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Glioblastoma Cancer Stem-like Cells – Implications for Pathogenesis and Treatment

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Abstract

Glioblastoma remains one of the deadliest forms of cancer. Infiltrating cancer cells in the surrounding brain prevent complete resection and tumor cell resistance to chemoradiation results in the poor prognosis of the glioblastoma patient. Much research has been devoted over the years to the pathogenesis and treatment of glioblastoma. The tumor stem cell hypothesis, which was initially described in hematopoietic cell malignancies, may explain the resistance of these tumors to conventional therapies. In this model, a certain subset of tumor cells, with characteristics similar to normal neural stem cells, is capable of producing the variety of cell types, which constitute the bulk of a tumor. As these tumor cells have properties distinct from those constituting the bulk of the tumor, a different approach may be required to eradicate these residual infiltrating cells from the brain. Here we outline the history behind the theory of glioblastoma cancer stem-like cells, as they are now referred to. We will also discuss the implications of their existence on commonly held beliefs about glioblastoma pathogenesis and how they might influence future treatment strategies.

Keywords

Glioblastoma; stem cells; gliomagenesis; brain tumors; GBM; cancer stem cells; CNS tumors

Glioblastoma (GBM) is the most common primary malignancy of the brain, as well as its most malignant, and is classified as Grade IV on the World Health Organization (WHO) scale [1]. Despite years of advances in therapy, the median survival after radiation and chemotherapy ranges from 12 to 15 months [2]. While recent advances in therapeutics for GBM have shown some limited promise [3–5], these tumors inevitably recur in the majority of patients and are nearly uniformly fatal. Despite surgery, infiltrating cancer cells that reside away from the main tumor mass are thought to be responsible for tumor recurrence as well as radiation and chemotherapy resistance [6, 7]. These residual infiltrating GBM cells were initially felt to be biologically identical to the cells in the main tumor mass; however,

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the tumor stem cell hypothesis now challenges prior GBM dogma. Clearly, there exists a strong need to understand this mechanism of GBM recurrence and resistance to common treatment modalities.

Recent research efforts hypothesize that much of the therapy resistance and ability to regenerate when the bulk of the tumor mass has been treated may rest within a small population of cells, known as cancer stem-like cells (CSCs) [8, 9]. Much of our knowledge of the existence of such cells arises from knowledge developed in the discovery of and understanding of hematopoietic cancer stem cells [10]. Using lessons from the hierarchical organization of the proliferative cells within the bone marrow of mice and humans, a model of hematopoiesis emerged which relied on a small population of self-renewing hematopoietic stem cells (HSCs). These multi-potent cells were able to give rise to progressively more lineage restricted, partially differentiated progenitors with reduced self-renewal capacity but increased proliferative activity, giving rise to mature blood cells. This led to a search for a multi-potent cell, which would serve as the origin of hematopoietic cancers, and the first “cancer stem cells” were thus identified [11, 12].

Recent studies have provided supporting evidence for the existence of CSCs in glioblastoma [13]. In addition to their tumor regenerating capacity, these cells have also been shown to be chemoresistant [14, 15] and radioresistant [16, 17]. Consequently, much of the ongoing GBM research is centered on better understanding how these cells contribute to the genesis of these tumors, recurrence, and how they might be specifically targeted.

Definition and source of neural stem cells and neural progenitor cells

The search for glioblastoma cells with stem-like properties built upon the knowledge gleaned from the study of neural stem cells (NSCs). NSCs are self-renewing multipotent cells with astrocytic features [18] that can generate most differentiated tissue components of the brain [19]. These cells were first discovered in the subventricular zone (SVZ) in mice [20]. When cultured in serum-free media supplemented with defined growth factors, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), NSCs grow in suspended cell aggregates called neurospheres, and self-renew. Upon exposure to differentiation signals such as serum, they can generate all of the different cell types within the adult brain. NSCs have been demonstrated in the human dentate gyrus [21], subcortical white matter [22] and the subventricular white matter [18, 23], which is the largest of these regions. Estimates number these cells as 0.77% of those within the SVZ, using Ki67 staining to identify mitotically active cells [23].

While it has been shown *in vitro* that NSCs can directly generate differentiated cells in the brain, these cells can also indirectly give rise to neurons, astrocytes and oligodendrocytes by creating fast cycling transit-amplifying progenitor cells. These progenitor cells were first demonstrated in the spinal cord of mice [24, 25]. Progenitor cells maintain proliferative ability similar to that of their precursor NSCs, but are committed to produce offspring of a neuronal or glial lineage only. There may be as many as ten million of these cells distributed throughout the brain, providing an ample reservoir of immature cells, which may be capable of malignant transformation (Figure 1, map of NSC and progenitor cell locations) [26].

Discovery and definition of GBM cancer stem-like cells

The discovery of multi-potent NSCs within the brain and their study has produced a better understanding of the possible cells of origin for GBM. It has also spurred a paradigm shift in the theories defining the mechanism for the generation and maintenance of the heterogeneous cell mass that constitutes a GBM. Since most GBM tumors occur late in life they are not considered developmental or congenital tumors; therefore, transformation of an

otherwise normal adult cell must occur to create the first pathological cell or tumor-initiating cell (TIC). Conceptually, GBM could arise through dedifferentiation of mature brain cells into more primitive cells or more directly from less differentiated cells. NSCs represent a population of cells from which the heterogeneous and aberrant cell populations found within GBM could be generated [27]. Early experiments were able to determine that there were in fact cells within gliomas which exhibited characteristics of normal NSCs, e.g. the ability to self-renew and generate a variety of progeny [28]. To isolate and grow these cells from excised patient GBM tumors, serum free media with growth factors are required, creating proliferating cell suspensions known as neurospheres. Interest in these cells grew considerably when it was shown that they carried genetic aberrations and could generate orthotopic tumor xenografts upon implantation in the brains of mice. The tumors engendered displayed more phenotypic similarity to the patient tumors from which they were derived and could be initiated from as little as 100 implanted cells [29] (Figure 1). The tumor cell populations identified in this way were defined as CSCs as they were shown capable of proliferation, self-renewal, and differentiation into cells of various lineages, [30].

One of the current challenges in the study of CSCs in GBM, is to distinguish them from the rest of the tumor cell population. Current studies focus on defining specific markers that will facilitate their identification, quantification in tumors, and isolation for experimental characterization. Early studies used the marker CD133 or the “side-population” method to purify CSCs. CD133 (or prominin I) is a transmembrane protein, which has been used as a marker for NSC and CSCs [31]. Initial studies delineated clear differences between CD133 positive and negative glioma cells, including a dramatic difference in tumor-forming capacity in xenografts [32]. While these studies suggested that CD133 may be necessary for CSC designation, further studies have shown that CD133 negative stem-like cells may also be tumorigenic [33, 34]. More recent studies suggest a high level of plasticity within this stem-like cancer cell population, as CD133 negative cell populations can produce CD133 positive cells after generating tumors in mice [35].

The “side population” method of sorting hematopoietic stem cells is based upon staining with the Hoechst dye 33342, which generates fluorescence in a subset of murine bone marrow cells, and was found to predict the expression of hematopoetic stem cell markers on these cells. This method has been used to identify GBM CSCs [36, 37]; however, recent evidence suggests that using this process does not effectively identify CSCs [38]. As CSCs form a small fraction of the GBM tumor bulk, many groups use a process of enzymatic and mechanical dissociation of patient GBMs, with subsequent re-suspension in stem cell media that favors the growth of CSCs [30]. As these methods are refined, we should expect that we will continue to learn even more about this population of CSCs and their origins.

GBM cancer stem-like cell niche

As much of GBM CSC research had its basis in NSC research, the location of a putative CSC “niche” was sought, in analogy to the subventricular zone where NSCs preferentially reside and self-perpetuate. Much research has been devoted to studying the local environment of NSCs, with the underlying premise being that the microenvironment plays holds the key to understanding how these cells retain their stemness, and there is definite evidence that heterotypic cell interactions with surrounding epithelial cells and associated factors play a significant part in their proliferation [39, 40]. Similarly, studies of GBM CSCs have shown they may be influenced by interaction with endothelium [41]. Co-culture experiments have shown that GBM CSCs preferentially associate with endothelial cells, proliferate more rapidly in the presence of secreted factors from endothelial cells, and more effectively produce orthotopic brain lesions when implanted with these cells [41]. One recent study examined the histopathological differences between single CD133+ tumor cells

scattered in different locations within the tumor mass versus those located in clusters in these vascular niches [42]. They found that the CD133+ cells within these vascular niches expressed more stem cell markers as compared to those single cells that expressed CD133. Nitric oxide produced by endothelial cells seems to support the stemness of CSCs in a Notch dependent manner [43] (Figure 2, Vascular niche). However, a separate body of literature suggests preferential proliferation of GBM CSCs in hypoxic environments [44]. In these studies, the hypoxia inducible factor (HIF) pathway is examined as an explanation of the resilience of the cells in these areas, with hypoxia increasing the proportion of CD133 positive cells grown in a culture of primary GBM cell lines in a HIF-2a dependent manner and HIF-1a being preferentially expressed in areas of necrosis [45, 46]. It remains unclear whether these two niches might provide similar stemness signals, or whether different populations of CSCs are present in the vascular versus the hypoxic niches, with the two areas exerting differing influences on the cells. The latter supports the idea that there may be two different stem cell niches which serve different functions for the tumor mass [42]. These niches may in fact be complimentary, in the sense that it is known that VEGF, whose production is increased in hypoxic areas in a HIF-dependent manner [47], stimulates the production of aberrant glomeruloid vessels with hyperplastic endothelial cells which may support the vascular niche.

Genesis of glioblastoma

The clonal origin of cancer postulates that tumors originate from the expansion of single cells having acquired sequential genetic and epigenetic alterations conferring them with the hallmarks of cancer, a series of phenotypic changes to normal cell physiology. The accumulation of multiple genetic mutations in a cell provide a survival advantage related to properties that include proliferation, evasion of growth suppression, and apoptosis [48]. Ongoing studies are focused on understanding what the inciting mutations might be in the genesis of glioblastoma, which are the cell(s) of origin and what the carcinogenic causes might be [49] (Figure 3, Cells of origin of GBM).

Under the stem cell hypothesis, the tumor-initiating cell (TIC) is the first genetically aberrant cell that can initiate the process of tumor development. The TIC may be responsible for the development of the bulk of tumor cells and over time can acquire sufficient alterations to engender a cancer stem-like cell (CSC). If we accept the tumor stem cell hypothesis, then there must be inciting events that generate these unique cells with significant expansion capacity and the ability to generate new tumors. To better understand the tumor initiating process, it is important to identify the cell(s) of origin for GBM subtypes and to define which are the early transformation steps that lead to the formation of TICs and their progression to CSCs (Figure 2). To address these important questions, two main approaches have been taken. First, it was hypothesized that comparison of gene expression signatures between CSCs and neural stem or progenitor cells in the brain might reveal similarities that may indicate cell lineage. A microarray study, which subdivided GBM into prognostic subgroups, showed that tumors with worst prognosis expressed markers of NSCs [50] such as Y-box protein 1 (YB-1) which is essential for normal brain development. YB-1 is highly expressed in the SVZ, is present in both NSCs and CSCs, and silencing YB-1 suppresses neurosphere growth and promotes differentiation [51]. Second, functional studies focus on experimentally testing the transformation potential of different genetic alterations on different cell types. The presence of a precursor lesion in a WHO Grade II astrocytoma of a patient with a germ-line p53 mutation was demonstrated using a p53 transcriptional assay in yeast [52]. One of the earlier studies linking neural progenitor cells to gliomagenesis demonstrated that rat oligodendrocyte-type-2 astrocyte progenitor cells, when transformed by c-myc and H-ras, created tumors which closely approximated human GBM when injected into rats [53]. Inducing the loss of p16 and p19 tumor suppressors in mice and transducing

constitutively active EGFR into their neural stem cells enables these cells to form GBM-resembling tumors when implanted in immunodeficient mice. Mature astrocytes with knockdown of p16 and p19 can also be dedifferentiated in serum free media with EGF, and these immature cells are also tumorigenic[54]. EGFR drives the acquisition of stem-cell like characteristics in these cells by inducing inhibitor of differentiation 3 (ID3)[55]. Another group was able to infuse PDGF into the subventricular zone of adult mice and induce what appeared to be early glioma formation with proliferation of the PDGFR-alpha positive NSCs located there [56]. Mice which lack p53 and have a conditional allele of the NF1 tumor suppressor gene that negatively regulates Ras signaling produce tumors with pathological features consistent with glioblastoma in regions of the brain containing NSCs [57]. We now have evidence that NSCs, glial progenitor cells, and mature astrocytes by dedifferentiation, could be the cell of origin of GBM.

New approaches to GBM treatment exploiting the CSC theory (Figure 4, Treatment strategies)

Current standard of care for GBM involves aggressive surgery, radiation and chemotherapy, yet provides only a modest survival benefit [2]. Most patients will eventually succumb to local recurrence of their tumor even if the patient undergoes a gross total resection. Making large inroads into the treatment of GBM will require new therapies designed to destroy the residual infiltrating tumor cells, which have the ability to reconstitute the entire initial tumor. Many in the neuro-oncology community believe the key to effective treatment of GBM will be directly targeting the CSCs within the tumor bulk which are resistant to standard therapies (Figure 3). Studies of neurosphere cultures and orthotopic xenograft tumors generated from these neurospheres have shown that these CSCs are radioresistant due to increased activation of DNA damage checkpoints [58, 59]. Enrichment of CD133+ cells was shown with radiation treatment, and inhibition of the Chk1 and Chk2 DNA checkpoint kinases removed the CD133+ survival benefit. In studying the HEDGEHOG-GLI1 signaling pathway, it was discovered that the most commonly used adjuvant chemotherapeutic agent, temozolomide does not block glioma CSC self-renewal [60], and this mirrors findings of multi-drug resistance in CSCs across tumor types [61]. Studies such as these highlight the need to develop new strategies, which target CSCs preferentially over therapies targeting the tumor bulk in order to prevent recurrence. To achieve this goal, a number of approaches are currently being tested.

Differentiation of GBM cancer stem-like cells (Figure 5, Differentiation pathways)

Bone morphogenic proteins (BMPs) are a group of growth factors, which have been shown to play an integral role in the differentiation of normal NSCs [62, 63]. This has led to efforts to understand the differences between the activity of BMPs on normal NSCs and CSCs. One such study showed that defects in the BMP receptor 1B function impaired astroglial differentiation [64]. As differentiation can reduce the self-renewing capacity of the CSCs, many studies have investigated various ways to affect this BMP-mediated pathway to induce CSC differentiation as a therapeutic approach. BMP4 was shown to reduce the ability of GBM neurosphere-derived cells to form xenografts in mice and, furthermore, infusion of BMP4 was able to prevent GBM cell growth and increased survival [65]. As noted above, YB-1 is highly expressed in the subventricular zone of mouse fetal brain tissues, and silencing YB-1 expression reduced growth of neurospheres derived from GBM patients by triggering differentiation [51]. Rapamycin triggers differentiation in GBM CSCs through mTOR inhibition, thus increasing the radiosensitivity of the CSCs and reducing their ability to form tumors in murine brains[66]. Rapamycin cotreatment with a PI3K inhibitor was

similarly found to promote differentiation and have a significant treatment effect [67]. In these ways and others, forcing differentiation of these CSCs may reduce their pathological potential.

GBM Stem-like cell Niche

Since the vascular niche may support the “stemness” of CSCs, many therapies aim to disrupt this relationship. Endothelial cells within the vascular niche express Notch ligands, which support the self-renewal capacity of CSCs. Inducing knockdown of these ligands from the endothelial cells decreases the propagation of CSCs in co-culture, so attacking these ligands could be a therapeutic option [68]. Bevacizumab, an anti-VEGF monoclonal antibody, has been shown to reduce aberrant blood vessel production in GBM and has been FDA-approved for the treatment of recurrent GBM [5]. Reducing the availability of supporting vessels could reduce the population of CSCs as well. While anti-angiogenic therapy has shown some promise, the success has been limited, and this may be due to the CSCs ability to create their own vascular niche. Recent studies have shown that many of the endothelial cells within GBM tumors contain the same genetic alterations as the malignant lesion, and orthotopic implantation of neurospheres in mice produced tumor xenografts with vessels composed of human endothelial cells [69]. It has been shown that treatment with bevacizumab or shRNA knockdown of VEGFR2 inhibits the maturation of tumor endothelial progenitors into vessels but does not prevent the differentiation of CD133+ cells into endothelial progenitors. However, inhibition of NOTCH1 signaling does prevent this vascular mimicry, which may lead the way toward future joint treatment [17].

Targeting Cancer Stem Cells

Ongoing attempts are underway to find signal transduction pathways that influence the tumorigenicity of CSCs that are unique to these cells, allowing treatments to be specifically targeted to these pathways [70]. IL-6, long believed to be implicated in multiple disease processes [71] and shown to be over-expressed in GBM [72] has led researchers to be interested in the signal transducer and activator of transcription 3 (STAT3), as this transcription factor can be activated by IL-6. STAT3 is constitutively active in some GBM cells, helping tumor cells resist apoptosis and promote local immunosuppression [9, 73]. The ubiquitous nature of STAT3 has made it a difficult target for therapeutics, but the bone marrow x-linked kinase (BMX), which associates with and activates STAT3, does show significantly increased expression in CSCs as compared to the bulk of GBM tumor cells. Furthermore, BMX shows minimal expression in normal tissues, making it an attractive therapeutic target [31].

Many groups are making use of nanotechnology to target CSCs. One group has used a virus-free, nanoparticle method of transfection to demonstrate significantly increased plasmid uptake in GBM CSCs as compared to normal fetal NSCs as a potential method of delivering therapeutic genes to these tumors [74]. Another group has treated GBM CSCs with iron oxide nanoparticles conjugated to an antibody against the glioblastoma specific target of EGFRvIII, promoting apoptosis in those cells [17], and increasing animal survival after convection-enhanced delivery (CED) treatment. Others have used curcumin nanoparticles to treat GBM CSC neurosphere cultures *in vitro* and demonstrated a reduction in clonogenicity and CD133+ cells [62].

Immunotherapy

Several groups are investigating both cell-based and antibody-mediated methods of treatment of GBM [75]. Given the tumor stem cell hypothesis, more investigators are turning these immune based therapies toward CSCs specifically. Recently, one group was

able to show killing of CSCs by lectin-activated NK cells and furthermore showed sensitivity of these cells to antibody-mediated cytotoxicity using cetuximab [76]. Another group has demonstrated that SOX6 is an effective human glioma antigen, capable of inducing SOX6 peptide-specific cytotoxic T lymphocytes, which showed efficacy in killing GBM CSCs [77]. The difficulty in advancing this treatment strategy will be continuing to identify cell-surface antigens which are specific to CSCs. Given the similarities between NSCs and CSCs, any treatments must be rigorously tested for toxicity to NSCs before they can be widely used.

Conclusion

The pathogenesis of GBM and the role of CSCs remain a controversial area in neuro-oncology. Fundamental questions remain as defining what it is, precisely, that defines a CSC, and defining once and for all which normal cell(s) within the brain is its precursor, which may vary based on GBM subtype, and determining the specific nature of the insults that induce the transformation process. In order to further target these cells effectively, we will need to determine what separates these cells from normal neural stem/progenitor cells within the brain, so that we can treat these cells without causing undue toxicity. As optimization of the retrieval and culture of these cells continues to evolve [30], along with the reliable creation of phenotypically accurate GBM orthotopic xenografts using neurospheres, we can expect that our understanding of and ability to treat these cells will grow significantly. It is our hope that making significant strides in the treatment of this small population of cells may make the difference that greatly increases the rates of progression free survival and overall survival in GBM patients.

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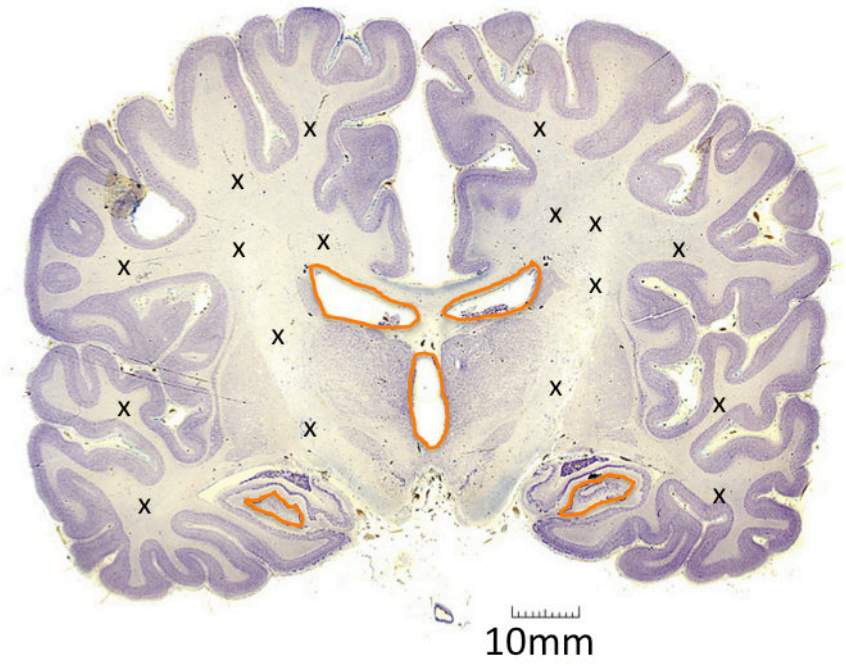

Figure 1.

Locations of multipotent cells within the brain. Orange areas denote regions that contain NSCs. X's show areas inhabited by glial progenitor cells (one group has also noted NSCs in these areas (22)) Coronal brain pathology slide provided by The Brain Biodiversity Bank at Michigan State University, with permission, funded by the National Science Foundation, https://www.msu.edu/~brains

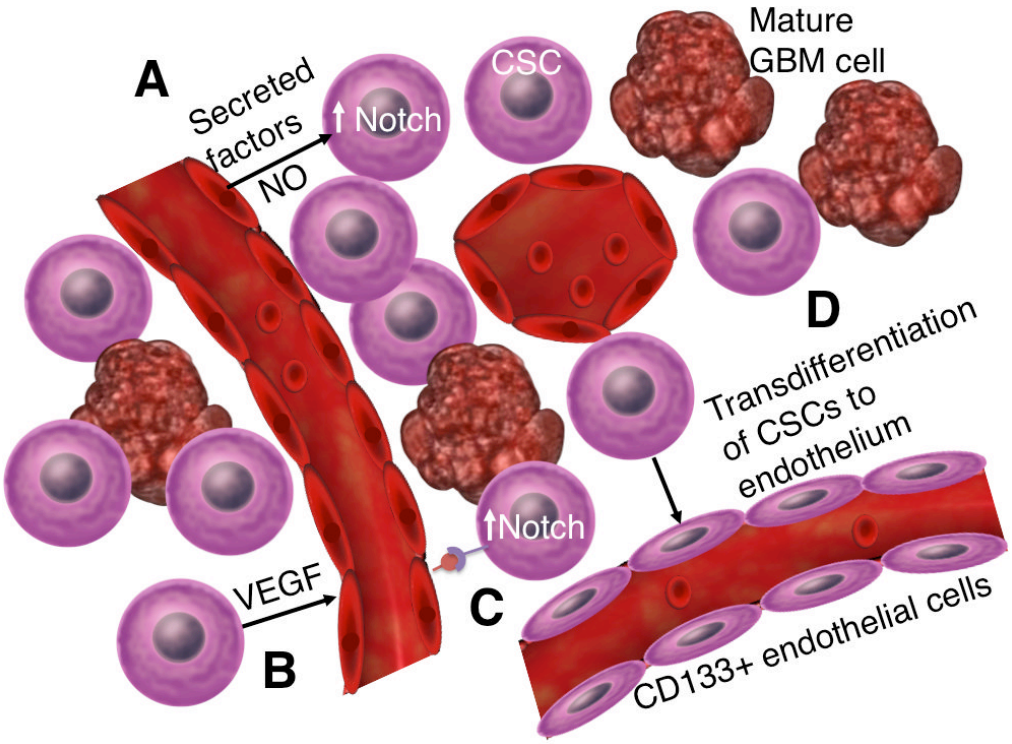

Figure 2.

This diagram illustrates the various symbiotic relationships between CSCs and its vascular niche. A. Endothelial cells have been found to secrete Nitrous Oxide among other factors which support the stemness of CSCs in a Notch-dependent manner. B. VEGF secreted by CSCs stimulates angiogenesis C. Endothelial cell Notch ligands also support the stem cell qualities of CSCs. D. CSCs may differentiate into CD133 positive endothelial cells, further enriching the vascular niche.

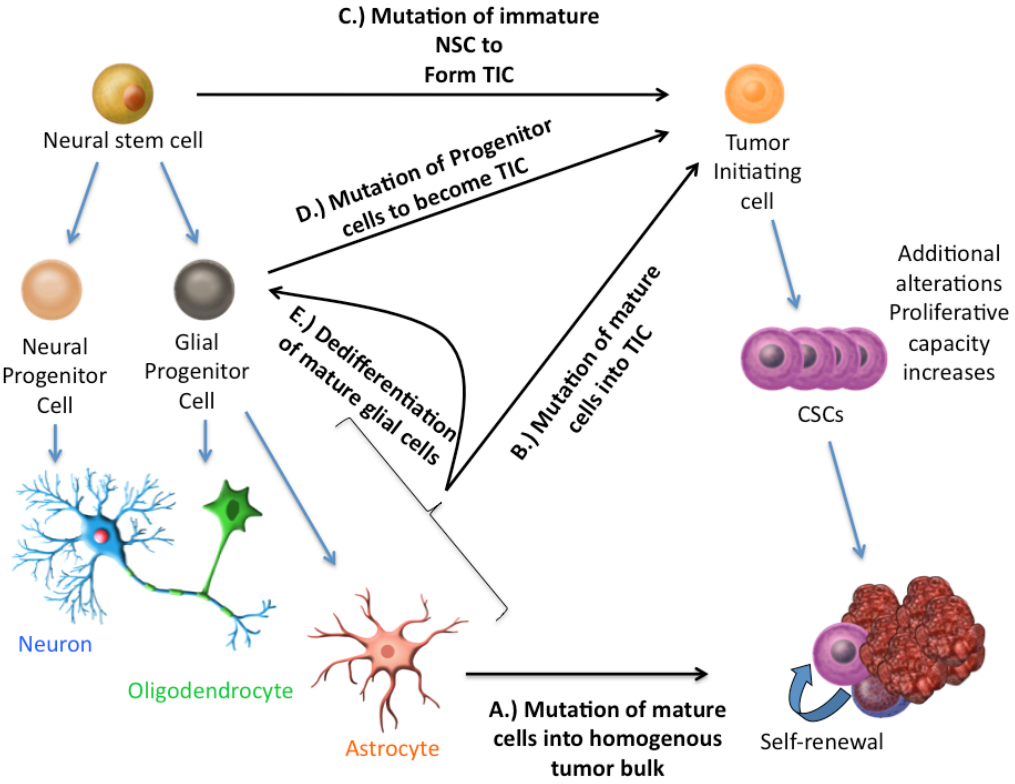

Figure 3.

An overview of the various theories of the genesis of GBM. Initially, it was thought that mature glial cells mutated to form the various equivalent tumor cells of the GBM A.), any of which would be capable of regenerating the mass. More recent theories for inciting events include B.) the mutation of mature cells into the TIC, C. the mutation of immature stem cells into a multipotent tumor initiating cell, which then develops into CSCs, D.) the mutation of the numerous glial progenitor cells into the TIC or even E.) the dedifferentiation of mature glial cells into a less differentiated state (progenitor cells) which then transforms into a TIC

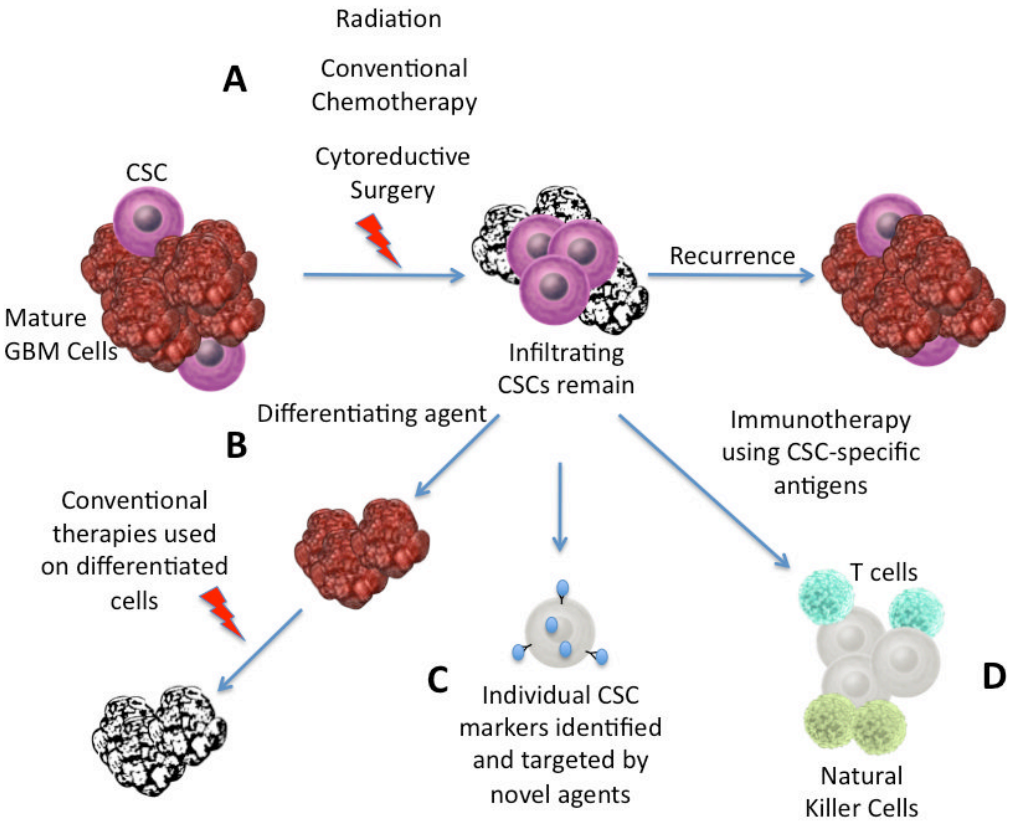

Figure 4.

Therapies targeting CSCs. A. Conventional therapies target the mature GBM cells, leaving the multipotent, self-renewing CSCs within the brain to cause recurrence. B. Differentiating the CSCs may enable conventional therapies to be more effective. C. Developing therapies targeted toward unique CSC markers may prevent these cells from evading treatment. D. Immunotherapy targeted at CSC specific surface antigens can use the body's innate defense mechanisms to kill these invasive cells

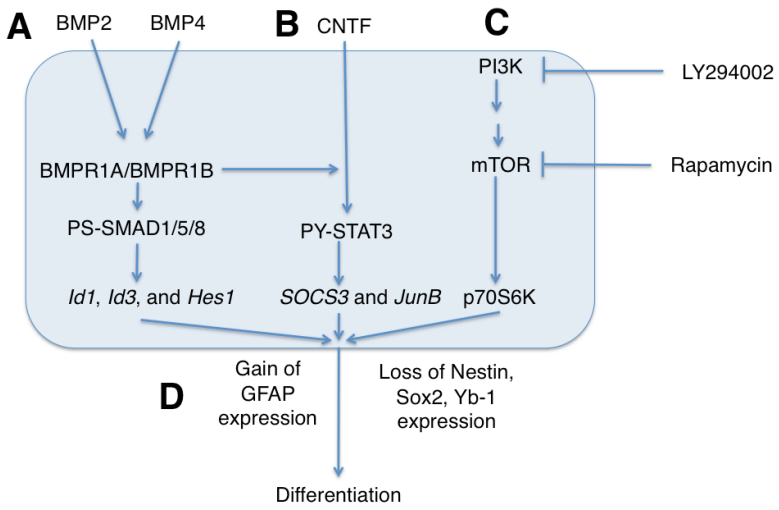

Figure 5.

Differentiation pathways of CSCs. A. The Bone morphogenic protein pathway, through the activation of the various bone morphogenic protein receptors increases the activity of PS-SMAD1/5/8, leading to an increase in *Id1*, *Id3* and *Hes1* expression, ultimately leading to differentiation. B. The STAT3 pathway is similarly activated by CNTF in a manner mediated by BMPR1B, also leading to differentiation. C. Inhibition of the mTOR pathway, either by direct inhibition with Rapamycin or by inhibition of the upstream PI3K pathway leads to decreased phosphorylation of p70S6K, also driving differentiation. D. As a result of the above pathways, the CSC differentiates, losing Nestin, Sox2 and Yb-1 expression and gaining GFAP