Laboratory Models for Central Nervous System Tumor Stem Cell Research

Imad Saeed Khan and Moneeb Ehtesham

Abstract Central nervous system (CNS) tumors are complex organ systems comprising of a neoplastic component with associated vasculature, inflammatory cells, and reactive cellular and extracellular components. Research has identified a subset of cells in CNS tumors that portray defining properties of neural stem cells, namely, that of self-renewal and multi-potency. Growing evidence suggests that these tumor stem cells (TSC) play an important role in the maintenance and growth of the tumor. Furthermore, these cells have also been shown to be refractory to conventional therapy and may be crucial for tumor recurrence and metastasis. Current investigations are focusing on isolating these TSC from CNS tumors to investigate their unique biological processes. This understanding will help identify and develop more effective and comprehensive treatment strategies. This chapter provides an overview of some of the most commonly used laboratory models for CNSTSC research.

Keywords Glioma stem cells • Tumor stem cells • Cancer stem cells • Laboratory models • Neurosphere culture • Matrigel-based assays • Orthotopic culture • Mathematical models • Animal models

Introduction

As evidenced by the name Glioblastoma Multiforme, investigators have long recognized the morphological variation depicted by brain tumors. Brain tumors have been described as complex organ systems comprising of a neoplastic component with associated vasculature, inflammatory cells, and reactive cellular and extracellular components. Tumor genetic analyses have also demonstrated regional

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M. Ehtesham (ed.), *Stem Cell Biology in Neoplasms of the Central Nervous System*, Advances in Experimental Medicine and Biology 853,
DOI 10.1007/978-3-319-16537-0_5

variation in gene expression and chromosomal aberrations. Using differentiation markers, multiple states and variations have also been shown in brain tumors [1].

There has been increasing interest in the role of tumor stem cells (TSC) in the pathogenesis of CNS tumors. This has given rise to the cancer stem cell tumor model that is predicated upon the presence of a small subset of cancer cells with the exclusive ability to divide and expand the TSC pool, and also to give rise of heterogeneous non-tumorigenic cancer cell lineages that constitute the bulk of the tumor [2].

TSC (also known as cancer stem cells or tumor-initiating cells) were first isolated from blood cancers. A small fraction of acute myeloid leukemia (AML) cells were shown to be capable of initiating and sustaining clonogenic growth and inducing leukemia in nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mice [3, 4]. Of note, these leukemic subclones shared the same cell surface markers (CD43+, CD38−) as hematopoietic stem cells, while the progeny of these clones, the blast cells, often expressed more differentiated lymphoid or myeloid lineage markers. More recently, cancer stem cells have also been isolated from gliomas, gliosarcomas, medulloblastomas, and ependymomas [5–12].

Importantly, increasing evidence suggests that while current cytotoxic therapeutics may kill the bulk of cancer cells, they are often not able to eliminate the critical TSC, which are protected by specific innate resistance mechanisms [13, 14]. The surviving TSC can then account for tumor recurrence or metastasis [15, 16]. The recurrent tumors are resistant to previously used therapeutic modalities and lead to a worsened prognosis for the patient. These considerations may explain the lack of success with current treatments for gliomas and stress the importance of studying the biological processes of TSC to identify potential therapeutic targets.

Unfortunately, no particular markers or gene expression signatures associated with TSC alone have been identified till now. For example, CD133 was previously thought to be a robust CNSTSC marker, but recent work has shown that the marker does not consistently distinguish tumorigenic from nontumorigenic cells [17–19]. While sorting techniques may be used to aid in the isolation and identification of cells, the stemness of a cell may only be confirmed with functional assays. These assays must be able to depict the TSC properties of self-renewal and lineage capacity. Two major assays are used for the enrichment of TSC: neurosphere cultures with multiple passes and animal propagation studies. We provide an overview of these assays, along with some of the other laboratory models used to study the properties of the CNS TSC.

Neurosphere Cultures

Neurospheres characterize three dimensional in vitro spheroid cell clusters that form when mitotic cells of the mammalian CNS are placed in a serum-free medium on a non-adhesive substrate. This assay was first developed for neural stem cells but has been used for TSC isolation and research as well. These spheres generally have the least differentiated cellular populations located on the surface, with cells expressing differentiation in the interior (Fig. 1) [1].

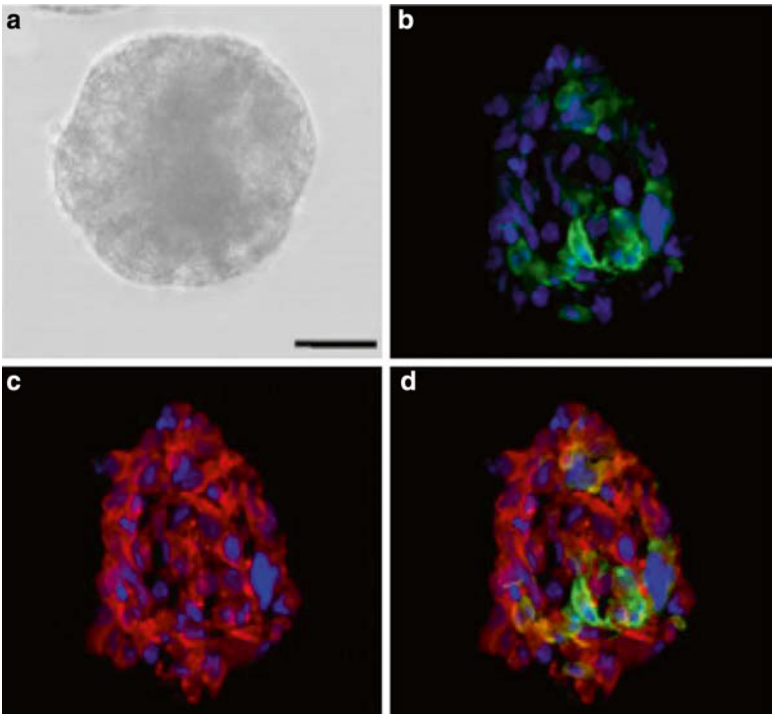

Fig. 1 GBM-derived neurosphere. (**a**) In bright field prior to the cryosectioning protocol described; (**b–d**) immunostained against GFAP (*green*) and Nestin (*red*). Dapi was used as a nuclear marker. The finding of more differentiated cells in the core of the neurosphere showing the cell heterogeneity within the neurospheres (with permission from Guerrero-Cázares H et al. Methods Mol Biol. 2009;568:73–83. Neurosphere culture and human organotypic model to evaluate brain tumor stem cells)

The neurosphere assay relies on a culture system that selectively supports the survival and proliferation of stem and progenitor cells that respond to epidermal growth factor (EGF), basic fibroblast growth factor-2 (FGF-2), or both, forming clonal aggregates called primary neurospheres [20]. EGF in mice has been shown to increase proliferation and survival of precursor cells in the subventricular zone (SVC), and also enhance the generation of astrocytes [21, 22]. FGF also increases neurogenesis, but is additionally protective against injury-induced degeneration [23–25]. Nakano and colleagues have studied the relative roles of stimulation of FGF and EGF receptors on self-renewal of neural stem cells and found that FGF induces a greater degree of self-renewal than EGF family members in embryonic cortical NSC [26]. Other growth factors that simulate the receptors of EGF and FGF also induce the production of neurospheres [27, 28]. Conversely, the removal of these mitogens has been shown to induce differentiation [29, 30].

Upon subculturing the primary neurosphere, renewal of the previous cells may be demonstrated by the production of secondary neurospheres [31]. Certainly, under these conditions, growth factor-responsive cells can be long-term passaged, maintaining

stem cell characteristics of multi-potency and stable proliferation [32] However it is important to consider that both TSC and progenitor non-stem cells have the ability to proliferate with each passage. To recognize neural stem cells it is imperative to display their characteristic nature of self-renewal: propagation in long-term cultures (at least five passes) and multipotency through the generation of its progeny [20].

The neurosphere assay can also be modified to investigate the function of various genes by introducing transient transfection [26]. Investigators have used small interfering RNA (siRNA) and small hairpin RNA (shRNA) to interfere with the expression of various genes to assess their roles in the pathogenesis of tumors. Using these techniques a growing body of work has described roles for HEDGEHOG-GLI1 [33], SMC1A [34], ASPP [35], hTERT [36], and FRAT1 [37] among others in the proliferation and growth of gliomas.

The effect of a particular treatment on neurospheres may be analyzed via a variety of methods. The number of clonal spheres a week after treatment corresponds to the proliferative capacity of neurosphere. Alternatively, the number of proliferating cells can also be assessed with flow cytometry based BrdU incorporation labeling [38]. We have also described quantitation of neural stem cell and TSC viability using a colorimetric assay for mitochondrial dehydrogenase activity [38, 39]. The size of spheres can also be quantified (proportional to their diameters) as a measurement of the effect of the treatment on asymmetric self-renewal total neural progenitor proliferation. Moreover, by conducting immunocytochemistry, the effect of on differentiation capacity of treated progenitors may be investigated [26].

The neurosphere assay is an attractive lab technique to isolate and study brain TSC because it includes the functional assay as the initial step [1]. A variant of the neurosphere culture, called the Cambridge Protocol, combines neurosphere and monolayer culture techniques in a bid to improve the efficiency with which cells can be derived from tumor samples under serum-free conditions [40]. The mainstay of neurosphere assay is the assumption that the neuroshere cells are clonal and there is no contribution from any other cell lines [41, 42]. However, generating single cells for neuropshere assays is very challenging and incompletely disintegrated cells may lead to sphere formation with chimerism. Additionally not all cells that form neurospheres are stem cells, as committed progenitor cells also have the same ability. Because clonality of spheres is of immense importance to stem cell research, the clonal relationships need to be confirmed with additional methods such as retroviral marking [41], using a single cell in a miniwell [42], or sparse, widely dispersed cells in methylcellulose [43, 44]. Once clonality has been established, stem cell lines can be effectively expanded and be cryopreserved. Of note, repeated cycles of freezing and thawing do not seem to affect the neural stem cell functional properties [1].

For proliferative studies, the diameter of neurospheres may also not be a very reliable marker [45]. While some of the earlier studies have shown the formation of large neurospheres within 7 days after culture, recent work suggests that it is virtually impossible to produce a large neurosphere in 7 days only [46, 47]; under stringent conditions large neurospheres are only detectable 2–3 weeks after culture. Certainly, a closer look has shown that spheres sometimes integrate free cells, or fuse with other spheres in the clonal medium, leading to chimeric spheres with rapid growth [45] (Fig. 2).

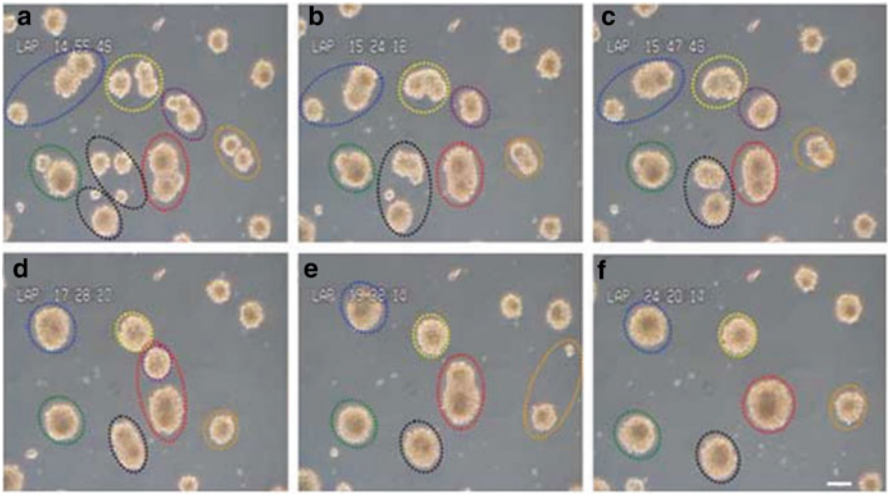

Fig. 2 Frequent, rapid, and multiple "coalescence" of secondary neurospheres. (**a**–**f**) Representative sequential frames from a time-lapse video microscopic recording, show 30 spheres at the beginning of the recording which "merge" with each other (21 mergers counted), resulting in 10 spheres within ~10 h, and for some clusters (for example, in the *upper panels*) within 1 h. "Merger" partners are *circled* using *different colors* to facilitate following their movements and changes over the 10 h of the movie. These cultures were not agitated or otherwise manipulated; the movement reflects the intrinsic locomotion of free-floating spheres. Scale bar, 100 μm (with permission from Singec I et al. Nat Methods. 2006 Oct;3(10):801–6. Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology)

Another concern with the neuropshere assay is the potential of serial neurosphere passages, which require extended periods of cell culture, to induce significant alterations in cellular biology and gene expression that do not accurately reflect in vivo conditions [1]. Additionally, immunocytochemistry in neurospheres is challenging due to their size, fragility, and floating condition [48]. Cryostat sectioning for neurospheres gives the best reported results without disturbing the spherical architecture [49]. Prior to the sectioning, the neuropsheres need to be suspended in OCT compound and placed on the top of a frozen OCT mold to freeze [48].

Matrigel-Based Assays

Matrigel-based assays have been used as in vitro assays to measure glioma stem cell invasiveness [50]. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth–Holm–Swarm mouse sarcoma. This tumor is rich in ECM proteins and contains all the major components of basement membranes [51]. The Matrigel assay can be used to test the various molecular mechanisms that govern the invasion and migration of the TSC.

Qui et al., investigated the invasive potential of glioma stem cells and found that they were significantly more invasive than their differentiated progeny cells [52]. The same group also used this model to show that glioma stem cell invasiveness is markedly decreased after IL-6 is blocked with neutralizing antibody, but significantly increased when exogenous IL-6 is added [53].

Inoue and colleagues isolated tumor sphere-forming cells from U251 cells and showed enhanced migratory and invasive ability on both Matrigel and organotypic brain slices compared to parental cells. Furthermore, knockdown of MMP-13 expression by shRNA was shown to suppress the migration and invasion of the glioma stem cells [54]. Matrigel invasion assay have also been used to study neural and mesenchymal stem cell tropism to malignant glioma [55, 56].

The utility of Matrigel assays is limited however, as they do not reflect the human brain matrix. Other in vitro invasion assays such as wound healing assay [57], microliter-scale migration assay [58], spot assay [59], and transwell migration assays [60] have the same limitations.

Organotypic Cultures

In the context of an invasion assay, the neurosphere culture does not account for the novel ECM configuration of the brain along with cell-to-matrix interactions within a tumor [60–68]. To address this issue, organotypic slice cultures have been developed (Fig. 3). The early slice cultures were derived from postnatal rodent brain and have been widely used due to their easy access for pharmacological intervention and live imaging [69, 70]. Ohnishi used rat brain slices obtained from the hippocampus or cortical regions of 2-day-old rats and maintained the brain slices in culture at the interface between air and the culture medium [55].

Juvenile rat and mouse brain slice cultures can be kept viable for more than | 8 weeks. Brain slice cultures have been developed using human tissue as well. Jung et al. used normal brain tissue specimens obtained from patients undergoing temporal lobectomies as a matrix to study glioma cell invasion [71]. Similarly, Chaichana and colleagues maintained intraoperatively collected human tumor and non-tumor explants ex vivo for approximately 11 days without any significant changes to the tissue cytoarchitecture [72].

Merz and colleagues described the use of ex vivo GBM slices in 6-well plates, and applied chemotherapeutic agents and irradiation, all the while allowing direct observation of the tumor response [70]. Similarly, Shimizu and colleagues have described a three-dimensional organotypic ex vivo system of surgical GBM specimens that preserves tumor cells in their original milieu [73]. Using this model, the group also described how Notch inhibition in explants resulted in decreased proliferation and self-renewal of tumor cells [74].

Organotypic coculture models are also valuable to study invasiveness of tumor cells [63, 64, 75, 76]. Aaberg-Jessen and colleagues implanted neurospheres derived from primary brain tumors into organotypic rat brain slice cultures and followed the

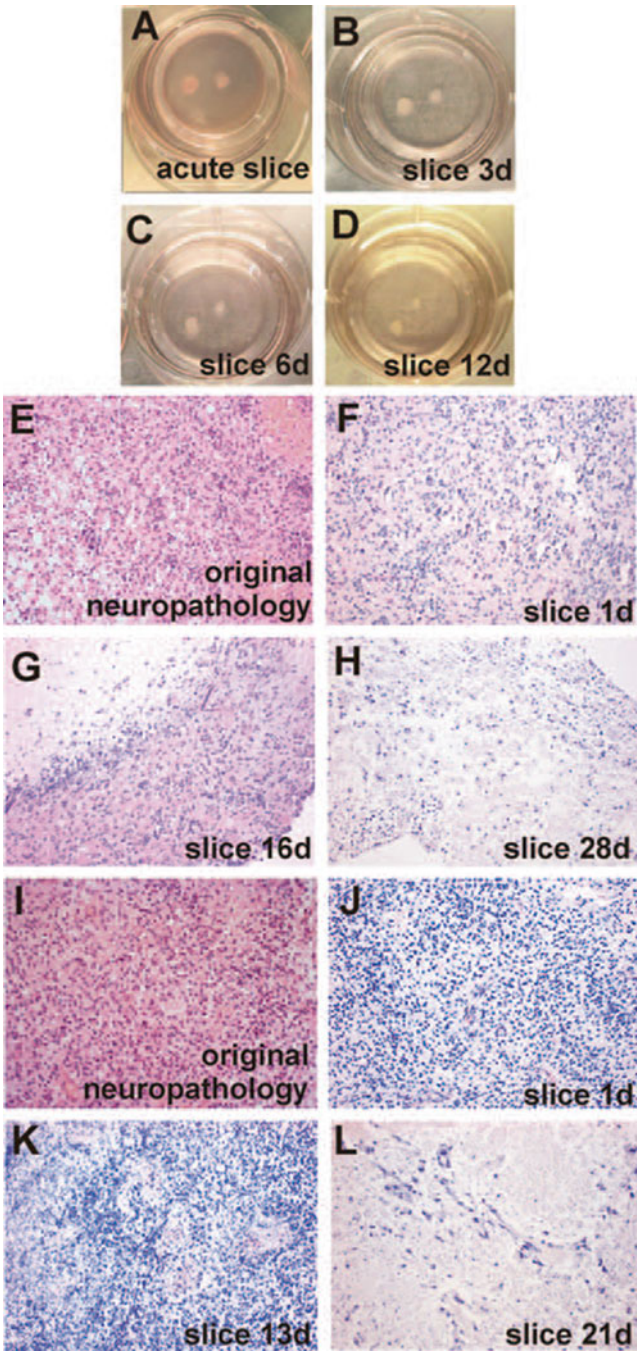

Fig. 3 Human GBM slices in culture. Slices were cultured on membrane inserts in six-well plates with no signs of degeneration\in acute (**a**) slices at 1 day or at 3 days (**b**), 6 days (**c**), or 12 days (**d**) in vitro. Original H and E neuropathology (**e** and **i**) and H and E-stained paraffin-embedded sections (8 mm; **f–h** and **j–l**) prepared from slices after various culture periods. Two different tumors (**e–h** and **i–l**) are shown. Note that typical features of individual tumors were maintained at least from 1 to 16 days (**f–g**) and 1–13 days (**j–k**) in vitro; massive cell loss was observed after 20 days in vitro (**h** and **l**). Original magnification: 1× in **a–d**; 200× in **e–l** (with permission from Merz et al. Neuro Oncol. 2013 Jun;15(6):670–81. Organotypic slice cultures of human glioblastoma reveal different susceptibilities to treatments)

invasive behavior of the tumor cells over time using confocal microscopy [77]. Likewise, Zhu et al. cocultured human brain microvascular endothelial cells (hBMEC) with GBM neurospheres and found increased NOTCH expression in endothelial cells leads to increased GBM cell growth and increased TSC self-renewal [78].

While the organotypic model has the advantages of more sophisticated cell-to-cell and cell-to-matrix interactions compared to other culture methods, there are a few limitations of the model that need to be considered. Although the vascular structures are preserved in the model, there is no blood flow along with nutrients, drugs or immune cells being carried through them. This limits its ability to test drugs that target blockage of the circulation system or modulation of the immune system [73].

Mathematical Models

Mathematical modeling represents a novel modality to develop predictive models for the biological behavior and treatment-response of TSC. Steel and colleagues accomplished some of the earliest work in mathematical modeling for gliomas [79]. Ganguly and Puri later formulated a predictive mechanistic mathematical model for brain TSC using a compartmental model [80]. Their group also formulated a model to understand the response to treatment of tumors with cancer stem cells. Their model predicted that the best response to chemotherapy occurs when a drug targets the abnormal stem cells [81].

A simple compartmental mathematical model for tumor growth, based on the TSC hypothesis using a chemical reaction approach has also been described [82]. Others have used multi-compartment models to predict growth potential of tumors with a heterogeneous cell population [83]. Mathematical models have also suggested that treatment modalities stimulating TSC differentiation and inhibiting TSC proliferation should be used together to get the best response [84].

Animal Models

Self-renewal and lineage capacity are the distinguishing features of any stem cell and any assay that aims to identify TSC needs the potential to show these characteristics. Serial transplantation in animal models is considered the best functional assay for these critical features [85–88]. In transplantation assays, tumor cells are xenografted into immunocompromised mice. Studies have typically used NOD/SCID mice [2], but BALB/c-nude [89] and Scid/bg mice [9] have also been used. The implanted tumors can then be assayed at various time points for tumor formation analysis.

To show self-renewal, the tumor cells need to be removed from the primary mice and transplanted into a secondary recipient animal. In the CNS, Singh and colleagues showed that only CD133+ brain tumor fraction contained cells that were

capable of tumor initiation in vivo. They used an in vivo limiting dilution assay, where progressively smaller numbers of tumor cells are implanted into the animals to demonstrate the minimum number of cells required to form a tumor. Injection of as few as 100 CD133+ cells produced a tumor in vivo, while orthotopic implantation of 10^5 CD133− cells did not [90]. Likewise, Galli et al. demonstrated that glioblastoma cell lines, established by culture in neurosphere conditions, showed TSC characteristics in vivo. Intracranial injection of 200,000 of neurosphere cells also generated tumors in vivo, and after repeat culture, initiated phenotypically similar tumors, in a secondary mouse [9].

Xenotransplantation may also be employed to investigate the tumorigenicity of serially passaged neurospheres. Utilizing an athymic nude mouse model our group implanted 50,000 neurospheres into the basal ganglia, and after 4 weeks observed the generation of diffuse tumor in the basal ganglia depicting tumorigenic nature of the implanted cells [38]. To be able to follow the growth of the implanted TSC, Lathia and colleagues described the used of serial in vivo intravital microscopy [91]. To compare the role of TSC and non-stem tumor cells in the growth of the xenotransplanted tumors, GBM specimen cells were transduced with a lentivirus to express fluorescent proteins; green or yellow fluorescent protein for TSC, and cyan fluorescent protein for non-stem tumor cells. Additionally high-molecular weight fluorescent dextran injected to highlight the vessels around the tumor. Using real time imaging, the study was able to show that TSC were primarily responsible for the propagation of the implanted tumors [91] (Fig. 4).

Interestingly, glioma stem cells are not unique to xenotransplanted human cancers. Wu and colleagues were able to isolate CD133+ cells from the GL261 cell line, a syngeneic mouse glioma model [92]. CD133+ GL261 cells expressed nestin, formed tumor spheres with high frequency, and differentiated into glial and neuronal-like cells. Furthermore, a much lower number of murine CD133+ cells were needed to initiate tumors on intracerebral implantation compared to CD133− cells, 100 vs. 10,000 cells, respectively [92].

Although nude mice serial transplantation assays are considered the gold standard to identify and enrich stem cells, there are still some issues regarding the interpretation of the experiments. The effect of removing TSC from the supporting matrix is not known, and might modify characteristics of the cells [2]. Additionally the presence of predisposing genetic mutations that give rise to mouse tumors may fail to adequately represent the heterogeneity in human cancers [93]

It may also not possible to estimate the proportion of TSC in the parent tumor based on the results from animal transplantation assays [93]. The site and type of host tissue also has an important effect on the TSC representation. Vascularization at the site of implantation, extracellular matrix constitution, growth factor availability and host immunocompetence are some of the factors that can effect tumor engraftment and the yield of TSC [94].

Recently, Zebrafish (*Danio rerio*) assays have also been used to study the behavior of CNSTSC in vivo [95, 96]. As a vertebrate animal, the zebrafish model depicts high levels of physiologic and genetic similarities to mammals [96]. The transparent embryos of zebrafish are inexpensive to maintain and allow easy visualization of

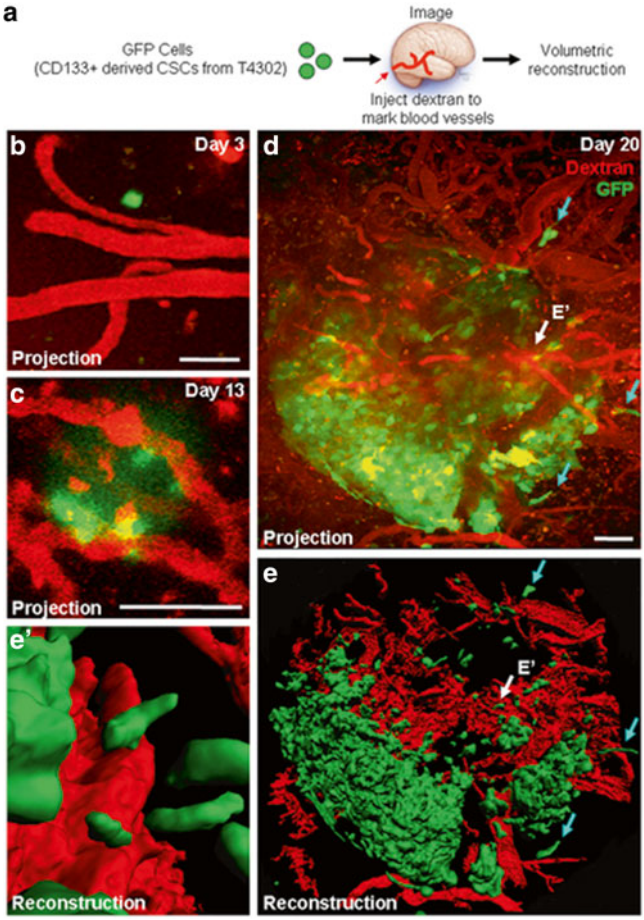

Fig. 4 Multiphoton microscopy reveals tumor propagation from cancer stem cells. Tumor formation in a xenotransplantation model was observed from GFP-labeled TSCs over time as shown in experimental design schematic (**a**). Projection micrographs (**b**–**d**) demonstrate tumor formation over time and three-dimensional reconstructions depicted in micrographs (**e**, **e**′) revealed tumor cells were closely associated with blood vessels (**e** shown with *white arrows* in **d**, **e**) and in peripheral areas (**d**, **e**, shown in *blue arrows*). Fluorescent dextran (shown in *red*) was injected into the circulation to illuminate blood vessels prior to imaging. Scale bar represents 50 μm (from Lathia JD et al. PLoS One. 2011;6(9):e24807. Direct in vivo evidence for tumor propagation by glioblastoma cancer stem cells (open access))

internal structures. A transparent adult zebrafish model has also been created to allow direct visualization of tumor engraftment and proliferation [97]. Investigators have used various imaging techniques to track the growth, invasiveness and response to experimental therapy, including fluorescence, bioluminescence and luciferase-based assays [96, 98, 99].

There are also some limitations of the zebrafish implantation model. The high mortality rate (>10 %) even with sublethal doses of radiation, poses a challenge for investigators. Post-transplant care also has to be optimized to minimize the risk of infection in the immunosuppressed recipients. Additional research is also required to examine the effect of background genotype on the behavior of the transplanted tumors, and to develop the ideal transgenic strain for particular tumors. Furthermore, injection of tumor cells in this model is mostly in the peritoneal cavity and while this may be a good model for metastatic tumors, the development of tissue-specific orthotropic injections will allow a more representative assay [100].

Conclusion

Increasing evidence points to a fundamental role for TSC in the initiation and propagation of several tumors. In the context of CNS tumors the development of treatment strategies specifically targeted towards TSC may hold a significant therapeutic promise. We have described some of the most commonly used laboratory models to investigate TSC to further this aim.

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