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Review article

Role of cancer stem cells in brain tumors

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ABSTRACT

Cancer stem cells contribute to tumor progression, resulting in their capacity to persistently self-renew and propagate tumors. Recent evidence suggests that brain cancer stem cells (BCSCs) are critical for tumor vascular development and therapeutic resistance. Here, we outline the crucial molecular mechanisms and interacting niches involved in BCSCs, which uncovering multiple potential targets for malignant brain tumors and may provide clues for developing novel antibrain tumor treatments.

1. Introduction

Both adults and children can experience malignant brain tumors, e.g., glioma, medulloblastoma, and ependymoma, yet current translational medicine has not resulted in significant improvement in survival. Glioblastoma (GBM) is the most frequent adult primary brain tumor and has an extremely poor outcome with only a median survival of 15 months [1]. Brain tumors, predominantly medulloblastomas, are comprised of a heterogeneous group of tumors and also a leading cause of cancer death in children. Even though therapies for primary tumor response have improved, these malignancies recur most of the time, and newer treatment modalities are urgently needed to target brain tumors. Based on extensive studies of brain tumors, it appears that targeting the regulatory signaling pathways, tumor microenvironment, and characterized stem cells form the basis for future development of targeted therapies [2–4]. Brain cancer stem cells (BCSCs) or brain tumor initiating cells (BTICs) belong to a sub-population of cells that possesses capacity for self-renewal, multipotency, and tumor

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propagation and has been attributed to increased angiogenesis and drug resistance [5–13]. There is mounting evidence that unveiled the molecular actions of BCSCs, leading to a significant number of potential targets. In this review, we will discuss the role of BCSCs in initiation and propagation in brain tumor as well as how to targeting brain tumor by directly or indirectly inhibiting BCSCs. Moreover, we will also highlight the niche of BCSCs, particularly in neurovascular interactions, as target for brain tumor therapies.

2. Cancer stem cells in brain tumor

2.1. *Characteristics and stem cells for malignant brain tumor*

Primary brain tumors (PBT) contain malignant heterogeneous groups that originate and arise from the brain and the central nervous system (CNS). According to the World Health Organization classification, the most prevalent PBT in elderly is gliomas, and the most malignant type of gliomas is grade IV, also known as glioblastoma multiforme (GBM) [14]. Both aggressive primary GBM and secondary glioblastoma multiforme as a result of a low-grade glioma progressing into highly malignance have very poor prognosis even with radiotherapy and chemotherapy [15]. More importantly, GBM possesses tissue-scattering distribution pattern accompanied by extensive diffusion within the brain, making it difficult for surgical resection [16]. In children, the highest incidence of brain tumor is medulloblastomas, which is commonly developed from the malignant transformation of progenitors of the external granular layer in the cerebellum, and is by far the most aggressive form of pediatric cancer with poor outcome. Compared with adult glioma, medulloblastoma is thought to develop from an embryonal tumor and shown to express several genes which involved in differentiation of neural stem cells, including Sox2, Bmi1, and Musashi 1[17,18].

The concept of cancer stem cells (CSCs) was first hypothesized in the studies of acute myeloid leukemia [19,20] and subsequently found in solid tumors, including brain tumors. Several groups have identified and characterized CSCs in clinical samples from patients with glioma and medulloblastoma [5,17,21–23]. Stem cells possess multipotent capacity to generate different types of mature cells in the tissue origin. Importantly, the key property of stem cells is the ability to self-renew to maintain a constant cell number in adult tissues throughout life. Stem cells acquire self-renewal capacity by executing asymmetric divisions to reliably reserve a copy of the mother cells, while producing mature progenitor simultaneously. Cancer stem cells have been proposed to arise from mutation in normal stem cells, and subsequently grow and differentiate to generate primary tumors. Similar to normal stem cells, cancer stem cells are able to self-renew, develop heterogeneous populations of daughter cells, and proliferate extensively [24].

2.2. *Enrichment of BCSCs*

Isolation CNS cancer stem cells in specimens by purification of $CD133^+$ cells from human glioblastoma and medulloblastoma allows generation of neurospheres and growth of tumor stem cell populations [23,25]. Neurospheres can be repeatedly separated into single cells, and these single cells can produce a new neurosphere. Evidence of self-renew is commonly observed from the ability of single cells to repeatedly produce neurospheres [26]. Additionally, as few as 100 $CD133^+$ human glioma cells which were transplanted into the brain of severely immunodeficient (NOD-SCID) mice developed gliomas, whereas no tumors formed from transplantation of 10^5 $CD133^-$ cells from the same tumor [5]. Subsequent studies revealed controversial findings that identified glioma stem cells as CD133 negative [27,28]. This controversy might be caused by the use of distinct methods and techniques to detect CD133 and factors that are able to affect its detection [28]. Albeit the contradiction, CD133 continues to be a frequently used marker for BCSCs, followed by several other BSCS markers, such as A2B5 [29], stage-specific embryonic antigen (SSEA-1/CD15) [30], L1CAM (CD171) [31], aldehyde dehydrogenase 1 (ALDH1) [32], integrin α6 (CD49f) [33], CD44 [34], and epidermal growth factor receptor (EGFR) [35].

Recently, Clement and colleagues [36] attempted an alternative method by utilizing intrinsic autofluoresce properties and distinct morphology to isolate human glioma stem cells without using molecular markers. For example, subpopulations of human gliomas with tumor initiating activities were identified by autofluorescence emission at 520 nm after excitation at 488 nm. Because the differences in marker expression or enrichment capacity of BCSCs varies from one laboratory to another, it is important to standardize the method of cell sorting by flow cytometric analysis for easier comparison of methods and data from different groups [37].

3. Niche of BCSCs

Gliomas appear to be highly vascular, and endothelial cells, pericytes, and astrocytes have been shown to serve as the functional unit for neurovasculature to foster tumor growth. Staining of BCSC markers and tumor vasculature from glioblastoma specimens showed a physically colocalized pattern, which appears in the angiogenic regions within glioblastoma [2,9]. Neural stem cells (NSCs) also share a defined vascular niche with medulloblastoma CSCs [9,38,39]. BCSCs are found adjacent to the neurovasculature in brain tumors, suggesting that the existence of molecular signaling and microenvironmental factors in the specialized perivascular niche make significant contributions to maintain BCSCs. Additionally, self-renew and proliferation of BCSCs can be promoted by tumor endothelial cells such that simultaneous injection of both CSCs and endothelial cells accelerates tumor initiation and progression [40], indicating that cell-to-cell signaling within perivascular niche is important to brain tumor development.

The maintenance of NSCs depends on their interactions with the extracellular matrix (ECM) [41], implying that ECM has a vital role in perivascular niche to regulate the maintenance of BCSCs. Although the components of the ECM have not been defined in gliomas perivascular, different groups have reported the expression of several laminin chains, including α2, α3, α4, α5, α3β1, and γ1, in brain tumors. Ljubimova and colleagues [42,43] also reported that expression of laminin depends on the tumor grade and is associated with

patient survival. More importantly, receptor integrin-α6 is highly expressed in BCSCs, and integrin-α6 is able to recognize several forms of laminin [41]. The interaction between integrin-α6-positive BCSCs and laminin in the perivascular niche may promote BCSC maintenance. Alternatively, the survival and tumorigenic ability of BCSCs were decreased by targeting integrin-α6 via lentiviral delivered short hairpin RNA (shRNA) [33]. Furthermore, ECM components that are located in the perivascular are capable of accelerating the BCSCs phenotype. More in-depth mechanisms remain to be identified and characterized.

In the paracrine regulatory pathway that associates endothelial cells with BCSCs functions is mediated by nitric oxide (NO). Accumulated evidence indicates that NO enhances tumorigenesis and gives rise to increased levels of endothelial NO synthase (eNOS) in gliomas [44]. The perivascular NO produced by eNOS has been suggested to facilitate glioma progression in a glioblastoma mouse model [45]. Thus, BCSCs may support their survival through mechanisms similar to autocrine regulatory pathway. For instance, Eyler and colleagues [46] demonstrated that inducible NO synthase (iNOS)-generated NO in BCSCs promotes glioma growth in xenograft mouse model. These findings suggest that NO comes from the endothelial cells or BCSCs and is a critical factor involved in modulation of BCSC maintenance.

4. Molecular signaling of BCSCs

A crucial issue in CSC biology is to delineate the regulatory signaling pathways that are involved in maintaining their phenotypes. Glioma CSCs were earlier reported among solid tumor CSCs and appear cellular hierarchy to initiate tumor formation [5]. Notably, glioma CSCs have been shown to promote tumor angiogenesis and are also highly resistant to chemotherapy and radiotherapy [5–7,13], and, thus, raise the importance of elucidating the underlying molecular mechanisms underlying in the regulation of BCSCs to develop more efficient therapies against BCSCs.

4.1. *Signaling of development and growth factor*

External signals from the microenvironment such as stromal, immune response, and other non-stem tumor cells persistently influence CSC actions. Thus, cell surface ligand-receptor systems also play an important role in the regulation of CSCs by extracellular and paracrine signals. There is accumulating evidence to suggest that receptor-mediated pathways control the physiologic functions of BCSCs.

4.1.1. *Notch signaling*

In both invertebrates and vertebrates, Notch signaling is crucial for regulating cell fate determination in many cell lineages through cell-cell communication. Notch proteins are transmembrane receptors, and their intracellular domains (ICD) can be cleaved by the γ-secretase complex for translocation into the nucleus to function as a transcriptional factor upon ligand binding. The significance of Notch signaling pathway has also demonstrated its high conservation during evolution. Notch can facilitate normal NSC proliferation that results in repression of their differentiation [47,48]. Notch has been implicated in brain tumor based on a significantly correlation observed between Notch-1 expression and its ligands, such as Delta-like-1 and Jagged-1 in high-grade gliomas and medulloblastomas [49,50]. Previous studies have indicated that Notch signaling potentially regulates BCSCs in medulloblastomas. Moreover, elevated expression of Notch in BCSCs has been shown to augment the sensitivity to inhibitors of the Notch pathway [51]. Notch proteins are also associated with CSCs, which can enhance the stem cell marker, Nestin in gliomas. The activation of Notch and K-Ras in mouse glioblastoma model yielded proliferative lesions that are located in NSC-occupied subventricular zone (SVZ) due to increased expression of Nestin and glioma formation [52]. Moreover, increased neurosphere-like colonies are also observed while Notch signaling is activated in glioma cell lines [53].

4.1.2. *Hedgehog signaling*

The Hedgehog pathway plays a key role in the regulation of embryogenesis, CNS development, and neural stem cell proliferation and differentiation [54,55]. Upon Hedgehog ligand binding to their receptors, Gli transcription factors and glioma-associated oncogene are activated and subsequently translocated into nucleus to turn on or off their target genes. Aberrant Hedgehog pathways are correlated with primary brain tumor, such as medulloblastoma and evaluated by genetic medulloblastoma models [56]. Moreover, Hedgehog signaling enhances CSCs self-renew and tumorigenicity in gliomas [57]. Increased glioma apoptosis was observed when treated with Hedgehog inhibitor cyclonamine or transduced interference RNA of Gli, which inhibits proliferation and self-renewal of glioma CSCs. Importantly, combining Hedgehog inhibitor and traditional chemotherapy agent, such as temozolomide (TMZ) for treating GBM, has been shown to improve cell death of BCSCs cell death and reduce tumor cell proliferation [57]. Several reports also demonstrated that cyclonamine treatment not only abolishes BCSCs resulting in the failure of tumor progression *in vivo* but also increases the sensitivity of BCSCs to radiation therapy [58]. Collectively, these findings indicate that the Hedgehog pathway is critical for BCSCs, and inhibitors that target this pathway may enhance the efficacy of standard treatment for brain tumor.

4.1.3. *Receptor tyrosine kinase*

The receptor tyrosine kinase (RTK) family is critically involved in growth factor-mediated oncogenesis, and among them, one of the first identified and best-characterized RTK in glioma is EGFR. Enhancement of EGFR signaling pathway is frequently observed in malignant glioma cells such as the constitutively active EGFR type III variant (EGFRvIII) and aberrantly amplified copy number of EGFR. In addition, glioma-like lesions are formed after transduction of Nestin-positive neural stem cells with EGFRvIII in orthotopic mouse model [59]. Consistent of this notion, Li and colleagues [60] revealed that transduction of constitutive EGFRvIII into phosphatase and tensin homolog deleted on chromosome 10-deficient neural precursor cells is sufficient to generate glioblastoma in which the transformed cells harbor tumor and stem-cell marker, CD133, and possess self-renewal ability. Additionally, EGF can promote formation of spheres and facilitate the self-renewal ability in $CD133^{+}$

sub-populations derived from three brain tumor patients [61]. Interestingly, gefitinib, which is a selective inhibitor of EGFR, induced apoptosis and significantly repressed $CD133^+$ BCSCs [61]. Together, these data suggest an important role for EGFR signaling pathways in glioma and BCSC biology.

RTK signaling can be propagated and amplified by the downstream cascades, including the serine/threonine specific protein kinase B (AKT)/phosphoinositide 3-hydroxykinase (PI3K) pathway. Upon RTK activation, AKT enhances cell survival, proliferation, and invasion. It has been demonstrated that glioma CSCs rely more on the AKT signaling pathway than the paired non-stem glioma cells. AKT inhibitors have been shown to reduce the number of viable BCSCs and glioma neurospheres, and attenuate intracranial tumor formation [62]. Collectively, targeting BCSCs subpopulation by inhibition of AKT via specific inhibitors can suppress brain tumor malignancy.

4.1.4. *Bone morphogenetic protein*

The major role of bone morphogenetic proteins (BMPs) is to mediate bone and cartilage development [63]. BMPs bind to the BMP receptors, which are transmembrane serine/threonine kinases, and subsequently activate the canonical regulatory proteins, Smads (Smad1/5/8). Phosphorylated Smads then interact with co-activator Smad4 to form a protein complex that translocates to the nucleus where it regulates transcription and gene expression. BMP signaling regulates NSC proliferation and apoptosis and mainly facilitates the differentiation of NSCs [64]. Interestingly, BMPs have also been shown to suppress the stem-like and cancer stem cell precursors of human glioblastomas [65]. BMP ligands can abolish BCSCs population by inducing stem cell to undergo differentiation into astroglial and neuron-like cells [65]. Glioblastoma treated with BMPs *in vivo* can effectively delay tumor growth and invasion [65]. These results offer a new therapeutic approach to treat glioblastoma by inducing BCSC differentiation rather than directly killing them.

Lee and colleagues [66] also demonstrated that BMPs could induce BCSC differentiation. Interestingly, they found that BMPs actually facilitate BCSCs proliferation rather than differentiation by EZH2-dependent epigenetic silencing of the BMP receptor 1B (BMPR1B) similar to early embryonic NSCs. However, enforcing the expression of BMPR1B can sensitize BCSCs to BMP-mediated differentiation. Therefore, an individual's epigenetic features may affect the response of BCSCs treatment.

4.2. *Epigenetic signaling*

Epigenetic gene regulation has a pivotal role in regulation of embryogenesis, stem cells, and human cancers. In cancers, aberrant epigenetic modulation is correlated with chromatin regulation of gene expression that maintains the embryonic stem cell (ESC) or progenitor cell state. Accumulated data suggest that cancer stem cells have the gene expression signature reminiscent of ESCs and that BCSCs are epigenetically deregulated.

4.2.1. *Bmi1*

The polycomb group (PcG) protein Bmi1 is an epigenetic silencer and has been implicated not only in the regulation stem cells in multiple tissues but also in mediating self-renewal of stem cells. Postnatal brain, and human brain tumor samples extensively expressed Bmi1, and defective stem cell compartments in the brain were observed in Bmi1-deficient mice [67]. Bmi1 also possesses oncogenic ability involved in several types of cancer, including glioblastoma. Bruggeman and colleagues [68] demonstrated that Bmi1 is not only required for astrocytes transformation and differentiation *in vitro* and *in vivo* but also essential for neural stem cells transformation and differentiation ability. Moreover, Bmi1 knockout neural stem cells can develop into low-grade tumor compared to wild type and are able to progress into high-grade gliomas. Fewer NSCs in Bmi1-deficient tumors express the stem cell marker, Nestin, indicating that there might be less number of BCSCs compared with the control. The repression of neurogenic capacity was observed both in transformed and non-transformed Bmi1-deficient glial cells, implicating Bmi1 as a key mediator involved in controlling NSC and CSC differentiation.

Consistently, there is direct evidence that shows Bmi1is highly expressed in enriched $CD133^+$ cells in human GBM [68]. Depletion of Bmi1 expression in GBM cell lines inhibited neurosphere and clonogenic formation. Furthermore, knocking down Bmi1 strongly inhibited brain tumor development even with to 1×10^5 cells inoculated in NOD/SCID mice. Gene expression profiling indicated that Bmi1 attenuates alternate tumor suppressor signaling pathway that can be activated to compensate for the deletion of INK4A/ARF, a inhibitor for cell cycle by arresting cell in G1 phase, and activation of AKT/PI3K. Disruption of INK4A/ARF, which is a tumor suppressor gene to regulate RB and p53 pathways, is one of the most common mutations existing in human GBM [69,70]. Also, the activity of AKT/PI3K is extremely increased in GBM and treatment of an AKT inhibitor enhances the sensitivity against CSCs due to decrease GBM malignancy [62]. These results support Bmi1 as an important in sustaining cancer stem cell renewal in human GBM.

4.2.2. *EZH2*

Enhancer of zeste homolog 2 (EZH2) is a catalytically active component of polycomb repressive complex 2 (PRC2) and participates in transcriptional silencing of specific genes via trimethylation of histone 3 at lysine 27 (H3K27me3). Induction of EZH2 is not only involved in hematopoietic and solid tumor progression but also associated with poor prognosis. Additionally, EZH2 plays a key role in stem cell maintenance, differentiation, and self-renew during development. Suva and colleagues [71] demonstrated that EZH2 is overexpressed and enriched in glioblastoma CSCs. The self-renewal and tumor initiating abilities of glioblastoma CSCs *in vivo* are dramatically inhibited when pharmacologic inhibitors or shRNAs are used to target EZH2 [71]. More recent studies also indicated that disruption of EZH2 reduces the expression of CD133 and proliferation of glioblastoma CSCs [72]. Together, these data support the potential of EZH2 as a valuable therapeutic target for GBM treatment.

4.2.3. *MicroRNAs*

MicroRNAs (miRNAs) are small noncoding RNAs that can modulate gene expression by targeting specific genes to silence protein expression. miRNAs play a significant role in cell fate determination and proliferation involved in

development and cancer biology. Several studies have been identified several miRNAs that specifically regulate brain development and neural differentiation through microarray analysis of miRNA expression in mammalian brain. For instance, the expression of miR-21 is significantly increased in glioblastomas, and attenuated its action triggers caspase dependent apoptosis [73]. Similar to miR-21, a recent study indicated that the miR-17-92 cluster is highly expressed in primary astrocytic gliomas and glioblastomas compared with the normal brain, and it is also implicated in the progression of low-grade to more aggressive brain tumors. Inhibitors of miR-17-92 were shown to suppress glioblastoma spheroids by promoting apoptosis and reducing proliferation [74].

Silber and colleagues [75] investigated the possible role of miRNA in the regulation of CSCs in glioma. Specifically, miR-124 and miR137 expression levels were dramatically reduced in anaplastic astrocytomas (grade III) and GBM (grade IV) compared with normal brain tissues. Ectopic expression of miR-124 and miR137 in GBM cells can reduce cell proliferation and promote differentiation of glioma stem cells. These data indicate that these two miRNAs could serve as a tumor suppressor in BCSCs. In addition, another study demonstrated that the expression of miR-451 is lower in glioma $CD133^+$ CSCs compared to $CD133^-$ non-glioma stem cells [76], suggesting the possibility of inducing the expression of miR-451 to destroy neurosphere formation and reduce giloma CSCs proliferation.

4.3. *Signaling in CSC niches*

Several studies have reported that the interactions and regulatory signaling pathways between BCSCs and perivascular endothelial cells are important for brain tumor progression and clinical targeting. BCSCs can not only receive signals from microenvironment but also propagate signals to affect the environment. The perivascular niche of brain tumor is the best example for such communication.

4.3.1. *Vascular endothelial growth factor*

BCSCs are able to produce the well-characterized proangiogenic factor such as vascular endothelial growth factor (VEGF) [6,11]. A paracrine role of VEGF generated from BCSCs is demonstrated through the inhibition of endothelial cell proliferation and tube formation when BCSC-conditional medium is supplied with VEGF-neutralizing or VEGFR2-blocking antibodies (bevacizumab). The significant suppression of human glioma growth in xenograft mice is observed while treatment with anti-VEGF or anti-VEGFR2 antibodies, that resulting in a reduction of blood vessels density [6,11]. In addition to undoubtable contribution of targeting effects of VEGF on endothelial cells, glioma cells also express VEGF and VEGFRs [77]. VEGF-VEGFR autocrine signaling can enhance glioma cells proliferation and viability, and blockage of this effect leading to increase the response to radiation-induced cell death [77]. Together, these results support that glioma progression relies on BCSCs-driven generation of VEGF through both autocrine and paracrine signal.

4.3.2. *Hypoxia inducible factors*

Microenvironmental stress, including nonphysiologic levels of oxygen, pH, and metabolites, can influence tumor development through distinct signaling pathway. For example, low oxygen concentration and hypoxia are crucial to maintaining BCSCs via hypoxia inducible factors (HIFs), in particular, HIF2α [2]. Under hypoxia condition or overexpression of a non-degradable form of HIF2α promotes non-stem glioblastoma cells to gain self-renewal capacity that leads to cellular transformation [78]. Under hypoxia, studies have shown that the signature genes such as lysyl oxidase (LOX), VEGF, HIG2, and prominin1 (CD133) of glioblastoma stem cell are overexpressed under hypoxia condition [79,80]. Importantly, response to hypoxia in BCSCs can be attenuated by digoxin, a HIF inhibitor [80], suggesting that HIF proteins could potentially become therapeutic targets for malignant brain tumors.

5. Targeting of BCSCs

5.1. *Direct BCSC targeting*

The standard treatment for brain cancer commonly uses both irradiation and chemotherapy, e.g., temozolomide (TMZ). However, resistance to irradiation and TMZ often occurs due to the enrichment of $CD133^+$ fraction in tumor [7,13,81]. Therefore, novel treatments for brain cancer that block function of BCSCs could potentially overcome resistance to standard therapy. Direct targets of BCSCs include Notch, Hedgehog/Gli, EGF/EGFR/AKT pathway, Bmi1, EZH2, and miRNAs, all of which have been shown to sensitize BCSCs to drug treatments and inhibit BCSC survival [82]. Also, as mentioned previously, another strategy to enforce BCSC and reduce their tumorigenic potential is to induce BCSC differentiation, such as through BMP signaling [65]. For instance, BCSCs treated with BMP by implantation of BMP-bearing beads in glioblastoma mouse model significantly attenuated their transforming capacities [65].

5.2. *Indirect BCSC targeting*

Strategies that indirectly target BCSCs focus on the niche or cell microenvironment that harbors key determinants to sustain growth and survival of BCSCs. In perivascular niche, bevacizumab, a well-known inhibitor of VEGF, inhibited tumor vasculature, decreased $CD133^+$ BCSC number, and significantly reduced tumor size [9]. Moreover, there are compelling data demonstrating that the hypoxia microenvironment is a distinct niche that enriches BCSCs through upregulation of HIF2α. Downregulation of HIF2α can reduce stem cell marker expression, neurosphere formation, and VEGF signaling [10,82]. These landmark studies not only characterized the importance of perivascular or hypoxia niches for BCSCs but also identified new therapeutic approaches that target them.

6. Conclusion

In the past decade, CSC research has provided distinct new views in cancer biology. In particular, the cellular hierarchy and tumorigenic ability of BCSCs are highly attractive as targets for therapeutic development against brain cancer.

Moreover, by addressing the regulatory molecular mechanisms and interactions between BCSCs and the niches that maintain and propagate them, researchers have provided extraordinary insights on potential therapies that directly or indirectly target BCSCs. While extensive investigations have broadened the understanding of brain cancer biology, there is still a lack of substantial improvement in brain cancer patient survival. Therefore, there is an urgent need for more in-depth investigations to unravel the underlying molecular mechanisms that support and maintain BCSCs as well as the development of novel therapies against brain cancer.

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