The role of cancer stem cells in glioblastoma

SWETHA J. SUNDAR, B.S.,¹ JASON K. HSIEH, B.S.,^{1,2} SUNIL MANJILA, M.D.,³
JUSTIN D. LATHIA, PH.D.,^{2,4,5} AND ANDREW SLOAN, M.D.^{1,3,5}

¹Case Western Reserve University School of Medicine; ²Cleveland Clinic Lerner College of Medicine;

³Department of Neurological Surgery, University Hospitals Case Medical Center; ⁴Department of Cellular & Molecular Medicine, Lerner Research Institute, Cleveland Clinic; and ⁵Case Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, Ohio

Recurrence in glioblastoma is nearly universal, and its prognosis remains dismal despite significant advances in treatment over the past decade. Glioblastoma demonstrates considerable intratumoral phenotypic and molecular heterogeneity and contains a population of cancer stem cells that contributes to tumor propagation, maintenance, and treatment resistance. Cancer stem cells are functionally defined by their ability to self-renew and to differentiate, and they constitute the diverse hierarchy of cells composing a tumor. When xenografted into an appropriate host, they are capable of tumorigenesis. Given the critical role of cancer stem cells in the pathogenesis of glioblastoma, research into their molecular and phenotypic characteristics is a therapeutic priority. In this review, the authors discuss the evolution of the cancer stem cell model of tumorigenesis and describe the specific role of cancer stem cells in the pathogenesis of glioblastoma and their molecular and microenvironmental characteristics. They also discuss recent clinical investigations into targeted therapies against cancer stem cells in the treatment of glioblastoma.
(<http://thejns.org/doi/abs/10.3171/2014.9.FOCUS14494>)

KEY WORDS • glioblastoma • cancer stem cells • pathophysiology •
treatment • clinical trials

GLIOMASTOMA (GBM) is the most common primary malignant brain tumor. It is extremely aggressive, with a median overall survival (OS) of less than 15 months after diagnosis even with maximal therapy.⁹⁵ Survival rates are dismal, ranging from 26% to 33% for 2-year survival and less than 5% for 5-year survival.^{49,53,96} The standard first-line treatment includes resection, if possible, followed by concurrent radio- and chemotherapy, typically temozolomide (TMZ), and then 6–12 months of adjuvant TMZ.^{38,68} Despite treatment, recurrence is nearly universal.⁹⁶ Glioblastoma demonstrates a great deal of phenotypic, morphological, and cellular heterogeneity and is thought to contain a population of self-renewing cancer stem cells (CSCs) that contributes to tumorigenesis and treatment resistance. Both intratumoral heterogeneity and the presence of these CSCs may contribute to the treatment-resistant nature of GBM and its propensity to recur in patients.^{15,70}

Abbreviations used in this paper: CSC = cancer stem cell; GBM = glioblastoma; HIF = hypoxia-inducible factor; NSC = neural stem cell; OS = overall survival; PFS = progression-free survival; SHH = sonic hedgehog; TGF- β = transforming growth factor- β ; TMZ = temozolomide; VEGF = vascular endothelial growth factor; VEGFC = VEGF C.

History and Definition

The concept of CSCs originated in 1994 with the observation that a fraction of cells in human acute myeloid leukemia can self-renew and reconstitute both the leukemic cell hierarchy and the clinical disease state in vivo after xenotransplantation.^{1,11,49,99} These self-renewing cells were later discovered to exist in various solid tumors as well, including those of the breast, colon, lung, brain, and liver.^{1,20,71,81,92} The CSC hypothesis proposes that individual tumors comprise a cellular hierarchy. Cancer stem cells reside at the apex of the hierarchy and possess the ability to self-renew and to divide to give rise to the variety of cells that populate a tumor. As tumor cells differentiate, their ability to self-renew is reduced and they lose their “stemness.” The hierarchy is dynamic with respect to cell type (CSCs, non-CSCs) and is maintained by the balance between self-renewal and differentiation.^{43,107} Viewed in this light, tumors can be thought of as aberrant organs comprising heterogeneous cell types derived from CSCs rather than simply an accumulation of diverse neoplastic clones.

Researchers who made the initial attempts to define CSCs described a qualitatively distinct population of pathological cells that was able to self-renew and ir-

reversibly and deterministically differentiate, resulting in the constitution of a tumor composed of phenotypically diverse cells. However, although physiological differentiation is thought to be an irreversible process, cells have been known to dedifferentiate in pathological conditions.^{22,90} Complicating the CSC paradigm, it has also been demonstrated that the differentiation of CSCs is not unidirectional. Stimuli such as hypoxia and acidic stress, as well as therapeutic agents such as TMZ, can induce some non-CSCs to adopt a CSC phenotype.^{5,16,39,60}

Furthermore, research on CSCs has failed thus far to discover universally informative biomarkers, mutations, or gene-expression patterns.³ Biomarkers used to identify and enrich CSCs have been shown to exhibit variable cell cycle-dependent expression.^{45,97} Initially CSCs were thought to be rare, but their frequency has been reported to vary among different cancers, and they may be quite common in some tumors.⁷⁹ Given the apparent ability of tumor cells to move in either direction along the tumor hierarchy (toward both differentiation and dedifferentiation), the highly variable molecular characteristics of CSCs, and their potential to change phenotype in response to internal and external signals, it is important to view CSCs as dynamic entities shifting fluidly among different molecular and functional states. It is also important to recognize that CSCs need not originate from aberrant stem cells and that different tumors may arise from stem cells or restricted-lineage multipotent precursors, whereas others may arise from nonstem cells or more than one cell type.

The current definition of CSCs is not universally agreed upon by researchers, but the working definition encompasses characteristics that CSCs are generally believed to possess: CSCs are oncogenic in their host or immunosuppressed xenograft recipients, they proliferate, they self-renew, and they are able to differentiate to give rise to heterogeneous populations of cells that make up the bulk of solid tumors.^{3,80}

The concept of CSCs and intratumoral hierarchy seems, at first glance, to be in opposition to the stochastic model of tumor growth, which suggests that tumor expansion is driven by the clonal evolution of acquired genetic mutations. In the CSC model, a dynamic population of cells (CSCs) is primarily responsible for tumor initiation, propagation, and maintenance, whereas in the stochastic model, many clones possess relatively equal levels of tumorigenicity. In 1988, Cavenee and coworkers, in the paper James et al.,⁴⁶ proposed dual models of GBM development, in which most GBMs represent the common final end point for progression from a variety of subtypes of lower-grade gliomas, whereas some GBMs arise spontaneously from single critical mutations (such as the loss of heterozygosity of chromosome 10q). However, it is important to note that these models are not mutually exclusive. For instance, CSC populations have demonstrated enhanced chromosomal instability, possibly highlighting a role for clonal evolution in their propagation.⁷² Conversely, the critical ability of CSCs to initiate and propagate tumors indicates the possibility of spontaneous formation of GBM through the acquisition of critical mutations leading to the development of

a CSC phenotype. The authors of another recent study suggested that interactions between multiple clones, including a subpopulation of cells that drove tumor growth by inducing microenvironmental changes, were critical to the tumor phenotype.⁶⁵ In reality, neither the CSC model nor the stochastic model is likely to exist in isolation, and the true mechanism of tumor formation lies somewhere between them.⁸⁰ These 2 models should not be thought of as necessarily exclusive but, instead, as complementary forces in tumorigenesis (Fig. 1).

Culture Conditions

Multiple difficulties exist when researchers attempt to analyze CSCs, among which is the unsuitability of standard culture conditions for CSC maintenance. Experiments on CSCs must demonstrate the cells' ability to establish a cellular hierarchy of both tumorigenic and nontumorigenic cells. However, standard culture conditions incorporating serum induce irreversible differentiation and loss of tumorigenicity in CSCs, as well as gene-expression patterns that diverge from those of the original tumor.^{23,25,57,85} The derivation of free-floating tumorspheres in stem-cell cultures^{57,91,92} without serum allows for the enrichment and study of CSCs but precludes side-by-side comparison with nontumorigenic cells and establishment of the cellular hierarchy. Furthermore, CSCs and nonstem tumor cells do not exist in isolation *in vivo* but, rather, are informed by the tumor microenvironment and cross-talk between cell types.^{13,39,52,64}

The establishment of tumorigenicity and cellular hierarchy is best assessed through the use of patient-derived xenografts, preferably after as few cell passages and as little time in culture as possible. Immunodeficient animal recipients are used most often, and xenograft assays putatively allow for recapitulation of the tumor microenvironment and cell-cell signaling found in human conditions.⁵⁷ However, xenografts typically require an immunocompromised host, which fails to replicate the immune system component present in native human patients. The xenograft condition may alter tumorigenicity, possibly artificially depressing the measured number of tumor-propagating cells by the introduction of a non-native environment or possibly inflating the number of tumor-propagating cells or altering the cellular hierarchy as a result of the absence of immune interactions with the tumor xenograft.^{52,103}

Glioblastoma and Cancer Stem Cells

Glioma cells were first grown under CSC conditions by Ignatova et al. in 2002,¹¹⁴ and further investigations subsequently demonstrated that GBM CSCs contributed to tumor maintenance and propagation,^{29,92,108} as well as treatment resistance.^{7,18,62} *In vitro*, GBM CSCs form neurospheres and demonstrate self-renewal capabilities, and when grown as *in vivo* xenografts, GBM CSCs form heterogeneous tumors that resemble the original parent tumor.^{29,92}

In addition to meeting this functional definition, GBM CSCs share much in common with neural stem

Fig. 1. Upper: The stochastic model suggests that tumor growth is driven by the clonal evolution of acquired genetic mutations and that many clones possess comparable levels of tumorigenicity. **Lower:** The CSC model suggests that there exists a self-renewing population of cells (CSCs) responsible for tumor initiation, propagation, and maintenance. These CSCs may originate from a mutated progenitor stem cell or from a more differentiated cell in the lineage that dedifferentiated to acquire stem-like properties.

cells (NSCs), although questions still exist about the true origin of CSCs.^{27,61} The similarity of the gene-expression profiles of GBM CSCs and NSCs provides support to the idea that CSCs are malignant variants of NSCs.^{43,113} There are many common pathways between CSCs and NSCs that are involved in neural development, including Notch, Wnt, and transforming growth factor- β (TGF- β) signaling.^{32,89} Oligodendrogenesis relies on platelet-derived growth factor (PDGF); increased PDGF signaling has been demonstrated to cause abnormal NSC proliferation and glioma formation.^{40,44,56} Putative CSCs selected using common NSC markers have demonstrated the ability to form orthotopic tumors in nude mice that more closely resemble human GBM than could isolated cells lacking these markers.^{22,91,92} Compared with the remaining tumor cells, CSCs are thought to be metabolically unique because of data demonstrating epigenetic DNA changes and a role for microRNAs in regulating gene expression.^{32,89} Despite evidence for shared signaling pathways, gene expression, and biomarkers between NSCs and CSCs, it still is not clear whether GBM CSCs originate from NSCs that mutated to acquire tumorigenicity or if they stem from mature cells that dedifferentiated and acquired the ability to self-renew.

CD133 is the best-studied CSC biomarker and is often used experimentally to identify and enrich tumor-propa-

gating and -initiating cells. Also known as prominin 1, CD133 is associated with normal NSCs and is expressed during embryonic development.^{21,76,109} In seminal experiments, tumor cells isolated from GBM that grew neurospheres in serum-free medium (indicating self-renewal capabilities) and grew tumors phenotypically similar to GBM were found to be CD133-positive, whereas tumor cells that lacked CD133 expression did not demonstrate self-renewal or tumorigenicity in xenotransplantation studies.^{22,50,91,92} Short hairpin RNA (shRNA) knockdown of CD133 in putative CSCs resulted in the loss of both of these properties, and after reexpression of CD133 in the same cells, the CSCs' maintenance ability and tumorigenicity returned.⁴ Despite the evidence outlining its crucial relationship with CSCs, CD133 is not a universal marker for identifying CSCs. Several studies have demonstrated that GBM cells that are CD133 negative are still capable of tumor initiation, and some GBM tumors do not contain any CD133-positive cells.^{9,14,30,63,95,101,105,111} It has been proposed that tumor-initiating CD133-negative cells may, in fact, actually express CD133 at low levels below experimental thresholds. In one study, CD133 demonstrated cell cycle-dependent expression, in which CD133-negative cells were found mostly to reside in the G₀/G₁ stage.⁴⁵ The subtleties of this relationship remain unclear, and the essential role of CD133 in CSC maintenance remains an

area of investigation. Microenvironmental interactions, such as those mediated by CD15 and local growth factors, may complement the function of CD133 in CSC maintenance and preserve stemness in CSC populations expressing very low or cell cycle-dependent levels of CD133.^{9,50}

CSCs show increased expression of SOX2, a transcription factor associated with multipotency via the TGF- β signaling pathway, which also promotes the self-renewal of CSCs.^{12,31,42} CSCs also typically have increased expression of Nestin, an intermediate filament seen in NSCs, although it is a better marker in mouse tumors than in human GBM.^{41,42} Integrin $\alpha 6$ is another biomarker that is highly expressed in CSCs, and silencing it through shRNA knockdown renders CSCs unable to self-renew or grow tumors.⁵¹ Epidermal growth factor receptor is expressed in more than 50% of patients with GBM and may increase tumorigenicity and activate the characteristics of CSCs that promote treatment resistance.^{47,66}

Additional biomarkers that have been studied in GBM include CD15, CD36, A2B5, L1CAM, CD44, and CXCR4.^{2,6,24,35,47,51,73,78,95} Although these markers are useful in furthering our understanding of CSC function and regulation and may be involved in targets for therapies against CSCs, no single marker can definitively identify or define CSCs (Table 1).

Tumor Niches/Microenvironments

Normal NSCs reside in particular anatomical regions known as niches, a microenvironment comprising somatic cells and the extracellular matrix.⁸⁷ The relationship between stem cells and these niches is not passive, and stem cells do not exist in a vacuum. Rather, NSCs interact dynamically with their microenvironment.⁸⁶ They actively influence their microenvironments and, in turn, are regulated by signaling from that same microenvironment.⁸⁶ Similarly, CSCs also exist in specific niches that play a role in the regulation of tumorigenicity. The microenvironment not only plays a role in helping to maintain CSCs and the tumor but can also affect response to

therapy. The tumor microenvironment and the CSC niche is an active area of investigation, and CSCs are thought to occupy, among others, perivascular, hypoxic, and necrotic niches, as well as tumor border regions, which affects the invasive properties of GBM.^{17,34,98,102}

Perivascular Niche

Perhaps the best established tumor niche for GBM stem cells is the perivascular niche.¹⁴ Many stem cells tend to be located close to the endothelial cells that line capillaries, particularly in the subventricular zone and the hippocampus.^{28,48,69,74,82,84,88} These niches contain vascular factors that seem to regulate stem cells. These endothelial vascular factors have not precisely been elucidated; however, studies suggest the involvement of vascular endothelial growth factor C (VEGFC) and brain-derived neurotrophic factor (BDNF).^{54,58} NSCs are not just influenced by the surrounding environment; they also actively regulate it by secreting VEGF and BDNF to promote angiogenesis and contribute to this dynamic, cyclical relationship.^{59,86}

Studies have shown that GBM contains abnormal perivascular niches, and histologically, highly disorganized vasculature is characteristic of this tumor. Abnormal vascularity was thought to occur in response to a rapidly growing tumor; however, the truth may be that these aberrant vascular niches are critical for maintaining the CSC population.^{7,13,26} It has been shown that the vascular density of GBM correlates with the amount of CSCs and even the patient's prognosis.^{13,87} Bevacizumab, an anti-VEGF antibody, is often used as part of salvage therapy for patients with GBM.¹⁰⁰ The interaction of CSCs with endothelial cells promotes activity in critical stem pathways such as Notch signaling, which contributes to their self-renewal abilities.¹¹² The CD133-positive CSCs are shown to express greater levels of VEGF, which leads to increased tumor vascularity over that of CD133-negative cells.⁸ CSCs may even be capable of differentiating into cells that functionally resemble pericytes, supplying the raw material necessary to continue supporting the perivascular niche.¹⁷

Hypoxic/Necrotic Niche

In addition to aberrant vasculature, GBM is histologically known to contain areas of intratumor necrosis that are surrounded by a rim of densely packed tumor cells, known as pseudopalisading necrosis.⁸³ These areas are believed to be another niche for CSCs to demonstrate increased self-renewal and differentiation that result from the hypoxia in the environment.⁶⁷ Oxygen levels drop further from vessels because of rapid uptake,⁷⁵ and hypoxia-inducible factors (HIFs) play an important role in embryonic stem cells and NSCs for promoting proliferation.^{34,67} Hypoxia has also been demonstrated to upregulate VEGF in CSCs and increase angiogenesis.³⁶ Hypoxia-induced activation of hypoxia-inducible factor 1 α (HIF-1 α) promotes self-renewal in CD133-positive glioma-derived CSCs, resulting in expansion of the CSC population, whereas the knockdown of HIF-1 α , or inhibition of the phosphoinositol 3-kinase (PI3K)-Akt or extracellular signal-regulated kinase 1/2 (ERK1/2) pathways, reduced this effect.⁹⁴ Hypoxia-

TABLE 1: Selection of molecular markers significant in the study of GBM CSCs

Authors & Year	Marker*
Singh et al., 2003 ⁹¹	CD133 (prominin 1)
Ogden et al., 2008 ⁷³	A2B5
Bao et al., 2008 ⁶	L1CAM
Gangemi et al., 2009 ³¹	SOX2
Ehtesham et al., 2009 ²⁴	CXCR4
Son et al., 2009 ⁹⁵	CD15 (SSEA-1, Lewis X)
Anido et al., 2010 ²	CD44
Lathia et al., 2010 ⁵¹	Integrin $\alpha 6$
Hale et al., 2014 ³⁵	CD36

* Markers are presented in chronological order of their discovery. Because of space limitations, we are unable to discuss all the significant markers or all the early studies in which the selected markers were identified.

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inducible factor 2 α and its target genes were also found to be preferentially expressed in GBM CSCs, and HIF-2 α has been found to colocalize with CSC markers.⁶⁰ Studies suggest that hypoxia may have the ability to induce CD133 expression and Notch signaling in CSCs, both of which are important for self-renewal. The pseudopalisading regions have shown CD133-positive immunoreactivity as well, further supporting the theory that these hypoxic and necrotic niches are involved in supporting CSCs.³⁷

Invasion/Tumor Edge Niche

The tumors seen in GBM are aggressive and highly invasive of surrounding brain tissue, forming large necrotic and hemorrhagic cavitations that can be several centimeters wide. The outer edge of the tumor and its invasive properties are believed to constitute another niche for CSCs. At the tumor boundary, TGF- β secreted from tumor macrophages may stimulate CSCs to expand the tumor by invasion of surrounding normal parenchyma.¹⁰⁶ Astrocytes also may play a role in GBM invasion through the activation of matrix metalloproteinases (MMPs) and initiating the sonic hedgehog (SHH) signaling pathway.^{19,55} MMPs may help impair the integrity of the surrounding normal parenchyma and its matrix, whereas SHH is known to promote the self-renewal of stem cells.¹⁹ GBM invasion is also regulated by the chemokine receptor type 4 (CXCR4), which is increased in CSCs.^{24,110} CXCR4 can help attract CSCs to nearby endothelial cells, reinitiating the cycle of invasion, tumor growth, and endothelium proliferation.^{24,36}

Cancer Stem Cells as a Therapeutic Target

That GBM recurrence is nearly universal with little improvement over 3 decades suggests that the approach to treatment needs to be fundamentally reevaluated. To develop effective treatments for this lethal tumor, there needs to be greater understanding of the CSC drivers of hierarchical GBM cell populations and how these cells

survive, proliferate, differentiate, and regulate their local environment. Such knowledge will likely lead to therapies targeting GBM CSCs.

CSCs are intriguing targets for GBM therapy, because they tend to be resistant to therapy and their cellular properties give them the ability to overcome our current treatment strategies. Recurrence is thought to occur when CSCs are left behind or not killed during treatment, because they are able to reinitiate tumor formation.¹⁶ An ideal GBM treatment that targets CSCs would distinguish between NSCs and CSCs and selectively eliminate only the CSCs.

Resistance of GBM to chemotherapy has been well studied, and there are several mechanisms by which treatment resistance may occur.^{103–105} CSCs are postulated to have intrinsic resistance to chemotherapy, and CD133-positive CSCs have demonstrated increased transcription of several antiapoptotic genes.⁶² There can be active transporters on cell membranes that will pump the chemotherapy drugs out of the cell, which reduces the medication's effectiveness.¹⁰ Parada and coworkers, in the paper Chen et al.,¹⁶ showed that Nestin-positive CSCs survived TMZ treatment and maintained the ability to regenerate tumors. Only when these Nestin-positive CSCs were specifically targeted did the tumor reformation cease.¹⁶ CSCs may also possess an increased ability to repair DNA, because CD133-positive CSCs were observed to activate kinases that made them resistant to apoptosis.⁸

Ongoing and Previous Clinical Trials

Given their critical role in tumor initiation, propagation, and maintenance, CSCs offer an attractive therapeutic target (see Table 2 for a summary of clinical trials). Immunotherapy with dendritic cells has been studied as treatment for many types of cancers, including gliomas. In an animal study conducted by Xu et al.¹⁰⁴ in 2009, a dendritic cell vaccine was created against tumor-associated antigens specific to CSCs. The results showed that such

TABLE 2: Selected clinical trials targeting CSCs for the treatment of GBM

Authors & Year or Clinicaltrials.gov Identifier	Trial Design	Agent (target)	Outcomes
Phuphanich et al., 2013 ⁷⁷	Phase I, single arm	ICT-107, an autologous dendritic cell–pulsed vaccine (tumor-associated antigens overexpressed on CSCs)	21 total patients; nontoxic; 33% immunological response rate; nonsignificant trend toward increased PFS, but not OS, for vaccine responders
Sloan et al., 2014 ⁹³	Phase 0/II, randomized	vismodegib (SHH pathway)	40 total patients; well tolerated; no difference in 6-mo PFS or OS time as single agent; achieved therapeutic intratumoral concentration; decreased SHH signaling; decreased CSC proliferation & self-renewal
NCT01280552	Phase II, randomized	ICT-107 (tumor-associated antigens)	in progress
NCT01122901	Phase II, nonrandomized	RO4929097 (g-secretase, Notch signaling pathway)	in progress
NCT01119599	Phase I, single arm	RO4929097 in combination w/ TMZ & radiotherapy (g-secretase, Notch signaling pathway)	in progress
NCT01189240	Phase I/II, randomized	RO4929097 w/ bevacizumab (g-secretase, Notch signaling pathway)	in progress

a vaccination induced cytotoxic T lymphocytes against CSC antigens, and in the 9L rat glioma model, survival was increased.¹⁰⁴ In 2012, a Phase I vaccination study of GBM treatment in humans was completed by Phuphanich et al.,⁷⁷ who used vaccines that targeted tumor antigens highly specific to CSCs. The vaccine developed was non-toxic, and for the 16 patients who received it, the median progression-free survival (PFS) time was 16.9 months and the median OS time was 38.4 months. A Phase II randomized clinical trial for this vaccine is currently ongoing (Clinicaltrials.gov identifier NCT01280552).

Another approach to GBM therapy may be to target signaling pathways critical to CSC renewal and proliferation (such as SHH or Notch) with small-molecule inhibitors.⁹² GDC-0449, known as vismodegib, can inhibit SHH signaling, and a recently completed Phase II clinical trial (Clinicaltrials.gov identifier NCT00980343) demonstrated that the drug reached the tumor, inhibited stemness, and downregulated the SHH signaling pathway, although there was little improvement in PFS or OS time with its use as a single agent.⁹³ A new trial targeting both the SHH and another metabolic target is currently in development (A. E. Sloan, L. Rogers, D. Peerboom, J. Barnholtz-Sloan, and M. Couce, Ohio Neuro-Oncology Collaborative, unpublished data, 2014). Similarly, Notch signaling is important for CSC self-renewal, and there are Phase I and II clinical trials currently investigating whether small-molecule inhibitors against this pathway can help treat GBM (Clinicaltrials.gov identifiers NCT01122901, NCT01119599, and NCT01189240). It has been demonstrated that the SHH and phosphatase and tensin homolog (PTEN) signaling pathways have a synergistic relationship in tumor proliferation, and there is interest in targeting both of these pathways simultaneously with small-molecular inhibitors. In vitro and in vivo tests have already demonstrated reduced GBM growth, so this may be a viable option for treating humans.³³

Conclusions

In conclusion, CSCs are dynamic entities with fluid molecular characteristics, functionally defined by self-renewal, differentiation, and tumorigenicity. Cancer stem cells contribute to GBM propagation and have inherent properties that render them resistant to current treatment options. Given their importance in these processes, novel investigation of targeted anti-CSC agents is a therapeutic priority for treating this deadly tumor.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Sloan, Manjila. Drafting the article: Sundar, Hsieh. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Sloan.

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Manuscript submitted August 15, 2014.

Accepted September 19, 2014.

Please include this information when citing this paper: DOI: 10.3171/2014.9.FOCUS14494.

Address correspondence to: Andrew Sloan, M.D., Department of Neurological Surgery, University Hospitals Case Medical Center and Case Comprehensive Cancer Center, 11100 Euclid Ave., HAN 524, Cleveland, OH 44106. email: andrew.sloan@uhhospitals.org.