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BRAIN TUMOR-INITIATING CELLS AND CELLS OF ORIGIN IN GLIOBLASTOMA

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Abstract

Glioblastoma Multiforme (GBM) is the most malignant and devastating primary brain tumour with a median survival of ~12-16 months. Although recent large scale sequencing projects have shed considerable light into the complexity of the disease, there remains much to be elucidated in the hopes of generating effective therapeutic strategies. Although these studies investigate the mutations and expression of bulk tumour they have limits with respect to cell of origin and the concept of brain tumour initiating cells (BTIC). Current research has challenged the old paradigm of the stochastic model as recent evidence suggests that a subset of cancer cells within a tumor is responsible for tumor initiation, maintenance, and resistance to therapy. To gain a better understanding of the different compartment of cells that GBM comprise of require careful and elegant experiments. In addition to studying GBM, exploring the role of normal neural stem cells and progenitors cells is essential to partially explain whether these GBM BTIC behave similarly or differently then their non transformed counterparts. Here we discuss the recent literature between the two models, candidate regions of glioma genesis, candidate cells of origin for GBM, and possible therapeutic avenues to explore.

Keywords

• Aberrant signaling pathways • Brain tumor-initiating cells • Cell of origin • Glioblastoma multiforme • Genetically engineered mouse models • Stem cell markers • Subventricular zone • Therapeutic resistance

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1. Brief introduction

Conventional approaches to the treatment and management of cancer have been to eliminate all tumor cells. This approach is primarily based on the stochastic model also known as the clonal evolution model in which all tumor cells have the potential to proliferate limitlessly, self-renew and drive tumor growth [1,2]. However, treatments tailored to this model have resulted in minimal gains towards survival post-treatment in several cancers including glioblastoma multiforme (GBM) [3]. Current research has challenged the old paradigm of the stochastic model as recent evidence suggests that a subset of cancer cells within a tumor is responsible for tumor initiation, maintenance and resistance to therapy [4,5]. This new concept of a subset of cells within the tumor that has stem cell like properties, limitless expansion potential and can drive tumor formation, has been designated the cancer stem cell hypothesis or hierarchical model (Figure 1). In addition, bulk tumor cells that are derived from this stem cell like tumor cells have limited proliferative capacity, are partially differentiated cells and cannot form tumors. The stem cell compartment of tumors have been termed cancer stem cells (CSC), tumor initiating cells (TIC), or tumor propagating cells (TPC) [6]. For simplicity, we will use the term tumor-initiating cells (TIC) and brain tumor-initiating cells (BTIC) to refer specifically to CNS tumor formation. Additionally, we would like to clarify that our definition does not encompass the term cell of origin. This term refers to the original cell or group of cells, which acquired neoplastic lesions to induce transformation. BTIC may be one possible candidate for the cell of origin.

BTIC have been isolated from several cancers including brain tumors such as GBM and medulloblastoma [7-9]. These isolated BTIC constitute a small fraction of the total population of tumor cells and are characterized by several hallmark features shown in Table 1. BTIC are able to propagate in an undifferentiated manner and are able to recapitulate the tumor when injected in low numbers (10^3-10^4 versus 10^5-10^6 in non- purified tumor cells) [7-10]. BTIC express markers including CD133, nestin, and SOX2, are similar to normal neural stem cells and are isolated/derived using several means as summarized in Table 2. In this review we will consider sources of neural stem cells and BTIC and aberrant signaling pathways within these cells. We will further discuss how targeting BTIC may provide a new strategy for therapeutic targeting and outline potential limitations and pitfalls of the cancer stem cells as therapeutic targets.

2. Definition and sources of brain tumor initiating cells (BTIC)

The isolation of a subpopulation of stem like cells from surgical specimens of malignant gliomas lent support to the cancer stem cell hypothesis, suggesting that GBM are composed of a heterogeneous collection of cancer cells with varying tumor initiation potential [9]. These glioma stem cells or BTIC have features of normal neural stem cell such as self-renewal, multi-potent differentiation, plus the ability to recapitulate the tumor phenotype in vivo

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in small numbers. However, these stem-like features cannot be taken as evidence of apparent neural stem cell origin. Currently the origin of BTIC is centered around three mayor underlying theories: 1) mature glia through acquisition of mutations will dedifferentiate to acquire unregulated "stem cell" like properties, 2) restricted neural progenitors, which have limited self-renewal potential need to acquire mutations, which leads to the gain of unregulated "stem cell" like properties, and 3) adult neural stem cells (NSC), which normally have tight regulation over their proliferative and differentiation potential, acquire mutations that render them tumorigenic [11,12] (Figure 2).

Several studies have supported the idea that committed glial cells could be the precursors of BTIC. Retroviral transduction of Ink4a/Arf-/- mature astrocytes with a constitutively active mutant EGF receptor (EGFRvIII), prevalent in human GBM, induces astrocyte dedifferentiation and GBM formation [13]. A phenomenon, that is also observed when GFAP-expressing cells are infected with platelet-derived growth factor (PDGF) expressing retrovirus using the RCAS/tva system [14]. In addition, overexpression of the transcriptional factor c-myc, in astrocytes results in the down regulation of the astrocytic marker GFAP and upregulation of nestin [15]. However, one of the most relevant pieces of evidence comes from the demonstration that adult fibroblasts can be reprogrammed to a pluripotent stem cell state by transfection of a small number of transcription factors [16]. Suggesting that permanently quiescent cells can be endowed of stem cell like properties arguably, similar process might be relevant during brain tumor pathogenesis.

The isolation of replication-competent, multipotent neural progenitors from the postnatal brain [17,18] provided new candidates for the origin of BTIC as this stem cell and progenitor elements might represent the path of least resistance to transformation, presumably because such cells have the machinery for self-renewal already activated.

Restricted neural progenitors, which have limited self-renewal potential will first need to acquire mutations which endow them with an increased self-renewal potential in order to experience additional mutations that would lead to transformation. Studies have shown that committed oligodendroglial progenitors can be induced trough the modification of extracellular signals, to gain stem-like properties [19], resulting in the reactivation of the primitive neural epithelial marker Sox2 [19], which is prevalently expressed in human gliomas [14]. These results suggest that similar mechanisms might be operative in the transformation of restricted progenitors in the adult brain.

Multipotent neural progenitors are found in specialized neurogenic niches such as the dentate gyrus and subventricular zone (SVZ) in the postnatal brain. The latter region has been suggested as a source of gliomas as many of them have a periventricular origin or are contiguous with the SVZ. This is further highlighted by studies reporting that viral and chemical carcinogenesis preferentially induce tumors when inoculated adjacent to the SVZ rather than when introduced to non-proliferative regions such as the cortex [20,21]. The SVZ is an extensive germinal layer adjacent to the ependyma, containing astrocyte-like

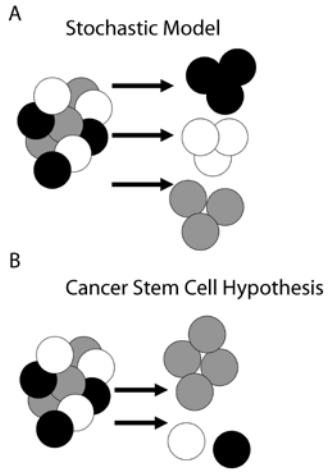

Figure 1. A. The stochastic stochastic model predicts that any tumor cell, given the chance, will be able to form a new tumor. B. The cancer stem cell model predicts that only a subset of cells (the grey cells in the figure) can generate a new tumor, while the other cells cannot.

Table 1. Hallmarks of brain tumor initiating cells.

Hallmark	Description
1. Self-renewal	Parent cell gives rise to two daughter cells which retain all the stem cell like properties of the parent cells
2. Multipotency	Potential to differentiate into different cell types and express markers of different lineages such as neurons, oligodendrocytes, and astrocytes.
3. Proliferation	The ability to produce many progeny cells, which form primarily, the bulk tumor

Table 2. Methods of isolating BTIC cells.

Method	Summary
1. Flow cytometry	Separating cells based on candidate stem cell surface markers into positive and negative fractions for the marker of interest [4,7,15].
2. Neurosphere assays	Growing tumor cells in neural basal cell medium with growth factors that enrich for neural stem cells [28,29]. Neurospheres can be passaged numerous times and display hallmarks from Table 1.
3. Adherent stem cell cultures	Growing isolated BTIC on adherent laminin-coated plates to allow for increased purity and stability, uniform growth factor delivery and reduce spontaneous differentiation [30].

stem cells (also known as type B cells in mice) [22]. These relative quiescent neural stem cells periodically give rise to lineage-restricted progenitors cells, which undergo limited mitosis before differentiating into mature cells [22]. A prevalent theory on the origin of BTIC asserts that they are a result of neural stem cell transformation, as there are several similarities between BTIC and neural stem cells. Both cells share multiple cell surface markers, exhibit a marked ability to migrate through the brain parenchyma and are able to form neurospheres *in vitro*, but most importantly, pathways regulating normal stem cell self renewal, proliferation and survival may also be operative in BTIC.

More recently, further experimental models in mice have validated the paradigm that BTIC might arise form neural stem cell transformation. Parada et al. [23] developed a mouse model were deletion of human astrocytoma-relevant tumor suppressors p53, Nf1 and Pten was targeted to NSC, through the use of an inducible nestin-Cre transgene, or alternatively using stereotactic viral delivery of Cre expressing adenovirus into the SVZ. This mice developed astrocytomas with a 100% penetrance, only when targeted to neural precursors cells but not differentiated glia [23]. Better mouse models investigating the role of the SVZ as a region of glioma generating potential will be essential to address these critical questions [24]. More importantly utilization of the powerful technique mosaic analysis with double markers (MADM) in mice harbouring *TP53* and *NF1* deletions have shown that oligodendrocyte precursor cells (OPC) can give rise to GBM and act as a candidate cell of origin over other neural stem cell-derived lineages [25]. MADM mouse transgenics and current BRAINBOW mouse models will allow for efficient labeling and lineage tracing that will allow for a better method of identifying candidate cells of origin in brain tumors such as GBM [25,26]. However translation of these results into humans may not be as easy. For example, the human adult SVZ contains a hypocellular gap that the mouse does not and the SVZ has a marked decrease of proliferation after 12-18 months after birth. These data suggests that the SVZ may be a region of interest of pediatric high grade gliomas (PHGG) more so than adult gliomas [27-30].

Although still controversial, the topic of the origin of BTIC is of great therapeutic interest, as the cancer causing genetic or epigenetic alterations may be quite different in the three cell populations outlined above, suggesting that the pathways to be therapeutically targeted might be dependent on the cell from which BTIC originated.

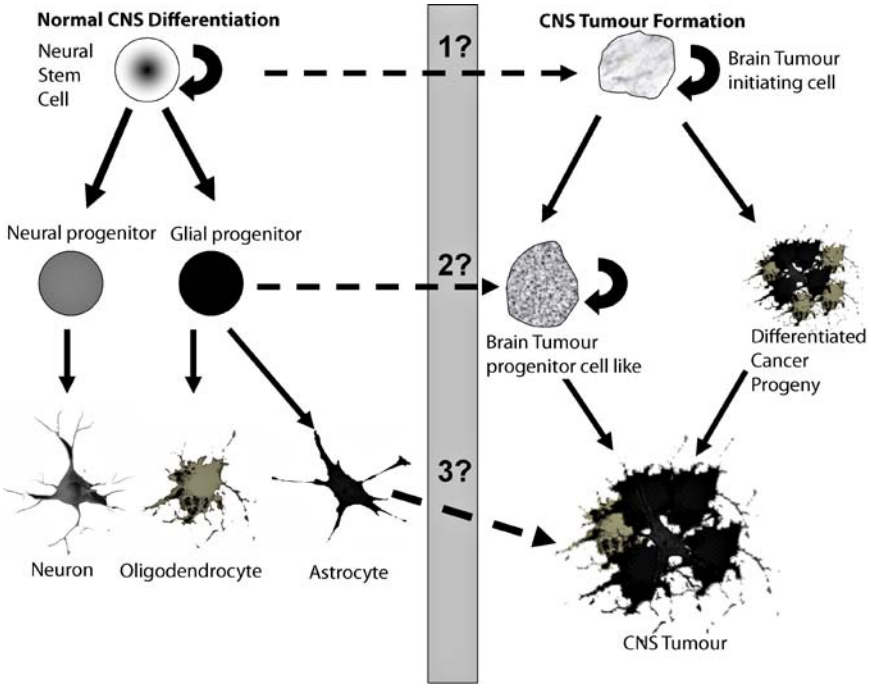

Figure 2. Normal CNS differentiations and CNS tumor formation. During normal neural stem cells (NSC) differentiation, NSC give rise to neural and glial progenitors which give rise to differentiated cells such as neurons, oligodendrocytes and astrocytes. BTIC have been theorized to come from terminally differentiated cells (1?), which acquire genetic mutations (?) endowing them with a proliferative advantage a slow accumulation of critical mutations result in its transformation. Normal neural stem cells give rise to progenitors with limited proliferative and self-replicative capacity. BTIC have been theorized to form as a result of neural stem cell (2?) or neural progenitor transformation (3?). Curved arrow represents ability to self-renew.

2.1 Cell surface markers of BTIC and abberant signaling in BTIC of GBM

2.1.1 BTIC associated markers

BTIC share many molecular markers once thought to be exclusively attributed to NSC, such as nestin [31], CD133 [32], musashi-1 [33] and stage-specific mouse embryonic antigen-1 (SSEA-1) [34], (Table 3). Unfortunately, at this time no cell marker is absolute in identifying BTIC. However, two cell surface markers, nestin and CD133 (prominin-1), have been of particular interest in the study of brain tumor organization. It has been observed that these markers are associated with grade of malignancy and are likely prognostic markers for brain tumor patients [35,36].

The intermediate filament nestin is expressed in all CNS lineages restricted progenitors and in astrocytes [37]. Evidence suggests that nestin expression is highly correlated with "stemness", whereby NSC progeny, which takes on a more committed role, results in a downregulation of nestin expression followed by upregulation of more committed neuronal and astrocytic markers [38]. Expression of nestin has been reported in BTIC, however its expression is variable and non-specific for BTIC [31].

The neuronal stem cell marker CD133 (prominin-1 in mice) has received particular interest as its expression has been associated with both, tumor initiation capacity and radioresistance [9,39]. Initial reports indicated that as few as 100 CD133+ cells collected from GBM surgical specimens, could form xenograft tumors in immunocompromised mice, that

Table 3. Markers of BTIC.

Marker	Summary
1. CD133	Used to isolate a subpopulation of tumorigenic cells from GBM operative specimens, capable of tumor initiation in vivo. Recent studies have reported its expression as a result of environmental stress, questioning its validity as a BTIC marker.
2. Nestin	Expressed in all CNS lineage restricted progenitors astrocytes and BTIC. However, the expression of this marker is variable an non-specific to BTIC.
3. Musashi-1	Expressed in normal NSC and BTIC. Its expression has been correlated with tumor grade and proliferative activity. Its role as a BTIC specific marker its non-conclusive.
4. SSEA-1	A neural stem progenitor marker shown to be express in GBM cells that fulfill the functional criteria of BTIC. Proposed to be a better BTIC marker in combination with CD133.

recapitulated the phenotype of the tumor from which they were isolated [9]. This is in sharp contrast to the 10^6 CD133- cells required for the same phenotype [9]. Although there has been overwhelming evidence that supports this issue, more recent investigations have called into question the reliability of CD133 as a marker of BTIC. Gringuer et al. reported that alternations in mitochondrial function among glioma cells induce CD133 expression [40]. Conversely, replacement of dysfunctional mitochondrial genes can reverse CD133 expression [40]. These results suggest that CD133 expression in gliomas is triggered as a response to environmental stress, questioning the reliability of CD133 as a BTIC marker.

Other markers that are share by normal NSC and BTIC include the RNA-binding proteins, musashi-1, which in normal stem cells is thought to repress the translation mRNA believed to be involved in the process of differentiation [5]. Increasing evidence points toward the involvement on musashi-1 in the process of tumorigenesis, as its expression has been reported in neurospheres derived form GBM operative specimens, and its expression is highly correlated to the grade of malignancy and proliferative activity in gliomas [41], however its role as a specific BTIC marker is questionable. More recently, Myung et al. identified the stage-specific embryonic antigen-1 (SSEA-1, also known as CD15/LeX) to be expressed in GBM cells that fulfill the functional criteria of BTIC, since SSEA-1-positive cells are highly tumorigenic, can give rise to both SSEA-1- positive and SSEA-1-negative populations and have self-renewal and multilineage differentiation potential [34]. However further validation that will identify SSEA-1 as a specific marker of BTIC is still pending.

As inferred above, neither of the makers by themselves seem to be specific for BTIC, it is possible that similar to hematopoietic stem cells, which are probably the most thoroughly characterized population, a combination of markers will ultimately best define BTIC.

2.1.2. Aberrant signaling pathways associated with BTIC

Multiple signaling pathways have been implicated to be disrupted in BTIC, interestingly they include those which serve to regulate the self-renewal, proliferation, and survival properties of normal stem cells. This include the hedgehog family of regulatory pathways which mediate the proliferation of progenitor cells through the activations of the transcription factor Gli, which promote cell cycle entry and DNA repair [42]. In the adult central nervous system, Gli1 is expressed by neural progenitors in germinal regions such as the SVZ and the dentate gyrus, were it is thought to play a role in the maintenance of this stem cell population [43]. Like the hedgehog pathway, the notch pathway has been linked to the biology of normal NSC as well as BTIC. Notch has been shown to be involved in the maintenance of "stemness", as its loss leads to enhance differentiation and reduced proliferation of neural progenitors in the SVZ in vivo. Both Gli and Notch are expressed in GBM [44, 45] and it is thought that these pathways may mediate the initiation and maintenance of these tumors as they do for neural stem cells.

Mitogens and their respective tyrosine kinase receptors such as EGFR and PDGR have been studied extensively in gliomas, as these are aberrantly expressed in most adult high-grade gliomas [46]. Both the PDGF and EGF receptors are expressed in progenitor populations in the SVZ. Overexpression of their ligand leads to reduced differentiation, followed by an increase in proliferation, leading to hyperplasia with some features of gliomas [47,48], suggestive of their role in glial progenitor differentiation and proliferation.

PTEN loss and Akt activation, were shown to correlate with aggressive and resistant phenotypes associated with BTIC [49]. Increased Akt through loss of PTEN leads to an increase in BTIC in mouse gliomas. In mouse medulloblastomas, combination of Akt pathway inhibitors with radiotherapy, significantly decrease the survival of the stem-like pool [49]. Besides signaling pathways and their direct downstream effectors, transcription factors such as BMI-1 have also been shown to play a role in stem cell self-renewal by repressing gene products of $P16^{Ink4A}/P19^{Arf}$ [50]. Bmi-1 oncogene/stem cell renewal factor is expressed in human GBM tumors and is highly enriched in CD133-positive cells. Stable BMI-1 knockdown using short hairpin RNA-expressing lentivirus resulted in inhibition of clonogenic potential in vitro and of brain tumor formation in vivo [51], suggesting its role in the regulation of BTIC self-renewal and proliferation.

3. Therapeutic promise of targeting the BTIC population

Traditional characterization of GBM based on proliferation, apoptosis, angiogenesis and invasion have been based on bulk tumor

but this may not provide the complete story in light of the BTIC hypothesis. Current and ongoing investigation into the BTIC hypothesis suggests that the BTIC population may be an attractive and effective target to eliminating a tumor. Conventional modalities of treatment including chemotherapy and radiation have been used to treat GBM. However, several studies have observed that the BTIC populations are intrinsically chemo- and radioresistant [52,53]. For example, BTIC expressing the CD133 candidate stem cell marker are significantly resistant to radiation compared to CD133-negative tumor cells. These cells exhibited elevated levels of the DNA repair proteins Chk1 and Chk2 compared to their non-BTIC counterparts [33]. Therefore, targeted therapies to Chk1/2 may be an effective strategy to overcome radioresistance. In addition to radioresistance, elevated levels of drug transporters ABCG2 and ABCA3 have been shown to promote temozolomide resistance in BTIC [53,54].

A novel approach for targeting BTIC may be promoting differentiation of these cells to limit their replication and self-renewal properties. Upregulation of bone morphogenic proteins (BMP) such as BMP4 has been shown to induce differentiation of BTIC, limit their self-renewal ability and drastically reduce proliferation [55]. The vascular niche that promotes and allows BTIC to grow has also become a potential target. Use of the anti VEGF antibody, bevacizumab has been shown to reduce the CD133+ BTIC population in vitro and in vivo illustrating that disruption of the BTIC niche may be an effective therapeutic strategy [56,57]. Our recent work on GATA transcription factors have shown that forced GATA4 expression can in part stimulate BTIC to differentiate and proliferate as these cells had higher *GFAP* and lower *NESTIN* expression. GATA4 was associated with the *GFAP* promoter and may be required for activation of the *GFAP* gene and thus promote differentiation. Also, these BTIC cells expressing stably expressing GATA4 were more sensitive to temozolomide, a chemotherapeutic that has clinical efficacy in treatment of GBM patients [58,59]. Interestingly, GATA4 did not impair expression or function of MGMT rather decreased expression of another DNA repair enzyme, alkyl-purine DNA-*N*-glycosylase (APNG), which promotes TMZ resistance through the base excision repair pathway (BER) [59]. Further experimentation will provide better insights for the role of GATA4 and GATA6 in brain tumor initiating cells.

Lastly, our unpublished results and the work of others have shown a novel component to the microenvironment of GBM. BTIC have been shown to differentiate in to tumor endothelium and thus provide additional support for tumour vasculature [60]. Although this requires mores investigation, it may provide novel therapeutic targets and strategies of targeting tumour vasculature. Lastly, bone marrow-derived cells are recruited to GBM at early stages of tumor development. Whether these non-neural stem cells are recruited for a protective response or to mount an immune response at the tumor remains controversial but may provide novel ways to selectively target niches that may be essential for BTIC development and maintenance.

Recent advances into the cancer stem cell hypothesis and BTIC have allowed for a new paradigm in terms of treating brain tumor patients. By selectively targeting the cells that promote resistance and propagate the tumor, it might allow for treatment with effective and promising results.

4. Current limitations to the stem cell hypothesis

Although targeting the BTIC population may seem like a viable and attractive option, there still are unanswered questions as to how effective targeting BTIC truly are. First, many of the drugs used to treat GBM and target BTIC namely 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), cisplatin and cytarabine have shown to be toxic to normal stem cells of the SVZ and the dentate gyrus of the hippocampus at clinically relevant doses [61]. Therefore direct targeting of BTIC with these compounds may also be harmful to normal NSC. Second, although differentiation of BTIC may be another avenue of treatment, the complete picture is still unknown. For example, although BMP4 can promote reduction of the BTIC population as mentioned above, in certain subset of GBM patients, BMP can paradoxically induce tumor proliferation and increased tumorigenicity [62].

Lessons learned from other cancer systems also have raised questions into the applicability of the hierarchical model and BTIC in certain situations. In breast cancer, tumor initiating cells shown to express CD24 or CD44 were genetically different within the same patients, suggesting that these cells originated from different subpopulations, providing evidence that had each subset of cells undergone different transformations to gain growth advantages, thus supporting the stochastic model [63]. A study demonstrated that supplementing the glioma cell line C6 with serum and under certain growth conditions, approximately 90% of C6 cells could proliferate, exhibiting exponential growth without spontaneous differentiation and the ability to self-renew. Again, this is more easily explained by the stochastic model and that the influence of microenvironment on conferring "stem cell-like" properties to non-stem cell tumor cells can occur. To further this, oncogenic stress and induction of certain transcription factors have been shown to reprogram differentiated cells or restricted progenitor cells to a more pluripotent state [64-66]. Therefore, whether the tumor initiating cell was originally a normal stem cell or arouse from dedifferentiation is still poorly understood.

Problems with xenotransplantation experiments can also limit the use of the BTIC and the cancer stem cell model. In most cases, only a small population of TIC has been shown to result in tumor growth when implanted into immunodeficient animals [67,68]. Using this system, TIC with certain phenotypes and markers would have been shown to be more likely grow into a tumor (CD34+ CD38- in leukemia, CD44+ CD24- in breast cancer, CD133+ in brain cancer), compared to cells negative for these markers. However, the environment of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice removes many of the tumor cell/immune cell interactions and introduces tumor cell to foreign milieu. How this environment of NOD/SCID mice contribute to tumorigenesis remains largely unanswered. Using NOD/SCID

interleukin-2 receptor gamma chain null mice (more immunocompromised than traditional NOD/SCID mice), one group found 27% of melanoma cells could form a tumor with a single cell transplant [69]. Again this observation supports the stochastic theory of tumor formation since a large population of cells has the ability to form tumors. Lastly, there is the difficulty with BTIC/CSC markers. Several studies have shown that the CD133+ compartment of BTIC form tumor compared to no tumor growth from CD133 - GBM cell implants in NOD/SCID mice [9]. However, subsequent studies found that not only can CD133- GBM cells form tumors in immunodeficient mice, but they can also give rise to CD133+ cells [70]. Identification of BTIC currently relies on several markers however, each marker has limitations and it is highly likely that BTIC express several different markers under varying conditions providing difficulty in isolating a truly pure population of BTIC from bulk tumor.

5. Conclusion

The concept of BTIC and CSC provide a new therapeutic target for effective treatment. However, only selectively targeting the BTIC population may not be an effective strategy. This is due to several reasons, including inducing toxicity to NSC and the ability of bulk tumor cells to acquire stem cell like properties. It is safe to argue that within a GBM lays a population of cells that exhibit BTIC properties in addition to bulk-differentiated tumor cells and cells that are progenitor like. Moreover, tumor microenvironment can influence any of these types of cells to become more plastic, stem cell like or more differentiated. Thus, targeting of all types of cells using combined strategies would be the most effective form of treatment and management of the tumor. Advancing our knowledge of the BTIC/CSC hypothesis and answering some of these outstanding questions may shed light and allow us to exploit this feature of GBM and other brain tumors.

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