

Isolation and Characterization of Stem Cells from Human Central Nervous System Malignancies

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Abstract Central Nervous System (CNS) tumors include some of the most invasive and lethal tumors in humans. The poor prognosis in patients with CNS tumors is ascribed to their invasive nature. After the description of a stem cell-like cohort in hematopoietic cancers, tumor stem cells (TSCs) have been isolated from a variety of solid tumors, including brain tumors. Further research has uncovered the crucial role these cells play in the initiation and propagation of brain tumors. More importantly, TSCs have also been shown to be relatively resistant to conventional cytotoxic therapeutics, which may also account for the alarmingly high rate of CNS tumor recurrence. In order to elucidate prospective therapeutic targets it is imperative to study these cells in detail and to accomplish this, we need to be able to reliably isolate and characterize these cells. This chapter will therefore, provide an overview of the methods used to isolate and characterize stem cells from human CNS malignancies.

Keywords Glioma stem cells • Tumor stem cells • Cancer stem cells • Stem cell sorting • Hoechst dye exclusion • ALDH1 assay • Neurosphere culture

Introduction

Tumors of the central nervous system (CNS) include some of the most lethal malignancies. While there has been sizeable development in the management strategies used to combat intrinsic CNS tumors there is still room for improvement. Glioblastoma Mutliforme (GBM) is the most common kind of primary brain tumor and carries a poor prognosis. With optimized surgical resection combined with chemotherapy and radiotherapy, the median survival rate is approximately 14 months [1].

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Due to the very invasive nature of the tumor, most of the treated patients eventually succumb to the disease.

Likewise, the most common brain tumor in pediatric patients is medulloblastoma. Treatment consists of maximal surgical resection followed by chemo-radiation and the median survival rate is a little more than 5 years. However, patients presenting with more advanced disseminated disease fare much worse. Furthermore, treated patients face a long-term prognosis that is fraught with increased risk of secondary malignances and cognitive deficits.

The poor prognosis of patients with CNS tumors along with the near absence of treatment modalities that have improved outcome significantly has led to investigators looking at the biology of the tumors more closely. This has steered us to the identification of a small subset of tumor cells that have stem cell like properties of cell renewal and lineage capacity [2, 3]. These tumor stem cells (TSCs) have been identified as the key mediators of tumor initiation, propagation and maintenance [4]. Moreover, recent evidence suggests that TSCs play an important part in angiogenesis and are relatively refractory to conventional chemo-radiation therapeutics [2, 5–8]. It is now thought that these cells may play an integral role in the recurrence of CNS tumors [9, 10].

Preparation for TSC Isolation

To be able to therapeutically target TSCs, it is imperative to be able to isolate and study their genetic and proteomic characteristics. Isolation of TSCs is a challenge because of their seemingly small number and the fact that they share some of the properties of downstream committed progenitor cells, which may also be able to repopulate the tumor [2]. The first step in isolating TSCs is to obtain a single-cell suspension of the tumor. This may be achieved with mechanical dissociation, chemical dissociation, or a combination of both.

To create a single-cell suspension it is important that the tumor be processed as soon as possible after the excision to retain viability. This may be a challenge in tumors excised from patients but is suggested that tissue processing should begin within 30 min of the removal. The tumor sample is then placed in the sterile Petri dish with HBSS +Ca+Mg, and using a dissecting microscope cleaned off all the necrotic tissue and blood vessels. The sample can then be mechanically dissociated with microscissors or the unsharpened side of a #22 scalpel blade. The cell slurry created is then dissociated using pipettes and passing through a 70 μ m filter. Alternatively, Trypsin–EDTA may be added to the clean sample to help dissociation. The decision to carry out mechanical vs. chemical dissociation is subject to investigator preference and the type of tumor used (for detailed methods see refs. [11, 12]).

Once the solid CNS tumor has been dissociated into a single-cell suspension, isolation of TSCs can be accomplished by three major categories: sorting cells based on cell surface markers, choosing cells with the expression of a particular protein of interest, and functional assays. As the recognition of TSCs is based on the multipotency and clonogenicity of the cells in question, TSC isolation methods are

best followed by *in vivo* functional assays to confirm identity [3]. This chapter further provides an overview of some of the most commonly used CNS TSC isolation and characterization methods.

Stem Cell Sorting

The two most important aims of cell sorting techniques are to preserve the viability of the cells and to get the purest selected fraction possible. Flow cytometry is a powerful tool to select cells expressing particular cell surface markers (Fig. 1).

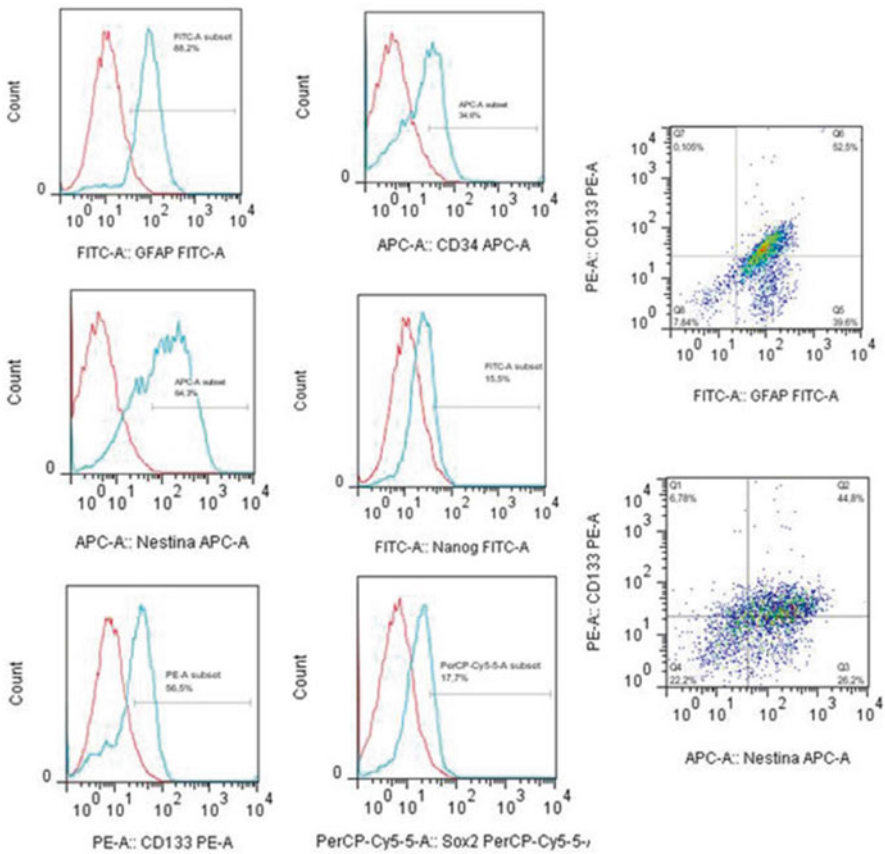


Fig. 1 Immunophenotyping of tumor subspheres by flow cytometry. Immunophenotypic characterization by flow cytometry assays showing the pattern of expression of markers GFAP (88.2%), CD133 (56.5%), Nestin (64.2%), Sox2 (17.7%), CD34 (34.0%), and Nanog (15.5%) in glioblastoma subsphere samples and the co-expression of CD133 with GFAP (52.5%) and Nestin (44.8%). Representative figure of five samples of glioblastoma. (From Pavon LF et al. *Front Neurol.* 2014 Jan 7;4:214. 2014. *In vitro* Analysis of Neurospheres Derived from Glioblastoma Primary Culture: A Novel Methodology Paradigm (open access))

This technique can be used to separate TSCs from the complex structure of a solid tumor. Another advantage of flow cytometry is the use of multiple markers to simultaneously positively or negatively select out the cells. Suitable cell surface antibodies conjugated to fluorophores (fluorescent chemical compounds that can re-emit light upon light excitation) are selected and then added to the sorting sample. The sample is then passed through the flow cytometer to sort out the cells with the surface marker of interest.

In the context of cell surface markers, CD133 (Prominin-1) is one of the most commonly utilized markers to identify neural stem cells (NSC) and TSC [13]. CD133 is a pentaspan transmembrane glycoprotein, which localizes on cellular protrusions [14]. Weigman and colleagues initially identified the glycoprotein by raising monoclonal antibodies against mouse neuroepithelium [15]. Around the same time, Yin et al. and Miraglia et al. also identified CD133 independently, using antibodies against CD34⁺ hematopoietic stem and progenitor cells [16, 17].

CD133 was initially used to enrich cancer stem cells in leukemia [18, 19], and has been observed in various other tumor stem cells from different cancers (review in [20]). Owing to its presence on NST, Singh and colleagues conducted *in vitro* and *in vivo* studies showing the presence of TSC in CD133⁺ cells in gliomas and medulloblastomas [21, 22]. Along with CD133, neurospheres derived from pediatric brain tumors may also express other surface markers, including Sox2, musashi-1, bmi-1, maternal embryonic leucine zipper kinase, and phosphoserine phosphatase [23].

Nestin is another important marker for CNS TSCs [24]. Initially described as an antigen of RAT401 against embryonic spinal cord, Nestin was later identified as a class VI intermediate filament protein [25, 26]. Alongside CD133, Nestin has been shown to correlate with the aggressiveness of gliomas in some studies [27, 28].

It is important to note that one of major issues facing stem cell surface markers is the fact that they are also found on other cell populations [29]. For instance, Nestin is also expressed by Bergmann glia and granule neuron precursors in the cerebellum [30, 31]. Similarly, CD133 has been observed in mature astrocytes, oligodendroglia, and neurons, as well as ependymal cells [32, 33].

Magnetic bead separation is an alternative to flow cytometry and allows the cells to be separated by incubating magnetic nanoparticles coated with antibodies against a particular surface antigen/marker. The nanoparticles attach to the cell surface marker of interest and the sample is then sorted into marker positive and negative groups by flowing the cells through a strong magnetic field.

The use of magnetic bead separation for TSC has been described in a variety of studies [34–36]. As this modality passes the cells as a group, in contrast to the flow cytometer that passes cells individually, a larger sample can be separated rapidly. This is of particular advantage in cases where the population of cells positive for the labeled antibody is very small, requiring a large tumor sample to be passed to get an adequate number of sorted cells. However, magnetic bead sorting can only separate one marker at a time and hence multiple runs may be required to sort out cells if multiple markers have to be used. This increases the time used for sorting and may decrease cell viability.

Hoechst Dye Exclusion

An alternative technique used to identify TSCs is by using the Hoechst dye exclusion in tumor cells. Hoechst stains are part of a family of blue fluorescent dyes used to stain DNA [37, 38]. Hoechst dye 33342 can be used in conjunction with flow cytometer to identify the side-population (SP) of cells. Goodell and colleagues were the first to identify a side-population (SP) from mouse bone marrow that was enriched with cells that had properties of stem cells [39]. The same group later isolated stem cells in humans [40].

Since then, several studies have described the use of this technique to isolate TSCs from a wide variety of cancers [41–48]. The unique property of SP cells is that they actively eliminate the dye leading to a low Hoechst staining (dye exclusion). The enhanced dye efflux in the side-population cells is due to an increase in the activity of multi-drug resistance proteins, primarily ABCG2 [49]. To carry out the protocol, Hoechst 33342 is added to the cell suspension to be tested. The cells can then be separated using flow cytometric sorting. Drugs such as verapamil, reserpine, or fumitremorgin C inhibit Hoechst exclusion and abolish the SP. Co-staining with antibodies helps with the identification and confirmation of the cells [11].

Whereas this technique has been useful in isolating stem cell enriched populations from CNS tumors and cell lines, it is important to note that on its own this technique isolates a population enriched for but not homogeneous for TSC. Additionally there is increasing evidence that the Hoechst exclusion alone may not be sufficient to isolate CNS TSCs [41, 42, 50]. Indeed, Broadley et al. found that while neurospheres were able to enrich for TSC from primary GBM cells and GBM cell lines, no SP was found when the neurospheres were analyzed. This led them to conclude that SP was not “necessary or sufficient” for a TSC phenotype in GBM [51].

ALDH1 Assay

Aldehyde dehydrogenases are a large group of enzymes that catalyze the oxidation of aldehydes [52], and are found in the cytosol, nucleus, mitochondria, and endoplasmic reticulum [53]. Of the ALDH group, the ALDH enzymes associated with NSC and TSC include ALDH1, ALDH2*2, ALDH3A1, ALDH4A1, and ALDH7A1 [52]. Of these, ALDH1 and ALDH3A1 in particular have been shown to play an important functional role in TSC. ALDH1 is critical in the Retinoid signaling pathway, which plays an important role in regulation of gene expression, morphogenesis and development of NSC and TSC [54–56]. ALDH1A1 and ALDH3A1 have also been shown to offer protection against alkylating agents, such as cyclophosphamide [57, 58].

Jones and colleagues were the first group to report a method to measure the intracellular ALDH1 activity in viable cells [59]. They used dansyl aminoacetaldehyde

(DAAA), a fluorescent aldehyde, in flow cytometry experiments to isolate viable mouse and human cells based on their ALDH content. However this technique can be mutagenic to the isolated cells as the DAAA fluorescence is excited by UV emissions. Additionally, the emission spectra of DAAA overlap with other fluorochromes, which makes it more challenging to carry out simultaneous analysis of other cell markers [59, 60]. Storms and colleagues modified the technique by using BODIPY aminoacetaldehyde (BAAA)—a fluorescent substrate for ALDH [60]. This assay is also known as the Aldefluor[®] Assay and has been shown to be useful method to isolate NSCs and TSCs without any ex vivo manipulation of the cells [61].

In the context of gliomas, Rasper et al. showed that high protein levels of ALDH1 facilitate neurosphere formation in established GBM cell lines, and even single ALDH1 positive cells could give rise to neurospheres [62]. Mao et al. characterized two mutually exclusive glioma TSC subtypes, and showed that ALDH activity was significantly elevated in Mesenchymal (Mes) TSCs but not in Proneural (PN) GSCs [63]. Additionally, inhibition of ALDH1A3 attenuated the growth of Mes but not PN GSCs, suggesting that ALDH1A3 pathways are promising therapeutic targets (Fig. 2).

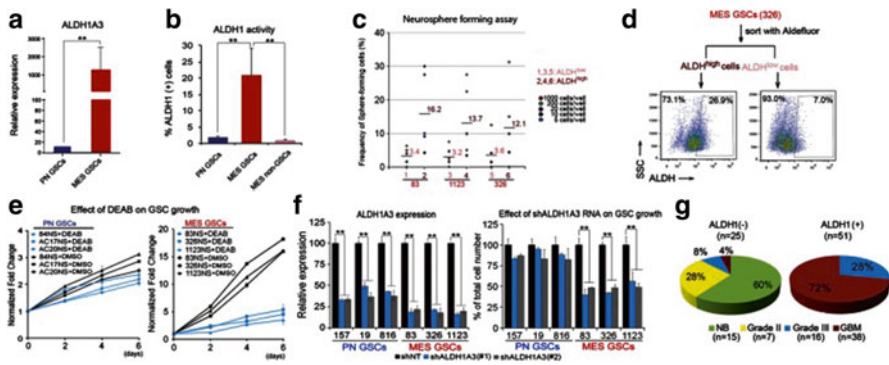


Fig. 2 ALDH1A3 is a functional Mes GSC marker. **(a)** qRT-PCR analysis of ALDH1A3 expression in PN and Mes GSCs (** $P < 0.01$). **(b)** FACS analysis using Aldefluor. ALDH activities in PN GSCs ($n = 3$), Mes GSCs ($n = 3$), and non-GSCs ($n = 3$) derived from Mes GSCs (** $P < 0.01$). **(c)** Frequency of sphere-forming cells between ALDH1^{high} and ALDH1^{low} Mes GSCs. FACS-sorted based on ALDH expression Mes GSCs were used in the assays (** $P < 0.01$). **(d)** FACS reanalysis: ALDH activity after 1-week postcell sorting of Mes 326 ALDH^{high} cells. ALDH^{high} Mes GSC spheres generated both ALDH^{high} and ALDH^{low} cells, whereas the majority of ALDH^{low} sphere cells retain as ALDH^{low} cells. **(e)** Effect of an ALDH inhibitor DEAB on cell growth of PN ($n = 3$) and Mes ($n = 3$) GSCs. DEAB abrogates the in vitro growth of Mes GSCs but has a marginal effect on PN GSCs. **(f)** Effect of shALDH1A3 knockdown on growth and ALDH1A3 gene expression of both PN and Mes GSCs. The growth of Mes GSCs is significantly reduced by shRNA-mediated depletion of ALDH1A3 compared with PN GSCs. RNA interference with 2 shALDH1A3 constructs significantly reduced ALDH1A3 expression levels in PN and Mes GSCs ($n = 3$ each, ** $P < 0.01$). **(g)** Pie chart indicating the number of samples that were analyzed in different WHO tumor grades of clinical glioma samples or normal brain tissues that are ALDH(+) or (-). Data in **(a-f)** are representative of three independent experiments with similar results. (With permission from Mao et al. Mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving aldehyde dehydrogenase 1A3. Proc Natl Acad Sci U S A. 2013 May 21;110(21):8644–9)

ALDH1 levels have also been correlated with outcome in patients with glioma. For instance, ALDH1A3 promoter methylation has been found to confer a favorable prognosis for patients with GBMs [64]. Liu and colleagues analyzed their samples of astrocytomas and reported an association of ALDH1 expression with pathological grade and patient survival [65].

Dye Based Isolation

The identification of a dye-retaining brain tumor population can also enable the identification of a subpopulation displaying the hallmarks of TSC. Label-retaining cell fractions that enrich TSCs have been isolated from various solid tumors, including breast [66], pancreatic [67], and skin tumors [68].

In the context of CNS tumors, Deleyrolle and colleagues used the properties of the pro-drug carboxyfluorescein diacetate succinimidylester (CFSE), which is converted by cellular esterase activity into a fluorescent compound covalently bound to proteins and retained within the cells [69]. CFSE dye can enable quantification of cell proliferation, as it is equally divided between daughter cells after division. They observed that a sub-population of cells diluted the dye significantly slower than the overall population, presumably due to a lower frequency of cell division. Using a limiting dilution transplantation assay in immunocompromised mice, these label-retaining brain tumor cells displayed elevated tumor-initiation properties relative to the bulk population, depicting their stem cell-like properties [69].

Spectroscopy

Other novel methods of TSC identification based on the biochemical composition of the TSC have also been described. One of these modalities is Fourier transform infrared spectroscopy (FTIR). Vibrational spectroscopy enables the label-free characterization of cells by probing the biochemical composition and numerous groups have described the classification of gliomas using this modality [70–72].

Wehbe and colleagues were able to depict the difference between normal and tumor vasculature of animal and human glioma using FTIR imaging [73]. More recently, Uckermann and colleagues described the identification of GBM TSC using FTIR [74]. They were able to discern biochemical differences between GBM cell populations with high and low content of TSCs that were likely related to differences in the RNA/DNA content.

Neurosphere Culture

While the previously mentioned techniques show great utility in the isolation of TSC, they are limited by their nature of using proxy characteristics to separate the cells. In this setting, neurospheres can help characterize TSC more definitively. This culture method relies upon the ability of TSC to initiate growth, self-renew, and display multi-potentiality through generation of its progeny [75].

Neurospheres refer to *in vitro* three-dimensional free floating spheroid cellular clusters in a supernatant that form when TSCs are exposed to a serum-free environment. The assay uses epidermal growth factor (EGF) and basic fibroblast growth factor (FGF), and has the property of selectively supporting the growth and proliferation of cells with stem cell-like functions. The presence of EGF and FGF in the culture medium is imperative to inhibit the differentiation of TSCs [76, 77]. Ignatova and colleagues were the first to show the formation of neurospheres with glioma using single-cell cultures in a methylcellulose (MC) matrix in the presence of EGF and FGF [78]. Since then, this technique has been commonly used to study the biology of TSC and the response to treatment [12].

The cells from the neurospheres may be subcultured and the primary clones can generate secondary neurospheres, representing the renewal of the previous population (Fig. 3) [79]. Stem cell renewal has been classified into two types: symmetric, where a division produces two daughter cells or two progenitor cells; or asymmetric, where a division produces one daughter and one progenitor cell [79]. Clonal analysis and serial subcloning assays are critical to definitely identify TSCs.

The neurosphere culture method has also been combined with other cell sorting methods to give a more robust result. Singh and colleagues in their seminal glioma TSC paper sorted the CD133+ cells prior to neurosphere cultures [22]. Similarly, Pavon and colleagues described a more vigorous method to isolate TSC wherein the isolation of neurospheres derived from GBM primary cultures was followed by sorting out the CD133+ cells to create further sub-neurospheres [80].

Neurosphere assays, despite their value, also have some limitations. The neurosphere assay is an *in vitro* phenomenon and does not occur *in vivo*. Additionally, the act of removing cells from their source and placing them in serum-free cultures may precipitate phenotypic or genetic changes that may not be representative of *in vivo* behavior. Also, it is important to remember that committed progenitor cells have the ability to produce secondary neurospheres, but they cannot continue to form neurospheres on continued passaging (unlike putative NSC or TSC) [81].

Neurosphere assays are also not useful to calculate stem cell frequency within a sample of tissue as a vast majority somatic cells do not remain viable in the serum-free culture medium and do not form neurospheres [75]. Finally, to establish clonality it is essential for a neurosphere to originate from clone only. However, as shown by Singh and colleagues using time-lapse video microscopy, spheres are highly motile structures with a high incidence of cellular aggregation leading to chimeric neurospheres [82]. In this setting, using a single cell in a miniwell [83], or sparse, widely dispersed cells in MC [84, 85] are viable options.

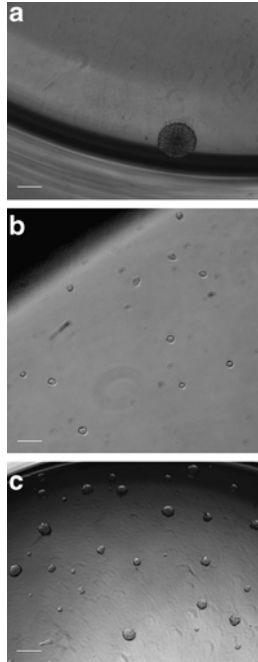


Fig. 3 Individual clonal neurospheres can be subcloned. (a–c) Representative examples of SVZ-derived NSCs. Individual clonal spheres generated by limiting dilution from serially passaged adult NSCs (a) can be dissociated and single cells replated in the presence of mitogens (b), giving rise after 7–10 days to secondary neurospheres (c). From 50 up to >200 secondary spheres can be obtained from each individual primary sphere, depending on the number of viable cells plated. The cloning efficiency for adult SVZ-derived NSCs under these culture conditions is in the range of 2–8 %. Bars = 100 μm (a), 25 μm (b), and 250 μm (c). (With permission from Gritti et al. *Methods Mol Biol.* 2008;438:173–84. Clonal analyses and cryopreservation of neural stem cell cultures)

Orthotopic Implantation

To assess the tumorigenicity of the TSCs, animal orthotopic models are considered the gold standard [86–89]. By allowing growth in *in vivo* conditions, investigators hope to recapture the cell-to-cell and cell-to-matrix interactions. Intracranial orthotopic implantation of TSCs (in the form of a single cell suspension or neurospheres) is mostly conducted in immunocompromised mice [3, 90]. This model is also particularly helpful to study the chemo- and radio-resistant properties of TSC.

Various nude mouse models have been used for implantation assays. The most popular model is the NOD-SCID mouse model, which has a completely knocked out immune system [3]. However this model is susceptible to developing spontaneous lymphomas as early as within a year of life [11]. Other mouse models include IL2 receptor- γ chain deficient, BALB/c-nude and Scid/bg [90, 91].

The most suitable model for *in vivo* studies is based on the aims of the study and by weighing the advantages against the disadvantages of using that particular model.

To depict self-renewal, the tumor is harvested from one animal and the cells are implanted into another animal. By using fewer and fewer cells in each passage, the enhanced tumorigenicity of TSCs is studied. For neurospheres, Singh and colleagues were the first group to use an *in vivo* limiting dilution assay to implant fewer and fewer TSCs at each subsequent passage to assess the fewest number of cells required to form a tumor in the animal [22]. As a general principle, compared to the marker-negative population, at least 50- to 100-fold fewer marker-positive cells should be needed to stimulate tumor formation in 50 % of the mice [11]

The mouse implantation model has some limitations as well. First, it is not possible to accurately assess the proportion of TSCs in original tumor mass. This is because the effect of *in vivo* conditions on the viability and tumorigenic potential is not known [92]. Second, in spite of being an *in vivo* model, it still has factors such as the extracellular matrix constitution, host immunocompetence, growth factors, and vascularity that may not be representative of the original tumor environment. Finally, the Zebrafish implantation studies for TSC analysis is a relatively newly described functional assay [93, 94]. TSCs are implanted into the peritoneal cavity of the animal in a transparent embryo and the growth and proliferation may be observed directly. Adult transparent zebrafish have also been created for the same purpose [95]. Additional work on developing tissue-specific orthotopic implantation can improve this model further [96].

Conclusion

With increasing evidence pointing towards the importance of TSCs in the biology of CNS tumors, it has become vital to be able to understand the unique biology of these cells and discover potential therapeutic targets. The first step in carrying out such investigation is to reliably isolate and characterize the TSCs. A variety of methods have been described in this regard and researchers need to be aware of the benefits and shortcomings of the methods chosen. In the future, improving technology is anticipated to drive the development of more reliable, accurate, and less labor-intensive assays.

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