Brain Tumor Stem Cells Remain in Play

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Traditional anticancer therapy includes the administration of harsh chemicals, often coupled with targeted radiation therapy. This strategy often delivers positive results in terms of reduced tumor burden, but the effects are rarely lasting and all too frequently the disease progresses. In addition, chemo- and radiotherapy do not discriminate between normal cells and cancer cells, but rather target all proliferating cells by attacking the normal cellular mitotic, replicative, or DNA repair and editing capabilities. For this reason, anticancer treatment is most often accompanied by debilitating adverse effects that can include nausea, GI malaise, anemia, and hair loss. In general, completion of chemotherapy is followed by gradual resolution of the adverse effects. One explanation for this lies in the fact that, in addition to targeting proliferating cancer cells, chemotherapy indiscriminately attacks and destroys the proliferating pools of normal progenitor cells (transient amplifying cells) within all organ stem-cell compartments. As a consequence, during treatment, production of new blood and intestinal epithelial cells is greatly diminished. In contrast, resting nonmitotic cells, including the relatively quiescent stem cells, remain relatively unaffected in the face of chemical drugs designed to target the machinery associated with cell division. After therapy completion, intestinal crypt stem cells and bone marrow hematopoietic stem cells can resume production of progenitor cells, and tissue homeostasis can be restored.

What about tumor recurrence? Prevailing theories suggest that acquisition of drug resistance results from the accumulation of new mutations caused by natural tumor progression as well as by the mutagenic effects of chemo- and radiotherapy. Indeed, studies of low-grade gliomas that have progressed to glioblastoma multiforme (GBM) after therapy have highlighted the potentially dramatic mutagenic consequences of the DNA alkylating agent temozolomide on tumor progression.¹ An alternative or complementary explanation for drug resistance emerges from the notion that at least some tumors retain aspects of normal tissue hierarchies, with cells at the apex of the hierarchy exhibiting properties related to stem

cells, such as relative quiescence and the ability to divide asymmetrically to give rise to transient amplifying progenitor-like pools of tumor cells.

The seminal work of Dick et al studying the hematopoietic system reintroduced the lapsed idea that limited subsets of primary tumor cells have the capacity to reinitiate tumor development on transplant into immunocompromised mice.²⁻⁴ These studies galvanized the search for and study of cancer stem cells (CSCs) in solid tumors including malignant brain tumors.⁵⁻⁸ In the context of glioma, Singh et al⁹ reported limited capabilities of human GBM tumor initiating cells in transplant assays as well as in primary spheroid culture assays, thus providing the first evidence for glioma stem cells. In addition to using a limiting dilution assay, these studies used the CD133 epitope as a means of enriching for CSCs.

GENETICALLY ENGINEERED MOUSE MODELS

One approach to addressing the question of tumor hierarchy has been the use of genetically modified mice that develop malignant brain tumors. After the original work of Singh et al⁹ indicated that CD133 enriched for tumor-propagating cells, a mouse model of hedgehog-driven medulloblastoma was tested for the presence of tumor-propagating cells in a transplant assay. These studies indicated that the transcription factor *Atoh1* and the cell surface marker CD15 could be used to enrich medulloblastoma CSCs.^{10,11} Notably, the CD15⁺ population represented a relatively large fraction of the tumor and included highly proliferative cells. Subsequent studies demonstrated that only a subset of these cells, which are quiescent and express the transcription factor Sox2, drives tumor propagation and may be responsible for relapse after therapy.¹²

In addition to the use of certain assays to propagate tumors after transplant, different assays have been used to identify and enrich brain tumor CSCs. The enhanced ability of stem cells to export fluorescent dyes causes them to separate into a side population in fluorescence-activated cell-sorting analysis. By analogy, an replication-competent ALV LTR with a splice acceptor-platelet

derived growth factor–driven glioma side population of tumor cells has been characterized as a stem-like population revealing the preference of these cells for the perivascular niche.¹³ Likewise, the ability to form spheres when cultured at low density is frequently associated with stem-like properties. Each of these approaches permits molecular and cellular analyses of enriched brain tumor stem-cell populations to reveal a variety of aspects of their metabolism and *in vitro* and *in vivo* cellular properties.

One caveat of these approaches is the necessity to remove tumor cells from their original microenvironment, dissociate them, and manipulate them before subsequent tumor-propagating assays. However, the consequences of such manipulations and their impact on the final outcome of studies cannot be fully assessed. A particular virtue of genetically engineered mouse models is the potential for studying autochthonous tumor development without tumor cell isolation, manipulation, and transplant. One such approach exploited a nestin promoter-enhancer–driven reporter transgene (NesTK-GFP) designed to mark quiescent adult neural stem cells in mice.^{14,15} This transgene further co-expressed the herpes simplex virus thymidine kinase gene. When the NesTK-GFP transgene was placed within a tumor suppressor genetic background that elicited spontaneous GBM, four key observations were made from study of the resultant GBM-harboring mice. First, all spontaneous tumors contained minor populations of green fluorescent protein (GFP)–expressing cells that did not express the proliferation marker Ki67. Second, ganciclovir administration, designed to kill NesTK-GFP cells entering the cell cycle, caused prolonged survival of the tumor-bearing mice. Third, temozolomide treatment of tumor-bearing mice specifically targeted the proliferating (bromo-deoxyuridine–incorporating) tumor cells, and renewed tumor cell proliferation after treatment completion was traced to the temozolomide-resistant GFP-positive cells.¹⁶ Fourth, within the resolution of the experimental system, no evidence was found to support the idea that after temozolomide treatment, a significant proportion of new proliferating cells emerged from outside the GFP-positive quiescent tumor cell compartment. These and other genetically engineered mouse studies¹² provide compelling evidence that in autochthonous brain tumors, hierarchical tumor growth emanating from CSCs is a property of tumor development and regrowth after therapy.

HUMAN GBM STUDIES

The hematopoietic system, where CSCs were first identified, offers several advantages compared with solid tumors. Preparation of cells does not require harsh treatments because the cells already exist in suspension. Moreover, transplantation of hematopoietic stem cells to reconstitute a depleted microenvironment and the ability of leukemic cells to outcompete nontumorigenic hematopoietic cells, have been well established for decades. In contrast, transplantation of human brain tumor cells requires intermediate *ex vivo* manipulations beginning with enzymatic dissociation and extending to incubation and/or culturing under artificial conditions for variable periods of time. The adoption of surrogate assays is unavoidable and therefore subject to potential complications and artifacts. However, in the absence of better alternatives, such approaches have yielded important insights into brain tumor biology.

It remains important to note, however, that despite the variability of assays and their relative merits, all these published studies generally use the same terminology of CSCs, and therefore, the brain tumor stem-cell field does not in general discriminate among differing experimental conditions. Thus, studies that use different assays or different paradigms to enrich for CSCs may yield entirely different results.

One frequently used system involves placing primary human GBM cells in suspension in serum-free defined growth factor conditions. The resulting tumor spheres have been termed glioma stem cells and have been used to study *in vitro* growth properties, chemoresistance, epigenetic characteristics, and tumor-initiating potential. Such cells have also been used successfully to perform small molecule compound screens that could identify drugs with specificity for these unique tumor cells.^{6,17-19} Other approaches to enrich for glioma stem cells use antibody-mediated selection for putative glioma stem cell–specific epitopes, such as the CD133⁺ population of GBM cells, through panning or fluorescence-activated sorting.^{20,21} These cells have been subjected to analysis for metabolic properties as well as for p53-dependent radio-resistance and immune avoidance, among other properties.²² CD133 enrichment has proved to be an effective method for enriching for tumorigenic glioma cells that exhibit a variety of *in vitro* properties that can be considered consistent with those expected for CSCs. Whether CD133 is truly an accurate or specific marker for CSCs remains an active topic of conversation in the field.

Next-generation sequencing has played an important role in deciphering the genomic landscape of GBM and other gliomas.^{23,24} Genomic studies have further categorized tumors according to transcriptional profiles and have used such data to infer the evolution of tumors.²⁵ An emergent technology is the use of single-cell sequencing to understand the intratumoral heterogeneity of human tumor samples. Early studies highlight the promise of such approaches. For example, initial studies of IDH mutant oligodendrogliomas reveal that these tumors include cells with glial properties as well as a subpopulation whose transcriptional profile is most aligned with that of stem cells.²⁶ A more recent study examined IDH mutant low-grade gliomas and oligodendrogliomas.²⁷ This study showed that at the individual cell level, features begin to emerge that suggest a developmental relationship of tumor cell lineages and the existence of more primitive stem-like signatures within both tumor types. These early studies do not identify cells with a quiescent signature that would be predicted by the CSC hypothesis. In mouse glioma studies, the relative proportion of CSCs is only a small percentage of tumor cells.^{10,12,28} Because these first single-cell studies likely do not reflect a full representation of all possible cell types in the tumors analyzed, it remains possible that relatively small cell subpopulations within the human tumor samples may remain undetected.

Whether brain tumors develop in a hierarchical model or not remains an important and unresolved question. The implications of this distinction are far reaching and would have an effect on the design, development, and evaluation of novel therapies. The dynamics of action by chemotherapy on normal stem-cell niches highlight the inherent resistance of stem cells. Potential analogies may apply to solid tumor resistance. To date, genetically engineered mouse models have provided some of the most compelling support for the CSC hypothesis. Studies using human primary tumor

stem-like cells add to the argument that a functional hierarchy likely exists. However, much needs to be accomplished before a final conclusion can be reached. Mouse models must continue to improve, to further validate physiologic relevance and to provide not only evidence for CSCs in mice but also novel tools that permit direct probing of the existence of CSCs in human tumors. Single-cell sequencing of murine tumors under unbiased conditions will likely provide important insights into the CSC question, although additional functional approaches are needed. Ultimately, the existence of a tumor hierarchy and of CSCs in brain tumors is relevant only if found in human tumors. The current methodologies for enrichment or identification of CSCs are not unified, and none of these in isolation can constitute proof of CSC existence. As single-cell sequencing efforts expand, the accumulation of data will make the information ever more granular. Direct comparisons of emergent data from single-cell sequencing of mouse and human tumors will either reflect convergence of evidence for CSCs or important differences that will require realignment of working hypotheses. Likewise, xenograft experiments that seek to identify tumor hierarchies using pulse-chase and

lineage-tracing techniques will either add to the evidence or detract from the functional hierarchy model. The question of whether brain tumor CSCs exist remains outstanding, but the importance of the problem is not diminished, and further developments may be just around the corner.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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