have been used to investigate the GSC features of primary or cultured cells. The gold standard in the field is to perform tumor cell transplantation assays in immunodeficient mice, requiring tumor cell dissociation and often with manipulation in culture outside the

tumor microenvironment before transplantation. The study of GSCs studied in their native microenvironment may better reflect their biological properties. The use of spontaneous mouse GBM models afford a reproducible and reliable system to genetically identify and label GSCs, elucidate their unique features, and examine the critical mechanisms involved in tumor recurrence.

IN VIVO GBM CSC MODEL

To directly test the CSC hypothesis in GBM, we used the human GFAP-Cre (*hGfap-cre*)-driven mouse GBM model together with a *Nestin-ΔTK-GFP* transgene, validated to label adult neural stem cells in the SVZ. This same transgene also labels a subset of quiescent tumor cells in mouse model GBMs. The presence of thymidine kinase (TK) allows the ablation of dividing TK transgene-positive cells with gancyclovir (GCV). Administration of GCV into mutant mice carrying the *Nestin-ΔTK-GFP* transgene was shown to significantly prolong the life span of tumor-bearing mice (Chen et al. 2012a). In vivo tracing with BrdU analogs IdU and CldU indicated that they were resistant to temozolomide treatment and responsible for tumor recurrence. Combination treatment of GCV and temozolomide also efficiently eradicated the type 1 dorsal tumors. However, mutant mice still died from secondary type 2–like ventral tumors that developed concurrently with the type 1 dorsal tumors. This may have been caused by the widespread expression of the *hGfap-cre* transgene used to initiate the tumors. Hence, a more restricted and preferably inducible cre expression would be required to circumvent this problem. On the other hand, the development of a GSC reporter provided a powerful means by which we can extensively determine the properties of GSCs.

USING THE NESTIN-GFP TRANSGENE TO CHARACTERIZE GSCS

The ability to label a GSC population in spontaneous GBM in vivo with the *Nestin-ΔTK-GFP* transgene opens the possibility of performing a relatively unbiased analysis for potential markers. FACS sorting of the Nestin-GFP$^+$ cells enriches a unique population of tumor cells derived from the mouse model. Whole transcriptome analysis between the GFP$^+$ and GFP$^-$ cells will potentially lead to a list of candidate genes for marker identification. It can also provide insight into the molecular characteristics of quiescent GFP$^+$ GSCs that can be used to design potential therapies. One potential issue in the CSC field is the resolution of the cellular and molecular data collected in different studies. The quiescent status of CSCs can affect the overall behavior of tumor cells significantly. For example, it might change the overall transcription level of GSCs. If this is the case, analysis of cells in the group level could be misleading. Advances in sequencing technologies and computational analysis have greatly reduced the cost and effort of studying biological events at the single-cell level.

Together with other single-cell technologies in DNA analysis, proteomics, and metabolomics, we will be able to draw a higher-resolution map for the unique features of GSCs to elucidate their various roles in tumor progression, maintenance, and resistance to therapies.

GSCS AND THERAPEUTIC RESISTANCE

How do GSCs contribute to resistance to current therapies? There could be several mechanisms at play. For example, like many normal stem cell populations, GSCs may have enhanced capacity to either block entry or efficiently export drugs. Certain membrane transporters, such as ABCG2, have been shown to be up-regulated in both normal stem cells and GSCs (Bleau et al. 2009), which may allow GSCs to pump out chemotherapeutic drugs like temozolomide. Another possibility is that GSCs harbor distinct features that current radiation and chemotherapy treatments are incapable of targeting. Most of these therapies were designed to act on dividing tumor cells. Hence, the less proliferative, the less likely these tumor cells will be effectively targeted by agents designed to target cell proliferation (cell cycle, mitosis, or DNA replication components). In addition, the relatively slow progression to cell cycle for GSCs would minimize the degree of mutation suffered compared to highly proliferative cells that would incur greater DNA damage during multiple rounds of treatment as they progress through cell division. Our studies with the *Nestin-ΔTK-GFP* transgene indicate that the GSCs are primarily in a quiescent state and exhibit relative resistance to the effects of chemotherapy (Fig. 2A,B). After drug treatment, quiescent GFP^+ GSCs re-enter the cell cycle to produce new cells that eventually repopulate the tumor (Fig. 2C). This leads to tumor recurrence after aggressive multimodal treatment that has been the norm in GBM patients. The challenge therefore is to identify CSC vulnerabilities. Such approaches can complement traditional therapies in order to effect more lasting tumor inhibition.

CONCLUSION

Our studies on tumor suppressor mouse models suggest important roles for the cell of origin and CSCs in the pathogenesis of glioblastoma. However, despite numerous advances, much remains to be accomplished to elucidate the various mechanisms involved. The direct link between cells of origin and CSCs is also not clear. Do cells of origin directly give rise to CSCs or is there a common pathway to CSCs, as in leukemias? How do these transformations occur? Do similar pathways govern tumor initiation, progression, and maintenance? What aspects of the cellular origin are maintained or acquired in CSCs? Do different functional GBM subtypes possess distinct GSC subsets? How can these be targeted? Continued research using animal and xenograft models as well as human data analysis will provide a clearer picture of their contributions in GBM formation, which will hopefully lead to better diagnostics and therapeutics for glioblastoma. The hope is that better functional stratification of GBM and direct targeting of GSCs will add to the direly needed armamentarium to prolong the lives of patients with GBM.

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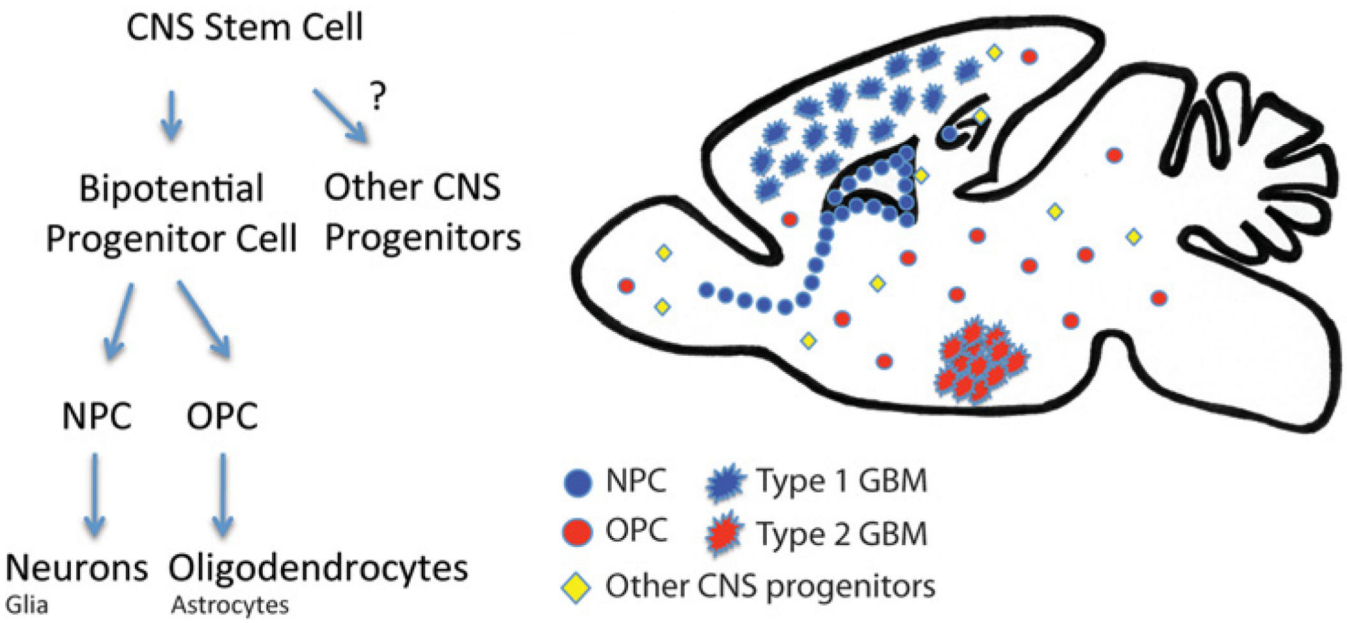

Figure 1.

Two independent populations of glioblastoma multiforme (GBM)-initiating central nervous system (CNS) progenitors exist in the adult mouse brain. (*Right*) GBMs can be initiated by tumor suppressor mutations in adult neuronal progenitor cells (NPCs) and adult oligodendrocyte progenitor cells (OPCs), which give rise to histologically and molecularly distinct tumors. NPC-driven type 1 tumors are infiltrative gliomas mostly found in the dorsal brain, whereas OPC-driven type 2 tumors have more defined tumor borders prevalent in ventral brain regions. (*Left*) Lineage hierarchy of CNS cells: Multipotent CNS stem cells undergo asymmetric cell division to produce bipotential progenitor cells that give rise to lineage-restricted progenitors that differentiate into mature CNS cell types. Other potential unique subsets of adult progenitor lineages may give rise to additional GBM subtypes.

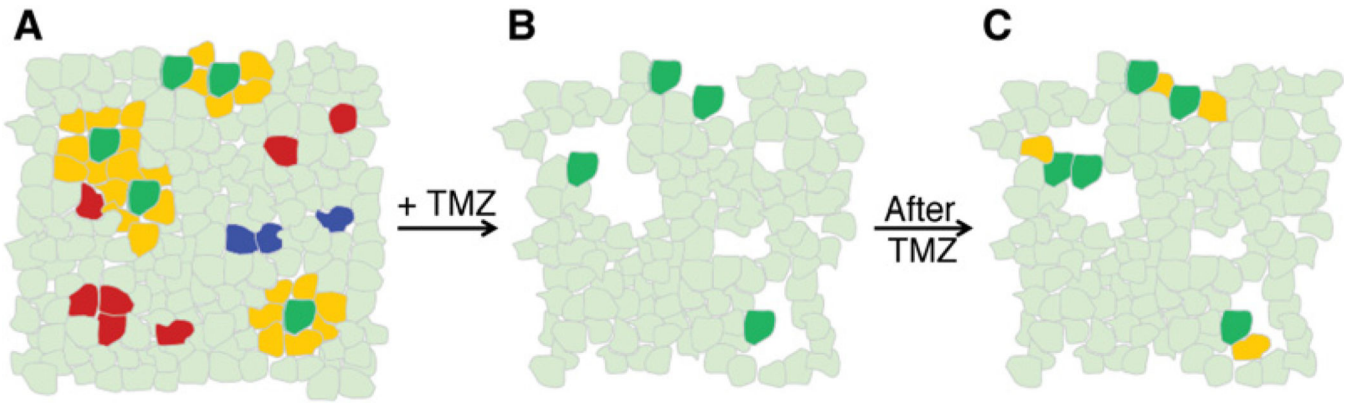

Figure 2.

A restricted cell population in a spontaneous glioblastoma multiforme (GBM) mouse model is responsible for tumor recurrence after chemotherapy. (*A*) A cartoon depicts the heterogeneity of glioblastoma. Dark green cells represent the quiescent stem cells; yellow, red, and blue cells indicate the actively dividing cells with different proliferative potential; light green cells, which represent the bulk tumor cells, have exited the cell cycle. (*B*) Upon temozolomide (TMZ) treatment, most of the dividing cells are eradicated. (*C*) Quiescent stem-like cancer cells re-enter the cell cycle to repropagate the tumor. They can divide symmetrically to form more stem cells (self-renewal) or asymmetrically to generate more proliferative cells.