REVIEW ARTICLE

The Utility and Limitations of Neurosphere Assay, CD133 Immunophenotyping and Side Population Assay in Glioma Stem Cell Research

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Abstract

The newly proposed glioma stem cell (GSC) hypothesis may re-model the way we diagnose and treat the tumor, which highlights the need for a complete knowledge on the genetic and epigenetic “blueprints” of GSCs. To identify the true “stemness” signatures, pure GSC populations are primarily needed. Reliable in vitro methods enriching for GSCs and thereby identifying the key stem-like characteristics constitute the preliminary step forward. We discuss in this review the current widely used methods for enriching and isolating GSCs, namely neurosphere assay, CD133 Immunophenotyping and side population assay, and detail their limitations and potential pitfalls that could complicate interpretation of corresponding results.

Keywords
CD133, glioma, neurosphere, side population, stem cell.

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INTRODUCTION

The recent identification and functional studies of cancer stem cells (CSCs) in multiple human cancers, including glioblastoma (GBM) and medulloblastoma, have provided strong evidence to support their critical roles in the initiation and propagation of human cancers (23). CSCs are defined as cells within a tumor that possess the capacity to self-renew and to produce the heterogeneous lineages of cancer cells that comprise the tumor (23). They share many of the characteristics of normal tissue stem cells, which could help to explain certain clinical features such as tumor hierarchy, recurrence, metastasis and therapy resistance (23, 69, 122).

The existence of brain tumor stem cells (BTSCs) is supported by evidence showing that proliferating progenitor cells and/or stem cells in the mouse subventricular zone can be transformed by carcinogenic substances or by manipulation of several key signaling pathways leading to gliomagenesis (51, 66, 120). Neural stem cells (NSCs) and BTSCs also share common markers; respond to the same external cues; share capacities for self-renewal and differentiation; and exhibit telomerase activity, apoptosis resistance and increased membrane transporter activity (94). In addition, both NSCs and BTSCs activate canonical signaling pathways such as Wnt, Notch and Hedgehog that regulate self-renewal and stem cell properties (94). Conceptually, BTSCs cover more diverse classes of brain tumors than “glioma stem cells” (GSCs) (alternatively referred to as glioma initiating/propagating cells), which form the focus of this review.

The GSC hypothesis may affect the way in which we diagnose and treat tumors. The therapeutic aim would change from eliminating the bulk of rapidly dividing, but terminally differentiated components of the tumor, to refocusing on the minority stem cell population that fuels tumor growth (23). Such a transition highlights the need for laboratory work to provide information on the genetic and epigenetic “blueprints” of GSCs, and to identify true signatures of “stemness.” Such studies require pure populations of cells. This is especially true for cells such as GSCs that are expected to be rare, as their expression signatures would be swamped by the majority of non-stem cells in whole tumor samples, resulting in an average signature for the mixed population, rather than a specific signature for GSCs (23). Thus, the development of reliable in vitro methods to enrich GSCs and enable the identification of key stem-like characteristics constitutes an important preliminary step. We discuss the current widely used methods for enriching and isolating GSCs, and the effects of their limitations and potential pitfalls on the interpretation of results.

UTILITY AND LIMITATIONS OF NEUROSPHERE ASSAYS IN GSC RESEARCH

Neurosphere assays were initially used by Reynolds and Weiss in 1992 to isolate neurospheres from the mouse striatum (92), and were subsequently used to successfully enrich tumor-initiating cells from brain tumors (35, 46, 49, 102, 131). These assays are...
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currently used as the standard in vitro method for identifying the presence of stem cells derived from both tumor and non-tumor tissues (17, 18, 90, 122). Similar sphere-forming assays are also used in a wealth of other stem cell systems, including skin (118), breast (73) and pancreas (97).

Neurosphere assays are carried out in selective serum-free media, in which stem-like cells are able to continually divide and form multipotent clonal spheres called neurospheres, while the more differentiated cells incapable of self-renewal and multipotency die off with serial passages (17, 46, 90). The assays thus provide culture conditions that permit competent cells to exhibit the cardinal stem cell property of self-renewal over an extended period of time, so generating a large number of progeny that can differentiate into the primary cell types of the tissue from which they were obtained (70).

These conditions usually include growth of cells in serum-free medium and on non-adherent plates, because unknown serum factors and adherence both promote differentiation (92, 122). In addition, mitogens including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) or both (38, 39, 92, 93) are also used to support the survival and expansion of cells that are responsive to these cytokines. Under these conditions, the generation of secondary spheres upon passage represents renewal of the previous population, and the growth factor-responsive cells can theoretically be passaged indefinitely with little change in the proliferation or differentiation potential between early and late passage cells (38, 93). In contrast, non-stem cells lack these features of self-renewal and multipotency, and are eliminated with serial passages (17). This demonstration of extensive renewal and generation of a large number of differentiated progeny provides the strongest evidence to date that neurosphere assays represent a bona fide method of isolating and enriching stem cells (90). Most importantly, in vivo studies have shown that neurosphere formation is a significant predictor of clinical outcome in glioma patients, independent of Ki67 proliferation index, and is a robust, independent predictor of glioma tumor progression (64). However, neurosphere assays, in common with other in vitro assays, are associated with some limitations.

Neurosphere assays represent an in vitro phenomenon that does not occur in vivo

Neurospheres could result from manipulation of the experimental environment, and reflect an artifact of the assay itself, rather than reflecting an event that occurs in vivo (90). In vivo, GSCs reside in a micro-niche composed of microvasculature readily accessible to the serum, and attachments to the stromal and more differentiated cells, and their biological behaviors are thus highly dependent on their microenvironment (15). Indeed, the two key elements of in vitro assays that are supposed to inhibit differentiation—serum-free medium and non-adherent plating—are now challenged by the development and utility of both GSC and NSC cultures that can be successfully isolated and propagated on adherent surfaces and even in serum-containing medium (41, 45, 57, 59, 88). Thus, the suitability of in vitro neurosphere formation to provide an environment that promotes stem cell self-renewal and proliferation is doubtful. The physical and geometric constraints of a growing spheroid limit the diffusion of culture media, and the presence of internal and external cells results in the formation of gradients of nutrients, oxygen and growth factors within the spheres, all of which could act as important determinants of stem cell proliferation, differentiation and death (8, 16, 127).

Conversely, Mori et al reported that the three-dimensional architecture of neurosphere cell aggregates could create a microenvironment that promotes the proliferation of stem-like cells (80). Proliferating cells are found in the hypoxic center, as well as at the periphery of neurospheres (79), suggesting that the inner compartment of neurospheres is not necessarily unfavorable for the survival and proliferation of stem cells. Indeed, some studies have shown that a hypoxic culture environment can promote neurosphere formation by enhancing and maintaining the self-renewal of GSCs, and can even confer self-renewal capabilities on non-stem tumor cells (44). Furthermore, given the heterogeneous composition of neurospheres, with the majority of cells being more differentiated or terminally differentiated (93, 112), it is reasonable to speculate that the architecture of neurospheres can provide an environment whereby stem cells can contact the surrounding non-stem cells, thus mirroring their in vivo niche. It has also been suggested that the cell aggregation in neurospheres may enhance stem cell proliferation by activation of the Notch signaling pathway (80).

Neurospheres are heterogeneous cell clusters

Neurospheres are complex, heterogeneous cell clusters that consist of stem cells, together with various progenitor cells and more differentiated cells (93, 112), whereas only the bona fide stem cells can exhibit extended self-renewal over serial passages (70). In contrast, the progenitor cells may be able to produce secondary or even tertiary neurospheres, but cannot continue to form neurospheres with extended passages (17). The majority of terminally differentiated cells within neurospheres are unable to reinitiate secondary sphere formation.

In NSC neurospheres, less than 1%–2.4% of cells can give rise to secondary neurospheres (90, 131), and the rate varies from 3% to 20% (69, 131) of cells dissociated from GBM spheres. Additionally, of the cells giving rise to secondary spheres, less than 6% are bona fide stem cells capable of producing spheres over extended passages, with the remaining 94% forming only short-term spheres of no more than six passages (70). These results suggest that less than 1% of cells in the neurosphere cultures are bona fide stem cells. As the use of progenitor cells or cells with non-stem cell characteristics could give rise to misleading results (18), the use of sphere cultures for elucidating and interpreting the biological and molecular characteristics of GSCs is questionable, and this limitation may have implications for practical research.

First, there is no experimental evidence to support a one-to-one relationship between neurospheres and stem cells (90). The majority of spheres are derived from non-stem cells with limited self-renewal capabilities which do not generally survive beyond six passages (70, 93). It is necessary to ensure that bona fide stem cells have been isolated by at least demonstrating self-renewal over an extended period of time (70, 90). Cultures that fail to survive more than six passages are more likely to be derived from progenitor cells (90), as suggested by the finding that spheres differentiated prior to establishing clonal ability (>6 passages) typically only display astrocyte characteristics (70).

Second, it is notable that individual cells dissociated from neurospheres show distinct proliferative potentials: some form abortive
single cells seeded in single mini-wells of 96-well plates), a large than one sphere. Notably, under stringent clonal conditions (ie, spheres may not be totally accounted for by intra-sphere cell prolif-
eration (101); (iii) The estimation of stem cell frequency based on the number of sec-
nondary neurospheres could significantly overestimate stem cell number because of the existence of confounding spheres derived from progenitors (90).

Third, besides being used to enrich GSCs, neurosphere assays are also widely adopted to estimate stem cell frequencies by count-
ing secondary neurosphere formation. Although self-renewal is the cardinal feature of stem cells, an ability to reproduce itself once or twice may not be adequate to satisfy this criterion (90). Therefore, an estimation of stem cell frequency based on the number of sec-
nondary neurospheres has recently been challenged by the finding of sphere fusion (or sphere aggregation) that occurs even under ostensibly clonal culture conditions (101). Sphere fusion appears to be a common, rapid and multiple event that occurs in both normal and neoplastic neurosphere cultures, regardless of age, species or number of passages (101). This intrinsic dynamic of neurospheres has several implications: (i) The individual spheres may not be clonally derived, but are rather chimeric entities, thus confounding the assessment of multipotency of single spheres, as well as the related molecular and genetic studies (20); (ii) The size of neuro-
spheres may not be totally accounted for by intra-sphere cell prolifer-
eration, but may be confounded by the cumulative growth of more than one sphere. Notably, under stringent clonal conditions (ie, single cells seeded in single mini-wells of 96-well plates), a large sphere (>100 µm) is detectable only after at least 2 weeks of prolif-
eration (101); (iii) The estimation of stem cell frequency based on the number of neurospheres is further complicated by sphere fusion (53, 101). The most rigorous method for extending the use of neurospheres to affirm clonal relationships is therefore to plate single cells in single wells (101), which involves arduous and time-
consuming work. Cordey et al (29) recently developed a micro-
well array using biomimetic hydrogel matrix to confine single cells in culture, and guide their extensive proliferation, thus providing a more efficient platform on which stem cell fate and neurosphere formation can be unequivocally attributed to a single founding cell.

**There is no standardized protocol allowing comparison of results**

Despite the importance of neurosphere assays and their wide utility in neural and neoplastic stem cell research, there is currently no standardized protocol that allows for a comparison of results, and different studies have used different growth factors and different concentrations of hormones (17). The serum-free media used by different laboratories include neurobasal medium (68), DMEM/F-12 (9, 32, 35, 93, 95, 113) and a methylcellulose-based medium (49). The use of mitogens further confounds the results, as combi-
nations of EGF, FGF, platelet-derived growth factor (PDGF), leuk-
emia inhibitory factor and even neural survival factor have been used in different studies (17). The use of hormones is also inconsis-
tent: whereas some studies use progesterone and/or insulin, other studies use neither (17). Other additives that are notably different include serum-free supplements such as BIT, N2, B27, antibiotics, antmycotics, selenide and putrescine (17, 31). This wide variation in the use of media components among laboratories significantly complicates data interpretation and limits the effective sharing of information among the research community (17).

**The efficiency of neurosphere assays for producing GSC lines is low**

Although the neurosphere culture technique has been used success-
fully to enrich tumor-initiating cells from brain tumors, long-term expansion of neurospheres from GBM has not been possible in all cases. The efficiency of successful isolation of established GSC lines from any given tumor is unexpectedly low (1%–30%) (7, 63, 87). This low efficiency has been attributed to the tendency of sphere cells to spontaneously undergo differentiation and/or apo-
tosis during serial passages (7, 64, 88, 127). Compared with neuro-
sphere cultures, adherent cultures of both NSC and GSC lines have recently been reported to be much more effective (88). By direct plating of cells onto a laminin-coated flask in serum-free media, adherent cell lines displaying stem cell properties and able to initiate high-grade gliomas following xenotransplantation were established from all GBM samples tested (88). This technique demonstrated 100% efficiency, compared with less than 30% for neurosphere cultures. It is argued that adherent cultures provide uniform access for cells to the key growth factors EGF and FGFR-2, which suppress differentiation and enable expansion of highly pure populations of stem cells (87, 88, 111). However, it is notable that these studies provided no quantitative assessment of the in vitro stemness phenotypes, and no comparison of in vivo tumorigenicity between cells from the adherent and neurosphere tumor cultures (91). Meanwhile, some authors have claimed a similarly high effi-
ciency of neurosphere assays for establishing GSC cell lines (91). Despite these controversies, it can be inferred that the prerequisite for sustained in vitro viability and self-renewal of stem cells resides essentially in the pro-mitotic growth factors, and does not necessarily depend on the detached culture and spheroid architecture.

Thus, although neurosphere assays represent a robust and widely used in vitro method for deriving and propagating GSC cultures,
the limitations mentioned above require a critical evaluation of their use in specific experimental settings. Some of the limitations (eg, the low incidence of bona fide stem cells capable of extended self-renewal) are based on NSC studies, and need to be confirmed by similar studies on GSCs. As GSC neurospheres demonstrate phenotypic characteristics distinct from their normal counterparts, for example, a higher proliferation rate and aberrant differentiation that produces cells co-stained for astrocytic and neuronal markers (121), further studies are needed to better define the characteristics of the neoplastic spheres.

THE UTILITY AND LIMITATIONS OF CD133 AS MARKER FOR GSCs

CD133 was initially identified in 1997 as AC133 antigen, a glycosylation-dependent epitope of CD133 selectively expressed on human CD34+ hematopoietic stem and progenitor cells (130). In the same year, a mouse homolog localized to the apical microvilli of neuroepithelial stem cells was found to display similar topological features to human AC133, and was named promin1, meaning “to be prominent” in Latin (126), thus CD133 is also known as promin1-1. Another glycosylated epitope of CD133, AC141, was also identified and found to be spatially distinct from the AC133 epitope (76). Thereafter, commercially available monoclonal antibodies (mAbs) to AC133 and AC141 epitopes were developed as CD133/1 and CD133/2, respectively, and have been used concomitantly for CD133 detection (11).

Since their initial discovery in hematopoietic stem cells, CD133 epitopes have been found to be widely expressed by a variety of other stem and progenitor cell populations, often in combination with a repertoire of other markers (77). With the more recent development of the CSC hypothesis, there is increasing evidence for the existence of CD133 epitope-expressing CSC populations in numerous tumor types, including ependymoma, prostate cancer, colon cancer, lung cancer, hepatocellular carcinoma, laryngeal carcinoma, melanoma, ovarian cancer, pancreatic cancer (11) and osteosarcoma (117). Given the activity and rapid progress in this field, it is likely that more examples of CD133 epitope-expressing CSC populations will be reported in the future (11).

GSCs were among the first cells to be defined as a small subpopulation of CD34+ cells. Quantitative analysis of CD34+ cells by flow cytometry has generally found them to be present at low and sometimes barely detectable levels in human gliomas, glioma sphere cultures and established glioma cell lines (25, 56, 100, 103, 124), consistent with the assumption that CSCs are a rare cell population in solid tumors. However, some studies have reported exceptionally high CD133+ fractions of 20%–60% in some human GBMs and/or GSC cell lines (56, 103, 105), in accord with immunohistochemical findings demonstrating that many GBMs contain more than 25% CD133+ cells (132). Notably, CD133+ tumor cells may be enriched during repeated in vitro sphere passages (69) or in vivo subtransplantations (100).

When isolated from human brain tumors, CD133+ cells display stem cell properties in vitro (enhanced capacity for proliferation, self-renewal, differentiation and neurosphere-like growth), and initiate tumor growth in vivo (6, 103). Only the CD133+ tumor fraction contains cells that are capable of tumor initiation in NOD−SCID mouse brains, and can reconstitute the phenotypes of the patient’s original tumor (69). Injection of as few as 100 CD133+ cells produced a tumor that could be serially transplanted, whereas injection of 10^6 CD133− cells failed to produce any tumor (103). The small fraction of CD133+ cells are preferentially resistant to chemotherapeutic agents and radiation, and express higher levels of mRNA for the ABC transporter BCRP1, the O6-methylguanine–DNA methyltransferase, markers associated with neural precursors, and negative regulators of apoptosis, and could thus be responsible for posttreatment recurrence (4, 13). Notably, and of clinical relevance, the proportion of CD133+ cells in a cohort of 95 patients with gliomas, as well as their topological organization in clusters, was a significant prognostic factor for adverse progression-free survival and overall survival independent of tumor grade, extent of resection and patient age (132). Furthermore, the proportion of CD133+ cells is an independent risk factor for tumor regrowth and time to malignant progression in WHO grade 2 and 3 tumors (132). These data provide a direct link between CD133 expression and patient outcome, further supporting the clinical significance of CD133+ GSCs. However, a paradox exists, as accumulating reports have demonstrated the existence of CD133− GSCs.

CD133− GSCs exist

There are several lines of evidence to suggest the existence of CD133− GSCs. First, CD133 is not detectable in some fresh GBM specimens (6, 56, 105) and established glioma cell lines, which can nonetheless form tumors in vivo (105, 124). This suggests that cells other than CD133+ ones must be responsible for maintaining tumor growth, and retaining tumorigenic capacity. Second, in vitro culture of cells derived from GBM specimens from different patients under serum-free conditions gives rise to two distinct phenotypes of cells that vary in their expression of CD133 and/or CD133 epitopes (6, 40, 56, 124). Both CD133+ and CD133− phenotypes have key stem cell characteristics and tumorigenic potentials, and both can reconstitute the CD133+ and CD133− populations, but they may differ in terms of other phenotypic features such as proliferation, angiogenesis, invasiveness and gene expression profiles (6, 40, 56, 124). Interestingly, no tumors can be formed by CD133− cells isolated from CD133+ GSC cell lines (6), suggesting that they differ from de novo CD133− cells that possess GSC properties. Lastly, while GSCs from different patients might be de novo CD133+ or CD133−, both GSC phenotypes might coexist in the C6 glioma cell line (133).

The hypothesis explaining the existence of heterogeneous GSC phenotypes can be summarized as follows: (i) GBMs are known to possess various genetic alterations, and might accordingly harbor heterogeneous GSCs (56). The differences in biological growth patterns and genetic expression profiles reported between CD133+ and CD133− GSCs might reflect two biologically different GBM subtypes (6). These respective GBMs might arise from different cells of origin, or from related cell types having acquired different molecular alterations (6). Indeed, comparisons between the phenotypic characteristics of neural stem/progenitor cells at various developmental stages and GSCs suggest that GSCs are derived from CD133+ radial glial-like cells (69, 85), or alternatively from CD133− neurogenic astrocytes, which produce CD133 during tumor development (85); (ii) CD133 expression may be regulated in a cell cycle-dependent fashion, with CD133− cells representing dormant or slow-cycling cells in which CD133 may have been specifically down-regulated during G0/G1 (110). The quiescent
CD133– cells might be a more primitive GSC phenotype preceding the development of CD133+ cells, while the CD133+ cells are more involved in brain tumor progression (124). The CD133+ GSCs in turn give rise to more differentiated CD133– progeny as the true effector cells characterizing the fast-growing tumor bulk, which are, however, distinct from the primitive CD133– phenotype; (iii) The genetic and epigenetic instabilities that are fundamental properties of tumor biology can induce cellular heterogeneity within the stem and non-stem cell populations of the tumor (23). The CSCs within an individual tumor may constitute a moving target because of progressive mutations occurring during the evolution of the tumor, and the cells driving tumor growth at any given time may not be identical to those responsible at another stage in tumor evolution or during metastasis (23). Thus, the heterogeneity of CSC markers may be a general phenomenon applicable to gliomas, as well as to other solid tumors (11). Finally, CD133 expression may be subject to environmental influences. For example, in vitro cultures under hypoxic condition or cellular stress can significantly increase the expression of CD133 in GSCs (37, 75, 86, 104). Therefore, CD133– cells may be positive under certain in vitro conditions, thus raising the question “When is a cell CD133+?” rather than “What is a CD133+ cell?”. The existence of CD133– GSCs thus complicates the identification of cancer cells as therapeutic targets, and markers other than CD133 are needed to more accurately define the population.

**The biological function of CD133 is not well understood**

The role of CD133 or its glycosylation status in regulating GSC phenotype remains an unanswered question (11). Because of its selective location in the apical membranous protrusions of the microvilli of epithelial cells, it has been suggested that CD133 may play an important role in the maintenance of apical–basal polarity and cell migration (26, 62). CD133 has also been ascribed a functional role as an “organizer” of plasma membrane topology (27). Interactions between CD133 and cholesterol within such novel membrane microdomains suggest that CD133 might be important for maintaining an appropriate lipid composition within the plasma membrane (77). It has been speculated that these membrane microdomains may be enriched in components involved in maintaining stem cell properties, and that cells become committed to differentiation upon their release, perhaps through asymmetric cell division (5). In support of this hypothesis, small CD133-containing membrane particles were found in the ventricular fluid within the developing mouse neural tube, and their appearance coincided with the regression of microvilli and the formation of large pleomorphic protruberances on the embryonic neuroepithelium (72). Consistently, these particles were released on differentiation of Caco2 cells in vitro (72). Notably, a single nucleotide deletion that results in a truncated form of CD133 causes retinal degeneration (74).

However, no targeted knockdown experiments have yet been performed, and a direct role for CD133 in maintaining the tumorigenic potential of GSCs remains to be defined. Future experiments designed to investigate the function of the CD133 molecule will be needed to clarify this issue. In particular, analysis of the tumorigenic capacity of CD133– GSCs following targeted knockdown of CD133 expression, would determine if CD133 expression is an absolute requirement for the tumorigenicity of these cells (11).

**The glycosylated epitopes of CD133 are not necessarily representative of the protein**

Most CD133-related experiments have made use of mAbs targeting the AC133 and AC141 epitopes (11). Despite the successful use of these mAbs to identify and purify GSCs, some features of these epitopes need to be considered when interpreting the results of these studies.

Although the AC133 and AC141 epitopes are known to be glycosylated and spatially distinctly located (76, 130), their exact molecular nature and expressions on the CD133 protein have not been determined (11), and discordant expression of the two epitopes is observed (36). Because of the restricted binding of the antibodies to the glycosylated epitopes, CD133 expression might be underestimated if non-glycosylated CD133 is present (77). Moreover, given the undefined nature of the glycosylated epitopes (ie, sugar structure and peptide context), it is possible that the AC133 and/or AC141 mAbs could recognize glycosylated epitopes on molecules other than CD133 (11). Indeed, using an antibody that recognizes human CD133 independently of glycosylation, CD133 antigen expression was found to be retained in differentiated Caco2 cells (34). Additionally, several studies have demonstrated that the AC133 and AC141 epitopes can be down-regulated independently of the CD133 protein or mRNA (28, 34), and the tissue distribution of CD133 mRNA is much more widespread than expression of the AC133 epitope (76). Notably, 28 alternatively spliced variants of CD133 exist, which are expressed in a tissue-dependent fashion and are regulated by methylation status (99). These alternatively spliced CD133 isoforms could lack the AC133 or AC141 epitopes. Thus, AC133 or AC141 antigens are not synonymous with CD133, and it may be incorrect to class AC133 or AC141 epitope-negative cells as CD133– without further verification of CD133 protein or mRNA levels (77). Alternatively, it is plausible that the glycosylation status of CD133 may be a more specific marker of GSC phenotype than CD133 protein levels (11), which is supported by the knowledge that a cell’s glycosylation state changes upon its differentiation and/or upon malignant transformation (28).

**Intrinsic technical limitations of CD133 fluorescence-activated cell sorting (FACS) analysis**

FACS represents a highly accurate and specific method for purifying CD133+ cells, producing cell populations with purities ranging from 92% to 97% (69). In contrast, an alternative isolation method, magnetic microbeads, demonstrates a lack of specificity because of the unspecific binding of glioma cells to the microbeads (25). However, in addition to the mentioned issues relating to the CD133 epitopes, some intrinsic technical limitations of CD133 FACS analysis make its application to GSC purification challenging (23).

First, trypsin digestion during the preparation of cell suspensions might modify the surface expression of proteins such as CD133. Trypsin is predicted to cleave the 865-amino acid CD133 sequence at 79 different sites, and many of these cleavage sites are within the glycosylated extracellular loops (94). The digestion process may therefore affect the way the cells are sorted and the ability of these molecules to play a role in the early stages of tumor growth following xenotransplantation (94). Additionally, immuno-
histrochemical staining of brain tumor sections has revealed a cytoplasmic staining pattern rather than membrane staining, questioning the reliability of flow cytometry, which is based on surface protein expression. This could result in an underestimation of the size of the original GSC pool, because cells with cytoplasmic CD133 will be refractory to cell sorting (94). Second, although phenotypes based on markers often use terms such as high, middle, low and non-expression to describe the properties of the sorted cells for CD133, these terms are subjective and can vary depending on the methods used for cell preparation, the gating setting and the antibody preparations used (23), possibly accounting for the highly divergent CD133 expression levels reported by different laboratories (25, 100, 124). Third, cell viability after long durations of staining and sorting procedures remains a challenging issue, especially given the generally very low percentage of CD133+ cells in the whole tumor cell population. Indeed, it has been shown that the CD133+ cells purified by FACS are not able to sustain long-term sphere formation during in vitro passages (25). Even using advanced sorting techniques to distinguish single cells from aggregates, cell doublets can occasionally sort together and need to be eliminated (23). As most flow cytometers are typically set to sort blood components using small-diameter streams at high pressures, which are often not tolerated by larger and more fragile cells, diameters of the liquid stream and sorting pressures must be frequently optimized for cells isolated from solid tumors (23).

Overall, from its initial identification as a promising and robust GSC marker to the recent reports of a paradox, CD133 nonetheless represents the most exploited surface marker in the field. Given that CD133– GSCs may exist, possibly related to the derivation of GSCs and/or representative of a more primitive developmental phenotype, together with its poorly characterized glycosylated epitopes with uncertain specificity and its elusive biological role, the use of CD133 expression as a marker for GSCs should be critically evaluated for each new experimental system, and the need for additional GSC surface markers that are directly involved in maintaining GSC properties is highlighted (11). As a variety of markers or a combination of markers are used to identify CSCs in many other cancers, GSCs are unlikely to be represented by one particular phenotype (124). Although some reports have suggested possible emerging candidate markers capable of distinguishing GSCs from non-stem tumor cells (3, 81, 105, 110, 115), none has yet been widely accepted and further verifications are needed. Any method used to identify putative GSCs must be verified and confirmed by functional assays, preferably by in vitro assays as the gold standard, before claims for “stemness” can be made. Furthermore, the use of gene inactivation to eliminate “stemness” or gene activation to engender “stemness” could be used to functionally link any marker with stem cell identity (23). It should be a goal of the field to develop cell surface markers and gene activity profiles that can be used to reliably identify GSCs, and this remains a challenge for future studies.

**THE UTILITY AND LIMITATIONS OF SIDE POPULATION (SP) ASSAY IN GSC RESEARCH**

Specific surface markers are commonly used for the identification and isolation of CSCs in a variety of tumors. However, this strategy fails in the absence of known markers, and normal stem cell markers are not necessarily suitable for the identification of CSCs (128). An alternative method of CSC isolation—Hoechst 33342 dye exclusion assay—exploits the different characteristics of stem-like cells and non-stem cells, and selects a subpopulation of cells known as an SP. The SP approach represents a valid marker-independent method of identifying CSCs (83).

Hoechst 33342 is a fluorescent dye that binds to the AT-rich regions of the minor groove of DNA (65). While uptake occurs in all cells, efflux is less permissive. Cells with the capacity to efflux the dye were first identified in the mouse bone marrow, and can be sorted using dual-wavelength flow cytometry as cells displaying low red and blue fluorescence after incubation with Hoechst 33342 dye (19). These are referred to as SP cells because they fall to the “side” of the bulk of the positively stained cells in FACS analysis plots (19). Since this original discovery, the SP technique has been adapted to identify putative stem cells and progenitors in a number of tissues and organs including umbilical cord blood (108), skeletal muscle (2, 78), mammary glands (24), lung (109), liver (98), epidermis (116, 119), forebrain (60), testis (67), heart (71), kidney (50), limbal epithelium (125) and prostate gland (10). In normal tissues, SP cells express high levels of stem-like genes including Notch-1, musashi-1 and ß-catenin (22, 83), and possess multipotent differentiation potential (128), thus functioning as stem cells in the tissues from which they were isolated (52).

Interestingly, with the resurgence of the CSC hypothesis, the Hoechst-based technique has now been used to assess the stem cell frequency in various cancer cell lines and primary tumors (22). SP cells have been identified in a variety of cancer cells at frequencies ranging from 0 to 20% of the total cell population (12, 21, 42, 47, 48, 61, 104, 112, 129). It has been demonstrated that SP tumor cells have an increased capacity for self-renewal (12, 21, 42, 83), and are highly enriched for cells capable of initiating tumor growth upon serial transplantation into NOD/SCID mice (22, 83, 128, 129). In addition, only SP cells are able to reconstitute cellular heterogeneity, and these cells are also more invasive than non-SP cells (22, 83). Importantly, the percentage of SP cells is correlated with tumor grade in primary mesenchymal tumors, suggesting that it may act as a predictor of patient outcome (129). Furthermore, SP cells show increased expression of genes involved in the regulation of stem cell function and cell cycle regulation, with more SP than non-SP cells residing in G1/G0. SP cells also show high levels of ABC drug transporter expression, a unique property of stem cells (22, 128).

Mouse C6 glioma cells contain 0.4% SP cells, which increases to 2% after 10 weeks’ culture in FGF and PDGF (61). Meanwhile, SP cells, but not non-SP C6 cells, can produce both SP and non-SP cells in culture, and form tumors in nude mice that contain both neuronal and glial elements, indicating that these cells have the characteristics of multipotent GSCs (61). In the human glioma cell lines U87MG, T98G, U251 and U373 (22, 47, 83), the proportions of SP cells vary from 0.04% to 1.34%. These SP cells are more tumorigenic and invasive than non-SP cells; are able to reconstitute cellular heterogeneity; and show increased expression of nestin, musashi-1 and ABCG2 (22, 83). Besides the established glioma cell lines, SP cells have also been successfully isolated from primary tumors in two transgenic mouse glioma models, which represent 0.15%–8% of the total population, and are enriched in GSC characteristics and self-renewal ability (12, 43). Remarkably, as few as 50 SP cells can initiate rapid tumor growth after
transplantation into host mice, whereas 500 non-SP cells are required to produce tumors with a similar frequency (43). However, to the best of our knowledge, no information is currently available regarding the isolation of SP cells from primary human GBMs or on the correlation between SP cells and glioma malignancy and prognosis. Meanwhile, the SP assay is associated with some shortfalls which must be borne in mind in GSC studies.

**SP phenotype and ABC transporters**

The SP phenotype is mediated by the ABC family of transporter proteins, and forced expression of these membrane transporters has direct effects on murine stem cells (14). ABC transporters belong to a 49-member gene superfamily of membrane pumps that catalyze ATP-dependent transport of various endogenous compounds and xenobiotics from the cell. These proteins participate in tumor resistance by actively transporting drugs across the cell membrane, serving to protect cells from chemotherapeutic agents (30). ABCB1, ABCC1 and ABCG2/BCRP are the three main transporters known to possess the ability to exclude drugs and are present in CSCs of different tumor types (12, 30). In addition to drug efflux, these pumps also possess the ability to exclude fluorescent dyes, for example, Hoechst 33342 (12).

ABCG2/BCRP is one of the major ABC transporters determining the SP phenotype (136). The strongest evidence linking ABCG2 and the SP phenotype comes from the almost complete loss of the bone marrow SP phenotype in ABCG–/– mice (135). Similarly, blocking of ABCG2 function in GSCs almost completely abolishes the SP tumor phenotype (12). Other supporting evidence comes from the findings that SP cells preferentially express ABCG2, and that ABCG2 expression is detected in a variety of known stem/progenitor cells (83). Reverse transcription PCR reveals much higher levels of ABCG2 mRNA in glioma SP cells than in non-SP cells, suggesting a contribution of ABCG2 to glioma SP phenotype (12, 61). However, it is notable that ABCG2 knockout does not abrogate the tumorigenic potential of SP cells from human glioma cell lines, suggesting that the tumorigenic potential of SP cells may be independent of ABCG2 expression (22).

However, it should be noted that only a fraction of SP cells express ABCG2, and that both SP and known stem/progenitor cells also express other ABC transporters such as ABCB1 (MDR-1), ABCC1 (MRP-1) and ABCA2, suggesting that these molecules may also be involved in mediating the SP phenotype (83). In glioma cell lines, SP cells demonstrate up-regulation of multiple ABC transporters despite ABCG2 knockdown, and it is plausible that SP cells are highly heterogeneous and contain several subsets of cells, one of which expresses ABCG2 (22). Interestingly, even Bcrp1/Mdr1a/b triple knockout mice still retain some SP cells in the bone marrow (55), implying that there is either a redundancy in transporter function and/or that the mechanism that determines the SP phenotype is not solely conferred by the expression of ABC transporters (128). Nevertheless, disruption of these transporters does lead to increased sensitivity to chemotherapeutics such as mitoxantrone, vinblastine and topotecan (54, 96, 135). It is of clinical significance that the SP phenotype represents the expression of a selection of drug transporters leading to the evolution of treatment-resistant cells in a variety of cancers (30). Taken together, drug transporters may not play an essential role in stem cell development, but may only impart xenobiotic resistance (106), with the exact mechanism remaining to be fully elucidated.

**Not all tumors contain SP cells**

Patrawala et al (83) studied a diversity of human cancer cell lines and their corresponding xenografted tumors, and found that 30% of them possessed detectable SP cells. Meanwhile, SP cells were identified in 15 of 23 tumors comprising neuroblastoma, breast cancer, lung cancer and GBM cell lines (47). SP cells were also identified in 23 of a cohort of 29 mesenchymal tumors ranging from benign to high-grade sarcomas (129). These findings imply that SP cells are not ubiquitous, and they may represent just one of several potential populations of tumor-initiating/propagating cells. Alternatively, it is possible that the assay is not always capable of detecting such rare cells among the total population. Lastly, it is notable that although SP cells are generally enriched in stem-like cells, that is not always the case (63, 116, 119).

**SP cells are heterogeneous**

SP-enriched stem cells are rare in normal tissues (0.01%–5%), and heterogeneous, varying with tissue type, stage of development and methods of preparation (83). Bone marrow SP cells contain not only hematopoietic stem cells, but also mesenchymal stem cells (58). Skeletal muscle SP cells are mostly composed of bone marrow-derived cells, with only a minor population of resident muscle stem/progenitor cells (2). Lung SP cells are also heterogeneous, comprising both CD45+ (ie, bone marrow derived) and CD45– cells (109). Similarly, testis SP cells may be enriched in spermatogonial and germinal, as well as mesenchymal stem cells (67). In mouse brain, SP cells consist mainly of endothelial cells (“low SP” cells) that very actively exclude Hoechst 33342. Importantly, the SP cells of mouse gliomas express both endothelial and stem cell markers (12, 84).

Besides the potential coexistence of different cell types, SP cells are heterogeneous in terms of ABCG2 expression and self-renewal capability. As mentioned previously, glioma SP cells can contain several subsets of cells, one of which expresses ABCG2, and the tumorigenic potential associated with SP cells might be conferred by the combined effects of other heterogeneous phenotypes in addition to ABCG2-positive cells, as ABCG2 knockdown could not abrogate the tumorigenicity of SP cells (22). A study on the ABCG2 phenotypes of U373 glioma cells using proliferation assays, clonal analyses, self-renewal and molecular studies (83) suggested a model in which the ABCG2– population contains primitive GSCs with higher self-renewal and proliferative potentials, but which are normally slow cycling. These cells subsequently give rise to ABCG2+ tumor progenitor cells that proliferate more actively, but possess reduced self-renewal and long-term proliferative capacities. The ABCG2+ tumor progenitor cells eventually produce ABCG2– cells, which are partially or even fully differentiated cells that constitute the bulk of tumor cells. Interestingly, even gating within different regions of the SP fraction per se has produced cells with differing “stemness” potentials, that is, cells in the lowest quadrant have increased “stemness” potential compared to cells in the upper quadrant (78). Based on these findings, it is reasonable to speculate that although SP cells isolated from tumors...
are enriched for tumor-initiating potential, they are unlikely to be exclusively composed of CSCs, and the exact nature of these cells has yet to be clarified (128).

**Toxicity of Hoechst 33342 to non-SP cells**

Hoechst binds to DNA, and Hoechst-labeled cells (primarily non-SP cells) may therefore undergo apoptosis when cultured for extended periods of time. This may lead to a potential bias in favor of improved viability of SP cells relative to non-SP cells. In support of this hypothesis, Hoechst labeling significantly decreased the viability of DAOY cells, while SP cells were resistant to this toxicity (106). A more recent study reported that brief exposure to Hoechst 33342 reduced the clonogenicity and proliferation of individual C6 cells (133). Meanwhile, nuclear Hoechst 33342 staining can have significant impacts on the induction of cell differentiation in both normal and neoplastic cell lines (1, 107). The DNA-binding affinity of Hoechst 33342 may thus interfere with cellular replication and differentiation, so confounding the ability to detect relevant biological differences between the SP and non-SP fractions (106). Non-SP cells may be deprived of their stem cell features by Hoechst 33342 staining, as a step in isolating Hoechst-negative SP cells using flow cytometry (133).

However, there is also evidence that Hoechst 33342 staining does not account for the increased “stemness” of SP cells (134). In a mouse glioma model, cell viability was measured 10 days after sorting and culture of the SP and main populations, as well as the total cell population. Cell viability was unaffected in all cases, regardless of whether or not they were stained with Hoechst 33342 (12). In addition, neither apoptosis nor any differences in cell cycle distribution were observed in Hoechst-incubated neurosphere cultures (12). Likewise, equivalent levels of cell death were observed in FACS-sorted SP and non-SP cells, most likely because of the sorting process per se, suggesting that selective cell death is not the major reason for the different tumorigenic potential of SP cells (43). Non-SP cells from primary mesenchymal tumors are able to form primary xenografted tumors, although they fail to engraft after secondary transplantation (129). In the MCF-7 breast carcinoma cell line, stained non-SP cells have similar in vivo tumorigenic potential and in vitro colony-forming potential to SP cells (134). These tumor-forming abilities of non-SP cells indicate that these cells remain viable after uptake of Hoechst dye (129).

However, the controversies regarding Hoechst 33342 toxicity suggest that the results of SP assays should be interpreted cautiously, and repeated viability assays are necessary to identify potential confounding factors.

**SP assays are highly variable between experiments**

SP cells have frequently been identified as a continuous tail emerging from the lower end of the non-SP group, using either a BD FACS Vantage or a Dako-Cytomation MoFlo. Discrimination between SP and non-SP cells is somewhat arbitrary and lacks consistency of gating strategies between experiments (22, 78, 83). Interestingly, cells of differing stemness potential have been isolated by gating within different regions of the SP fraction (78). Thus, inconsistencies in gating strategies can result in cross-contamination of the SP and the non-SP fractions, leading to the production of confounding data (128).

Furthermore, dye efflux is a dynamic process, and small differences in staining times, dye concentrations and cell densities can affect the isolated SP phenotype (106, 128). The experimental conditions used in different studies differ (12, 43, 61, 83, 106, 129); for example, Hoechst concentrations have varied from 2.5 to 5.0 μg/mL, verapamil concentrations from 50 to 100 μM and assays can involve verapamil pre-incubation for 10–30 minutes before Hoechst staining, and/or co-incubation for 90 minutes along with Hoechst. Moreover, the cell densities have differed from 1 to 2 × 10^6 cells/mL. More stringent gating strategies and more consistent staining protocols are therefore needed to allow the true nature of SP cells to be elucidated.

In summary, despite its limitations and pitfalls, the SP assay represents a valid functional method for prospectively selecting CSCs, independent of known surface markers. However, some issues remain to be answered, including the existence and frequencies of SP cells in primary cultures of human gliomas, and their correlation with malignancy and prognosis. Some of the limitations mentioned above (eg, Hoechst toxicity, inconsistency of gating and staining protocols) have been more extensively explored in other CSC systems, and need to be further clarified in GSC studies. However, it should be noted that all isolation strategies have their limitations, and a combination of different methods may be required to enhance the purity of isolated GSCs (128).

**COMPARATIVE CONSTITUTIONS OF POPULATIONS OF GSCs ENRICHED BY THE THREE METHODS**

**Neurospheres and CD133+ cells**

Numerous studies have revealed that isolated glioma CD133+ cells preferentially show neurosphere-like growth (35, 46, 49, 102, 131). Meanwhile, the CD133+ cell fraction correlates closely with the neurosphere-forming capability of the cells dissociated from fresh GBM biopsies (103). However, under stringent clonal conditions (one cell per well), only a small proportion (2%–5%) of CD133+ tumor cells are able to form primary neurospheres, and the majority are unable to sustain long-term sphere formation in in vitro passages (25). Conversely, tumor spheres also represent cultures with a higher percentage of CD133+ cells relative to adherent cells growing in serum-containing media, but the percentage of CD133+ cells is not prominent, ranging from 0.4% to 15.3% (25, 43, 69, 89). Notably, CD133+ cells may be enriched during repeated in vitro passages (69, 89), and in our established cell lines they can represent ≥90% of the total cells (data not published). The cause of the enrichment remains to be clarified, but could be because of: (i) conversion of CD133− to CD133+ cells through the process of dedifferentiation; (ii) expansion of a pre-existing CD133+ population; or (iii) in vitro aberrant events associated with long-term passages. Interestingly, the CD133+ and CD133− tumor cells might have similar neurosphere-forming abilities, but differ in the phenotypes of the spheres formed, that is, floating versus adherent spheres. The two types of spheres show distinctive gene expression profiles and differ in terms of other phenotypes, such as proliferation and invasion, while being similarly tumorigenic in nude mice in vivo (6, 40). Neurosphere cultures thus correlate only marginally.
with CD133+ populations. Neither represents a pure population of stem-like cells, but both comprise heterogeneous cell populations moderately enriched for GSCs.

**Neurospheres and SP cells**

The comparative constitutions of neurospheres and SP cells are similar to those of neurospheres and CD133+ cells. The proportion of SP cells in the C6 cell line is enriched from 0.4% to 2% when shifted from adherent, serum-containing growth to sphere culture (61), and the fraction can be 10%–20% in spheres derived from a mouse glioma model (12). Conversely, SP cells are enriched for sphere-forming ability compared to non-SP glioma cells (12), with the percentage of neurosphere-forming cells being as high as 60%–70% (43, 61). Unfortunately, the sustainability of the tumor neurospheres formed by SP cells after repeated passages has not been reported in these studies.

**SP cells and CD133+ cells**

 Likewise, the SP and CD133+ phenotypes overlap, but do not represent identical populations. SP glioma cells have been reported to contain 5%–17.6% CD133+ cells (12, 89), which is much higher than the non-SP fraction. In contrast, the CD133+ cells harbor both SP and non-SP phenotypes (106). However, relative analyses of the two phenotypes are scarce in the literature, possibly because of the rarity of both populations among tumor biopsies and cell cultures.

In summary, none of the three methods discussed may be suitable for tagging the entire GSC pool, and the populations of cells isolated by each of these methods represent heterogeneous, rather than uniform collections of cells that are moderately enriched for GSCs, and which marginally overlap with each other (the relationships are illustrated in Figure 1). The potential pitfalls associated with each method further complicate the interpretation of any results. It is possible that a combination of methods might produce a purer GSC fraction than those obtained using any individual method. Additional assays or markers need to be developed to define the GSCs beyond the reach of each of the three ellipses.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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